Behavioural and neurochemical effects of monosodium glutamate on striato-hippocampal region of the brain and the protective role of *Myristica fragrans* Houtt.

Thesis submitted to
the University of Calicut for the award of
the degree of

DOCTOR OF PHILOSOPHY IN PHYSIOLOGY

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Under the guidance of

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DECLARATION

I, Sreejesh P G., hereby declare that this thesis entitled **"Behavioural and neurochemical effects of monosodium glutamate on striato-hippocampal region of the brain and the protective role of *Myristica fragrans* Houtt."** is being submitted to the University of Calicut, in partial fulfillment of the requirement of the degree of Doctor of Philosophy in Physiology under the faculty of Science. This thesis is the result of my work carried out in the department of Life Sciences under the guidance and supervision of Dr. E. Sreekumaran, Associate professor of Physiology and Head of the Department, Department of Life Sciences, University of Calicut. This thesis or any part thereof has not been submitted for any other degree, diploma or any other similar title of any other university.

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Sreejesh, P. G

Dedicated to

All those who supported me in this endeavour

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LIST OF ABBREVIATIONS

ACh	:	Acetylcholine
AChE	:	Acetylcholinesterase
ADHD	:	Attention deficit hyperactivity disorder
ADI	:	Acceptable daily intake
ALT	:	Alanine amino transferase
AMPA	:	A-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANSA	:	1-amino-2-naphthol-4-sulphonic acid
AST	:	Aspartate amino transferase
BBB	:	Blood-brain barrier
CA	:	Cornu ammonis
CAs	:	Chromosome aberrations
CAT	:	Catalase
CBMN	:	Cytokinesis-blocked micronucleus
CD	:	Conjugated dienes
CDNB	:	1-chloro-2,4-dinitrobenzene
CNS	:	Central nervous system
COX	:	Cyclooxygenase
CPCSEA	:	Committee for the purpose of control and supervision on experiments on animals
CRS	:	Chinese restaurant syndrome
DG	:	Dentate gyrus
DLT	:	Dark/light transition test
DPPH	:	1, 1- diphenyl 2-picrylhydrazyl

DTNB	:	5’-dithio-bis [2-nitrobenzoic acid]
EAAT	:	Excitatory amino acid transporter
EC	:	Entorhinal cortex
ECFs	:	Extracellular fluids
EFSA	:	Europe’s food safety authority
eNOS	:	Endothelial nitric oxide synthase
EPM	:	Elevated plus maze
FASEB	:	Federation of American societies for experimental biology
FDA	:	Food and drug administration
FEP	:	Free-exploratory paradigm
FSH	:	Flolicle stimulating hormone
FST	:	Forced swim test
GABA	:	Gama-aminobutyric acid
GAD	:	Glutamic acid decarboxylase
GPe	:	External globus pallidus
GPi	:	Internal globus pallidus
GSH	:	Glutathione
GST	:	Glutathione-S-transferase
H&E	:	Hematoxylin and Eosin
HDL-C	:	High-density lipoprotein cholesterol
HP	:	Hippocampus
HPA	:	Hypothalamic pituitary adrenal
IAEC	:	Institutional animal ethics committee
IP	:	Intraperitoneal
IP3	:	Inositol-1,4,5-trisphosphate
LDH	:	Lactate dehydrogenase

LDL-C	:	Low-density lipoprotein cholesterol
LH	:	Luteinizing hormone
LPO	:	Lipid peroxidation
LTP	:	Long-term potentiation
MB	:	Marble burying
MDA	:	Malondialdehyde
MDR	:	Multidrug-resistant
MSG	:	Monosodium glutamate
MSH	:	Melanocyte-stimulating hormone
MSN	:	Medium spiny neurons
NBT	:	Nitroblue tetrazolium
NCBI	:	National Center for Biotechnology Information
NE	:	Norepinephrine
NKB	:	Neurokinin B
NMDA	:	N-methyl-d-aspartate
NOR	:	Novel object recognition test
NO	:	Nitric oxide
OF	:	Open field
PMS	:	Phenazine methosulphate
RIP	:	Resident-intruder paradigm
ROS	:	Reactive oxygen species
SB	:	Subiculum
SCEs	:	Sister-chromatid exchanges
SN	:	Substantia nigra
SOD	:	Superoxide dismutase
SPSS	:	Statistical package for social sciences

ST	:	Striatum
TBARS	:	Thiobarbituric acid reactive substances
TH	:	Tyrosine hydroxylase
TM	:	T-maze
TNFα	:	Tumor necrosis factor alpha
TST	:	Tail suspension test
VGLUT	:	Vesicular glutamate transporter
VMN	:	Ventromedial nuclei
WHO	:	World health organization
ZF	:	Zona fasciculate

CONTENTS

ABSTRACT

Monosodium glutamate (MSG) is a sodium salt of L-glutamate, a well-known flavor enhancer. Because of its capacity to increase the palatability, it is most widely used in all households and fast foods nowadays. Glutamate is one of the most abundant excitatory neurotransmitter of brain and plays an important role in learning and memory. The present study was designed to investigate the effect of MSG on neurobehavioral, neurochemical and histological alterations on striato-hippocampal region of the brain in *Wistar albino* rats and explore the possibility of recovery from MSG toxicity, after 60 days of MSG administration followed by a 60 days of non-MSG treatment. It also investigates the ameliorative effect of decoction of mace from *Myristica fragrans* Houtt against the MSG induced neurotoxicity.

Male *Wistar albino* rats, approximately 3- 4 weeks old rats were used for the study. They were divided into group I and group II. These groups were again subdivided into control group, 100 mg MSG, 400 mg MSG, 2 g MSG and 4 g MSG group treated orally with various doses of MSG/ Kg body weight for sixty days. Immediately after 60 days of MSG treatment, the group I rats were used for the analysis of neurobehavioural changes including anxiety like behaviour assessed by open field test (OF), elevated plus maze test (EPM), dark/ light transition test (DLT), marble burying test (MB) and free exploratory paradigm (FEP), depression like behaviour assessed by tail suspension test (TST) and forced swim test (FST), aggression by resident intruder

paradigm (RIP), social interaction by three chamber sociability and social novelty tests, spatial memory and working memory was assessed by T-maze test (TM) and novel object recognition test (NOR) and sensory motor activities by hot plate and rotarod experiments. The striato-hipoocampal biochemical changes including lipid peroxidation (LPO) assessed by melonaldehyde (MDA) and conjugated diene (CD) level, antioxidants including glutathione (GSH), superoxide dismutase (SOD), catalase (CT) and glutathione-s-transferase (GST), Na^+/K^+ATPase, acetylcholinesterase (AChE), glutamine, glutamate and GABA level was assessed. The histological changes in striato-hipoocampal region of the brain were also assessed by hematoxylin and eosin staining. In group II, after sixty days of MSG treatment, the rats were kept free from MSG for further sixty more days and later used for the above experiments. The attenuating effect of mace of *Myristica fragrans* Houtt against MSG toxicity was also studied. For this, the rats were treated with 4 g MSG/ Kg body weight along with 200 mg mace/ Kg body weight for 60 days, after that the rats were used for behavioural and biochemical studies.

The results showed that deleterious effect of MSG were dose dependent and more severe in group I which was fed MSG for sixty days, whereas the effect is mild and showed a recovery in group II which were also fed MSG for sixty days and later kept free from MSG for an additional sixty days. The high dose of MSG (2 g and 4 g) fed rats in group I exhibited neurobehavioural alterations including anxiety and depression like behaviour associated with reduced aggression, less social interaction and memory impairment while sensory and motor

impairment was not observed. The biochemical assays also revealed that high dose of MSG showed a significant positive correlation with MDA and CD levels accompanied by depletion of GSH, CAT, SOD and elevated GST concentration in the rat brain which resulted in an increased oxidative stress thereby leading to cell suppression or conductive damages. The oxidative stress was developed in all the dosages of MSG, whereas in mild dosage administration only showed a slight variation and high dose of MSG showed a significant difference. A significant elevation in the level of acetylcholinesterase, an enzyme to split acetylcholine, and depletion of Na^+/K^+ ATPase level was also observed in high dose MSG fed rats. Significant elevation in blood glutamate level was also observed in MSG treated rats but it was not reflected in the brain neurochemicals such as glutamate, glutamine and GABA and it did not cause any morphological change in the striato-hippocampal region of the brain. However in group II, there appeared a reversing effect i.e., reduction in anxiety and depression like behaviour, improvement in memory and increase in social interaction in rats. The reduction in lipid peroxidation markers MDA and CD associated with increase in antioxidant status, decline in acetylcholinesterase activity and improved Na^+/K^+ ATPase level was also observed. The rats treated with the decoction of mace of *Myristica fragrans* showed an ameliorative effect against the MSG induced neurotoxicity. It reduced anxiety and depression like behaviour and improved memory performance. It showed a decline in the lipid peroxidation and oxidative stress by scavenging free radicals associated increase in antioxidants and decreased acetylcholinesterase level.

Thus, this study concluded that dose dependent long term administration of MSG has a capacity to cause behavioural changes such as anxiety, depression, cognitive impairment, altered aggression and social interaction associated with increased oxidative stress and antioxidant depletion by indirect mechanisms. But it is not a perpetual and reversible process, during the life time. Due to normal diet and natural healing mechanisms, the body ameliorates the toxic effects of MSG and showed gradual positive response and recovery in behavioural and biochemical parameters, however, it was a time consuming process. Decoction of mace from *Myristica fragrans* also has the capacity to improve antioxidant status and scavenging radicals associated with increase in acetylcholine level, thereby improving cognition and other behavioural parameters. Thus the use of mace in the food improves our antioxidant status and improves neurochemical status and thereby preventing the MSG induced toxicity to a certain limit.

INTRODUCTION

Food is a substanceconsumed to provide nutritional support and building block for an organism. It is usually of plant or animal origin and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins, minerals and water. Throughout the world each nation, religion or even a single individual follow their own style and culture in their food habits. Many cultures have a decipherable type of food or a specific set of preparations using various spices and flavors, which are unique to that culture, or even the culture of a country. Because of its importance in life, humans quickly found ways to keep food scrumptious and edible for extended periods of time. Food additives have been used for thousands of years. As there is no exact history of when and how food additives were discovered, it is assumed by researchers that additives were first used when humans learnt to preserve their crops on different harvesting times. The earliest food additives and preservatives included salt, vinegar, sugar and spices. A food additive is a substance or a mixture of substances, other than basic food stuffs, present in food to preserve flavor, colour or enhance its taste, appearance, shelf life or other qualities. It has become the part of food either directly or indirectly at some stage of production, processing, storage, packaging or even in the home foods (Bruce Eaton, 2007; Chaudhary, 2010).

Depending up on the nature and the function of food additives, it is divided into different groups such as acidity regulators (acetic acid), anticaking agents (sodium bicarbonate), antioxidants (ascorbic

acid), food coloring agents (sunset yellow), emulsifiers (polyglycerol ester), flavor enhancers (monosodium glutamate) etc. (Erich and Gert-Wolfhard, 2000). The role of food additives has become more prominent in recent years, due to the increased production of prepared and processed foods, necessary to guarantee adequate food supply for a growing population. At the same time, researchers and others have confusion and concern of whether these additives added to the food stuff is in fact safe or dangerous.

Monosodium glutamate (MSG) is one of the most commonly used flavor enhancer and a controversial food additive, found in almost all types of fast foods, packed Chinese food, soups, canned vegetables and processed meats. MSG is sodium salt of a naturally occurring non-essential amino acid L glutamic acid and is the most abundant amino acid found in nature. It is a white odourless crystalline powder represented by the formula $C_5H_8NNaO_4$•H2O. It has an E number E621 (Appaiah, 2010; Perry, 2017; NCBI, 2017). In solid from, it contains separate sodium cations and glutamate anions in zwitterionic form. It contains 78.2 % glutamic acid, 12.2 % sodium and 19.6 % water (Samuels, 1999). It is soluble in water and stable under food-processing conditions (Sano, 2009).

Glutamate, one of the most abundant amino acid is present in many proteins, peptides and most animal and plant tissues of nature. It is also produced in the body and binds with other amino acids to form a structural protein. When glutamate binds to protein molecule,it is tasteless and does not provide *umami* taste to the food. However, protein hydrolysis during fermentation, aging, ripening and heat

cooking process will liberate free glutamate. Glutamate is a crucial component of the taste of cheese, sea foods, meat broths and other foods. A high level of free glutamic acid, is present naturally in different foods, such as meat, poultry, cheese, fish sauce, seafood, seaweed, vegetables, soy sauce, fermented beans and tomatoes (Ninomiya, 1998; Yoshida, 1998; Jinap and Hajeb, 2010).

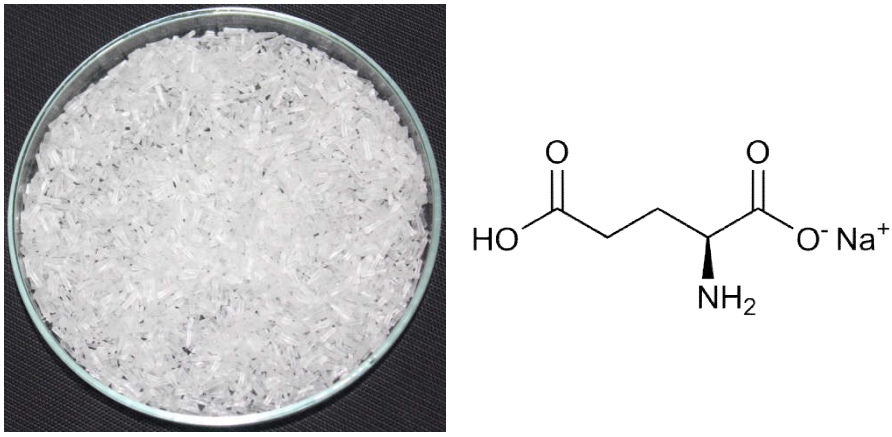

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Figure: 1.1: (a) Photograph of monosodium glutamate (b) Structure of Monosodium glutamate.

Glutamate is the source for the unique flavor called "*umami*", a pleasant savoury taste and one of the five primary tastes. MSG adds the *umami* taste to the food through the stimulation of taste receptors by free glutamate and also enhances and intensifies the palatability of other tastes particularly salt, sweet and sour. Other salts of free glutamate, which also provide the *umami* taste include monopotassium glutamate, calcium glutamate, monoammonium glutamate, magnesium glutamate, natrium glutamate, hydrolyzed protein, calcium caseinate, sodium

caseinate, yeast extract, torula yeast, yeast food, yeast nutrient, autolyzed yeast, gelatin and textured proteins(Tsimidou and Boskou, 2003).

The flavour enhancing property of MSG is first identified by a Japanese scientist Kikunae Ikeda at 1908 and extracted it from a seaweed Laminaria japonica, which is traditionally used to enhance flavor to Japanese foods. Later he took patent to monosodium glutamate and started commercial production in the name of "Ajinomoto" (Nakamura, 2011). Earlier, MSG was synthesized from highly rich protein sources like seaweeds and wheat gluten by treating with hydrochloric acid and in later years, it was produced from acrylonitrile by direct chemical synthesis. Currently, MSG is produced by bacterial fermentation using sugar as a carbohydrate source from corn starch, sugar cane, sugar beets or molasses and hydrolysates from corn or tapioca. During fermentation process, *Corynebacteriumglutamicum*, *Brevibacteriumlactofermentum* and *Brevibacteriumflavum* are cultured in high carbohydrate with ammonium medium and excrete L-glutamte in the medium. Later sodium is added to from monosodium glutamate. Today, the world production of MSG has reached to 2 million tons per year by fermentation process (Sano, 2009).

Natural and processed foods contain diverse types of *umami* compounds, including glutamate, aspartate, monophosphates of inosinate or guanylate and inosine 5′-monophosphate and guanosine-5′-monophosphate, while free glutamates are most effective in enhancing the palatability. The *umami* receptors include 2 glutamate-

selective G protein–coupled receptors, $mGluR_4$, $mGluR_1$ and the taste bud expressed heterodimer $T1R_1$ and$T1R_3$. Each of these receptors is expressed in small number of cells in the anterior and posterior taste buds. Taste sensations from these receptors are carried by chorda tympani and glossopharyngeal nerves and activate the insular cortex, the caudolateral orbitofrontal cortex and the rostral anterior cingulate cortex (De Araujo *et al*., 2003; Chaudhari *et al.,* 2009).

The presence of MSG in food increases the palatability or alters and magnifies the desirable taste, thus is an essential part of human diet and commonly found in Asian cuisine associated with Chinese restaurants and frequently in Western diets. The MSG ingestion is growing worldwide with average daily intake estimated as 3-4 g/day. Europe's food safety authority (EFSA) has set a safe level for glutamate food additives and strictly sticking on the acceptable daily intake (ADI) of 30 mg/ Kg body weight, yet instead no established ADI for glutamic acids and glutamates as food additives in the EU but there is a maximum permitted level of 10 g/ Kg of food. They also recommended that the dose above ADI associated with MSG symptom complex known as Chinese restaurant syndrome (CRS) including head ache and increased blood pressure (Mortensen *et al.,* 2017). The Food and Drug Administration (FDA) has determined MSG as safe for the general population and not set a specified ADI. World health organization (WHO) in 1974 suggested an ADI of MSG in a healthy diet is 0-120 mg/ Kg body weight. The consumption of MSG varies between country to country and population to population and it may be up to 10 g/ day. In US the average daily intake of MSG is around 550

mg/day and in UK it is around 580 mg/day for the general population and 4.68 g/day for extreme users, whereas in Japan and Korea it is around 1.2–1.7 g/day. LD_{50} of sodium glutamate in rats ranges between 15 and 18g/ Kg of body weight (Rhodes *et al.,* 1991; Walker and Lupien, 2000; Beyreuther *et al.,* 2007; He *et al.,* 2008).

Chinese restaurant syndrome

The Food and Drug Administration categorized MSG as a safe substance in 1959. However, the FDA commissioned a report that an unknown percentage of the population might react to MSG and develop MSG symptom complex. The Chinese restaurant syndrome is a pattern of symptoms that affects some people after eating Chinese food. CRS was first reported by Kwok in 1968, a complex of symptoms experienced after eating Chinese meal at Chinese restaurants. He described a recurring symptom complex consisting of numbness in the back of the neck that radiated to the arms and down the back, accompanied by generalized weakness and palpitations. In 1995, the Federation of American Societies for Experimental Biology (FASEB) published a report of a comprehensive analysis of the safety of MSG and established the following symptoms that constitutes the syndrome including burning sensation at the back of the neck, forearms and chest, headache, chest pain, facial pressure/tightness, nausea, palpitation, numbness at the back of neck, radiating to arms and back, tingling, warmth, weakness in face, temples, upper back, neck and arms, drowsiness and weakness (Walker and Lupien, 2000).

In 1969, Schaumburg *et al.,* observed that people administrated soup with MSG showed MSG symptom complexes which were associated with headache, burning sensation, facial pressure, tightness and chest pain. He also described that all individuals are prone to MSG but the intensity and duration of the symptoms were dose related. In 1972, Kenney & Tidballidentified that people who experienced MSG symptom complex only after ingesting 3–5 g of MSG, an amount much higher than that of normally found in the typical western diet. In 1997, Yang *et al.,*conducted a double blind placebo study on allergic persons showed that a group of symptoms including angioedema at first called the Chinese restaurant syndrome and are better referred to as the MSG symptom complex. Wilkin in 1986 noticed that MSG is regarded as a provocating agent for flushing, edema and associated symptoms which are the components of Chinese restaurant syndrome. In 1987, Moneret-Vautrin observed an intrinsic asthma and mild bronchospasm in a small population administrated with a high dose of MSG and the possible mechanism might be either due to stimulation of the synthesis of acetylcholine, or a high vagal hyperactivity. In 2000, Geha *et al.,* observed that people who consumed MSG showed symptoms such as numbness, weakness, sweating, dizziness, flushing and headaches, which are the components of MSG syndrome complex.

Systemic effects of MSG

MSG is conceded as a toxic substance for both human and experimental animals. The neonatal ingestion of MSG severely damaged hypothalamic nucleus resulting in obesity, increased total cholesterol and defective endocrine system of the body. Earlier studies

reported that chronic consumption of MSG produced various side effects including oxidative stress associated with hepatotoxicity, cardiotoxicity, nephrotoxicity, respiratory toxicity, neurotoxicity and metabolic or digestive problems (McEntee and Crook, 1993;Dawson *et al.,* 1997; Martínez-Contreras *et al.,* 2002).

Neurotoxic effect

Glutamate, the most abundant excitatory neurotransmitter present in the human brain is essential for memory, learning and other cognitive functioning, even though increased amount of glutamate in the brain may be excitotoxic and cause neuronal death (McEntee and Crook, 1993). In addition to neuronal death, dendritic hypotrophy, neuroendocrine disturbance, obesity, retinal degeneration and epileptic seizures were also observed in MSG ingested rodents (López-Pérez *et al*., 2010). It is believed that the high amount of MSG consumption cause neuronal death by excitotoxicity, which is due to the release of intercellular calcium ions by the hyper activity of glutamate receptors (Olney, 1969; Gonzalez-Burgos *et al.,* 2001; Varga *et al.,* 2011). Robinzon *et al.,* (1974) examined that chicken administered with MSG (4 g/ Kg B.w, i.p) at the ages of 5, 70 or 120 days developed neurotoxicity but was severe in early age groups such as 5 and 70 days than 120 days and it is revealed that MSG neurotoxicity is mainly an age dependent process and more severe in early age or neonatal groups because of the poor development of blood brain barrier. Ross *et al.,* (1975) reported that postnatal treatment of MSG in mice showed a decrease in choline acetyltransferase and a huge increase in AChE activity. Köhler and Schwarcz, (1981) showed that microinjection of

300 μg MSG/ 0.5 μl into the hippocampal cells of the adult rat caused local damage to the marginal neurons with associated increase in oxidative stress.

Wallace and Dawson, (1990) showed that an acute, subconvulsive dose (500 mg/ Kg i.p.) of MSG alter neurotransmitter level, especially norepinephrine (NE) and dopamine content of hypothalamus and cerebellum. Administration of monosodium glutamate in the neonatal period renders the rat to be α-MSH deficient in later life, which is a crucial hormone regulating behaviour like thermoregulation, stress resistance and immunity. These results suggest that both α-MSH and melanin are controlled by dopaminergic system in the brain and excessive excitation of glutamatergic system may destroy the dopaminergic neurons or they get transformed to excitatory neurons (Yehuda *et al.,* 1991). Reports show that neonatal MSG administration cause significant changes in choline acetyl-transferase activity, muscarinic receptor binding and GABA release in brain especially in HP and ST regions (Ortuño-Sahagún*et al.,* 1997; Beas-Zárate *et al.,* 1998).

A systemic administration of MSG in rodents showed that a decline in learning and memory associated with decline in neurotransmitter dopamine and serotonin, decline in total glutathione, increased lipid peroxidation rate and neuronal death in arcuate nucleus of hypothalamus (Park *et al*., 2000; Abu-Taweel *et al*., 2014). Beas-Zárate *et al*., (2001) observed that neonatal administration of MSG (4 mg/g body weight given subcutaneously) cause elevated glutamate level and excitotoxic effects on rat brain. Elevated glutamate level

increased glial cell activity, increased expression and structural changes of NMDA receptor subunits especially NMDA R1, R2A and R2B along with neuronal damage. Beas-Zárate *et al*., (2002) also observed that MSG administration increased the NR2C and NR2D subunit gene expression levels in the hippocampus and striatum of adult rats but not in cerebral cortex, which lead to alternate changes in neuronal circuits in these regions as a compensatory change. Beas-Zárate *et al*., (2002), reported that neonatal injection of 4 g MSG/ g body weight for the first seven days produced loss of pyramidal cell, adversely altered dendritic arborization and dendritic spine density in the CA1 region of the hippocampus at 60 days of age. It could be altering the structural and functional integrity of hippocampus. The neonatal administration of MSG in newborn rats observed excitotoxic damage in neurons and associated increase in the activity of astrocytes and microglia cells in the fronto-parietal cortex (Martínez-Contreras *etal*., 2002)**.**

Ureña-Guerrero *et al*., (2003) showed that neonatal administration of MSG increased the level of glutamic acid decarboxylase (GAD), a crucial enzyme for the synthesis of GABA, in HP and ST region while decreased in cerebral cortex of the brain. López-Pérez *et al*., (2005) explained that over-stimulation of the glutamatergic system by neonatal exposure to a high glutamate concentration induced a partial loss in Tyrosine Hydroxylase (TH) positive neurons in the substantianigra (SN) and an important reduction in dopaminergic markers expression in the striatum, suggesting that early excitotoxicity could contribute to developmental

alterations in the nigrostriatal pathway, which may be associated with various disorders of the basal ganglia. In most cases neonatal elevation of glutamate as a result of MSG administration, caused neuronal death through apoptosis. Another study conducted by Olvera-Cortes *et al*., (2005) on neonatal rat pups treated with MSG through subcutaneous injection at postnatal days one, three, five and seven and later at four months of age, the rats were impaired to place learning acquisition and an elevated escape latencies. The expression of place learning acquisition is a major task of HP through NMDA receptors. Chaparro-Huerta *et al*., (2008) showed that neonatal MSG administration elevates the Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 level and neuronal death in ST and mildly in frontal lobe. These pro-inflammatory cytokines activate the signaling p38 MAPK pathway involved in apoptosis of neurons. Several studies showed that high dose of L -glutamate cause over expression of the NMDA receptor sub-units and excitotoxic effects on glutamatergic post-synaptic receptors. It also causes behavioral abnormalities, glial reaction and neuronal death.

Neonatal administration of MSG leads to poor development of blood brain barrier (Boonnate *et al*., 2015), reduced serotonin and cognitive functioning (Moneim *et al*., 2018), pyknotic Purkinje and granule cells with inflamed cells in the cerebellum (Hashem et al., 2012). Onaolapo et al., (2012) reported that subchronic MSG doses was anxiogenic and showed a slightly retardant effect on spatial working memory. Dief et al., (2014) reported that subcutaneous administration of MSG for 10 days in *Wistar* rats lower the

hippocampal AMP-activated protein kinase (AMPK) and enhances β-amyloid accumulation causing neurotoxic effects. It also causes the elevation of Fas ligand in the hippocampus, which is a mediator of apoptosis. Neuro-behavioural studies conducted on dark light chamber and hole board test indicated an increased latency and anxiety like behaviour. O'Brien, (2016) reported that consumption of 50 mg/ Kg MSG enhances dural blood flow to 20 -25 % in both females and male adult rats. Consumption of MSG elevate blood glutamate level and excite the peripheral glutamate receptors such as NMDA, AMPA, kainate and mGluR5 receptors in the nerve fibers innervated in the dural blood vessels, which may cause headache in young healthy individuals.

Obesity and hepatotoxicity

Obesity is a complex, multifactorial and a preventable disease affecting along with overweight. It is world's third most common disorder and at the end of 2030 around 38 % of the world's adult population will be overweight and 20 % will be obese. The economic development, automated transport, industrial civilization, reduced physical activities, change in life style and a dietary transition from homely nutritious food to processed and high calorie foods fuelled the increased prevalence of obesity (Hruby and Hu, 2015; W. H. O, 2018). Obesity and overweight are clinical because it increases the risk for several diseases including cardiovascular diseases (CVDs), hypertension, stroke, diabetes mellitus and ultimately lead to mortality (NHLBI, 1998).

Obesity is mainly because of over eating or prolonged imbalance between energy intake and energy utilization. Hypothalamic ventromedial nuclei (VMN) is controlling the energy homeostasis along with the integration of feeding and metabolic mechanisms (Resch et al., 2011). The bilateral lesion of VMN causes hyperphagia and increased intake of lipid and carbohydrate diet (Yadav et al., 2009). However,Gold (1973) suggest that lesion to nearby ventral noradrenergic bundle produce obesity rather than VMN lesion.

Obesity the most common clinical characterization of MSG treatment is not related to hyperphagia. A clinical trial conducted by Anderson and co-workers showed that MSG increase fullness and reduced desire to eat (Anderson et al., 2018). In rodents, experimental studies showed that arcuate nucleus and median eminence of the hypothalamus was the most prone area for MSG toxicity and obesity but fail to show hyperphagia (Olney, 1969; Seress, 1982; Dawson and Annau, 1983). Hyperphagia is a direct function of a hormone Ghrelin, acting through the arcuate nucleus of the hypothalamus (Tamura et al., 2002).

Animal studies indicate that high dose of MSG has the capacity to induce hypothalamic damage in the brain and leptin resistance, essential for food intake and energy balance, can cause over weight and obesity. Dawson et al., (1997) showed that neonatal administration of MSG for fourteen days damaged the arcuate nucleus of hypothalamus and subsequent increase in body weight and neuroendocrine dysfunction. The suggested mechanism behind the

elevated body weight is mainly due to the suppression of leptin. Leptin is a hormone secreted by adipocytes to regulate appetite; fat storage and energy balance, acting through the receptors (leptin receptors) present in the arcuate nucleus of the hypothalamus. Holzwarth-Mcbride et al., (1976) showed that postnatal administration of MSG from 5^{th} to 10^{th} day decreased the perikarya in the arcuate nucleus of adult mice resulted in endocrine and reproductive deficit along with obesity. Subcutaneous injection of MSG to 4 days old mice at a dose of 0.2 mg/ Kg body weight showed an immediate peak upsurge in glutamate level at arcuate nucleus than any other parts of the thalamus and hypothalamus. This transient accumulation of subcutaneously administered glutamate might be selectively destroying the hypothamic anterior neuronal perikarya(Perez and Olney, 1972).

Pepino et al., (2010) reported that obese women showed low sensitivity to the monosodium glutamate taste and favor higher concentrations compared to normal weight women. He et al., (2008) conducted an experiment on 752 healthy Chinese, aged 40–59 years. The participants consumed homely food with an average intake of 0.33 g MSG/day. The results showed that MSG users had a significant prevalence of overweight than non MSG users.

Ayman et al., **(**2013) recorded that oral administration of MSG at a dose of 0.6 mg/ Kg tissue body weight severely alter the cyto-architecture of liver including dilatations of the central veins, distortions of the hepatocytes, marked reduction in both carbohydrates and proteins, vacuolated cytoplasm, swollen mitochondria, vesiculated rough endoplasmic reticulum with pyknotic nuclei and significant

variation in the expression of ki-67 and p53 proteins. The neonatal administration of monosodium glutamate to rodents lead to obesity in the adult animal, characterized by increased visceral adipose tissue, serum leptin, free fatty acids, HDL-cholesterol, total cholesterol levels, blood glucose, lactate dehydrogenase (LDH), aspartate amino transferase (AST), alanine amino transferase (ALT), hepatic and cardiac lipid peroxides and decline in antioxidant enzymes such as glutathione (GSH) superoxide dismutase (SOD) and catalase (CAT) levels and Tumor Necrosis Factor (TNFα) levels (Luz et al., 2010; Collison et al., 2013; Kumar and Bhandari, 2013). The characteristic changes in the lipid profile and obesity might be a direct result of hepatic and adipose tissue gene expression by increasing the transcription of genes involved in lipid mobilization and storage together with an attenuation of the expression of several lipid catabolizing genes (Collison et al., 2009).

Another study conducted by Miranda and coworkers in 2017 showed that maternal MSG-induced obesity at a dose of MSG 4 mg MSG/ g body weight subcutaneously led to an obese male offspring characterized by hyperinsulinaemia, hyperglycaemia, dyslipidaemia, hyperleptinaemiaand impaired leptin signaling while cross-fostering with control normalize the lipid profile, but not hyperinsulinaemia and hyperglycaemia. Administration of monosodium glutamate in the neonatal period causes development of obesity in the four month old rats. Under monosodium glutamate induced obesity there are metabolic changes in salivary glands of rats such as marked elevation in the activity of NO-synthase and the maintenance of nitrites, significant

decrease of activity of ornithine decarboxylase and α-amylase, increasing activity of general proteinases and significant decreasing of general proteinases inhibitors maintenance and significant increase of oxidative modified proteins (Beregova et al., 2014). MSG elevate oxidative stress in liver and lead to elevation of liver enzymes, lipid peroxidation and lipid profile (EL-Kholy et al., 2018). A study showed that the neonatal treatment of MSG induces obesity, elevated insulin secretion and acetylcholinesterase level. MSG also increases the activation of parasympathetic tone, which cause hyper secretion of insulin from the pancreas (Balbo et al., 2000).

MSG increases insulin resistance and thereby increases blood insulin level, blood glucose plasma levels and also an increase in the plasma total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and free fatty acid levels. It also reduces the cortisol induced lipolysis (Zhang et al., 2010). Monosodium glutamate induced obese rats displayed adipose tissue hypertrophy, elevated levels of insulin, leptin and slightly elevated serum glucose. The condition which causes increased blood sugar level and insulin level is called as insulin resistance, may be it is because of the activation of phosphotyrosine protein dephosphorylation and reduced level of protein tyrosine phosphorylation, which reduces the development of Gαi2 G-protein, i.e., essential for the activation of insulin signaling to GLUT4 and transportation of glucose (Baculikova et al., 2008). Another study evidenced that an intravenous administration of MSG produces obesity, increased blood glucose, insulin, total cholesterol and

triglyceride levels (Nagata et al., 2006), but it did not show any polyphagia which may be due to the excessive stimulation of the vagus nerve (Balbo et al., 2000). Neonatal administration of MSG produces hypothalamus induced obesity (Braga et al., 2001). Another study showed that 4 g MSG treated group showed an increase in the body weight, but mild doses did not show any increase in the body weight (Sari et al., 2018).

Other systemic effects

A bilateral synchronization of arcuate neurokinin B (NKB), kisspeptin or dynorphin neuron signaling in vital segments of the reproductive axis and production of Gonadotropin-releasing hormone (GnRH).Krajewski and co-workers (2010) demonstrated that MSG decimate the neurons present in the arcuate nucleus project to GnRH terminals in the median eminence of the rat and diminishes the expression of NKB, kisspeptin, dynorphin, NK3 receptors and estrogen receptor α (ERα). Das and Ghosh (2010) reported that a subcutaneous injection of 2 g MSG/ Kg body weight in newborns of Swiss Albino mice cause degeneration of spermatogenic cells and hypertrophied Leydig cells. The seminiferous tubules and interstitial tissues were filled with a hyaline material, necrotic and exfoliation of spermatocytes and spermatids with pyknotic nuclei, congested blood vessels and vacuolar degeneration was also observed. MSG caused an elevation in lipid peroxidation level along with significant decline in SOD, CAT as well as GPx activities in testis tissues. It is possibly due to the damage of basomedial hypothalamic nucleus and associated disruption of the hypothalamic–pituitary–gonadal axis leading to low

serum luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and progesterone level, while it is more prone in males than females (Sun et al., 1991**;**Das and Ghosh, 2010**;** Alalwani, 2014; Hamza & AL-Harbi, 2014; Igwebuike et al., 2010). The ovary of MSG-treated rats (4 mg/g body weight) had a vacuolated and clustered cortical stromal cells with reduced number of primary follicles, secondary follicles and Graafian follicles. Graafian follicles are distorted and the several other follicles were atretic with collapsed oocytes, disorganized follicular granulosa cells, pyknotic nuclei and vacuolated cytoplasm, exfoliatedzonagranulosa cells with congested and hypertrophied medullary blood vessels. Functioning of uterus is also impaired (Ahmed, 2011; Ali et al., 2014**;**Mustafa, 2015**;** Mondal et al., 2018**).**

Destruction of hypothalamic nucleus by the neonatal MSG administration also cause dysfunctioning of hypothalamic–pituitary-thyroid axis and characterized reduction in growth, obesity and hypogonadism(Miskowiak and Partyka, 1993). The histological changes of thyroid tissue including compressed follicular epithelium and enlarged follicle with the accumulation of colloid, suggested hypothyroidism (Dhindsa et al., 1981). In addition to that in 2015, Khalaf and Arafat explained that neonatal administration of MSG also cause decreased follicular diameter, irregularity in follicular shape with discontinuity of basement membrane, follicular hyperplasia with multiple pyknotic nuclei, exfoliated cells in the colloid, dilatation of rough endoplasmic reticulum, increased lysosomes and vacuolation of mitochondria with lost apical microvilli of follicular cells of thyroid

tissue. Neonatal subcutaneous injection of MSG at a dose of 4 g/ Kg body weight were more vulnerable to develop anxiogenic and depressive like behaviors in rodents, which might be the direct result of hypothalamic pituitary adrenal (HPA) deregulation and malfunctioning of serotonergic system in the brain. It also damages the micro-architecture of zonafasciculata (ZF) of adrenal gland (Seo et al., 2010; Quines et al., 2014; El-Helbawy et al., 2017).

Ataseven et al., (2016) explained the genotoxic effect of MSG on human peripheral blood lymphocytes. The team observed that MSG significantly and dose dependently increased the frequencies of chromosome aberrations (CAs), sister-chromatid exchanges (SCEs), cytokinesis-blocked micronucleus (CBMN) and DNA damage in all treated groups. MSG intoxication cause significant reduction in haemoglobin (Hb), red blood cell (RBC) and white blood cell (WBC) without alteration in packed cell volume (Egbuonu and Opara, 2017; Mohammed, 2017).

A study conducted in Saudi Arabia by El-Mawla and Osman, (2011) showed that 6 mg MSG/ Kg body weight has the capacity to cause damage like hypertrophy and degeneration of epithelial cells of renal tubules with infiltration of mononuclear cells and enlarged vascular glomeruli. Light microscopic examination of the cortex of kidney revealed severe dilatation of Bowman's capsule and shrinkage of glomeruli, loss of brush border of proximal convoluted tubules and dilatation of both proximal and distal convoluted tubules were noticed. The electron microscopic examination revealed that there was partial loss of brush border of proximal convoluted tubules with destruction of

most cytoplasmic organelles and thickening of basement membrane, lumen of renal tubules were filled with cell debris and the nuclei of podocytes and endothelial cells were condensed and irregular with thickened basal lamina along with increased lipid peroxidation and altered antioxidant system (Paul et al., 2012a; Afeefy et al., 2012). Another study showed that neonatal MSG induced hypertension in obese rats was due to the augmented renal sympathetic activity (da Silva Mattos et al., 2012). Another study revealed that MSG reduce the elimination of sodium, potassium and water through urine, which causes hypertension accompanied by increased oxidative stress, increased lipid peroxidation, glutathione peroxidase, glutathione reductase, superoxide dismutase, decreased reduced glutathione, reduced nitric oxide excretion and histopathological changes in kidney (Contini et al., 2012).

Hassan et al., (2014) reported that consumption of MSG distorted cytoarchitechure of thymus and spleen associated with increase in serum interleukin -1, lipid peroxidation, reduce CD3-positive T-lymphocytes, serum interleukin -10, glutathione, catalase and superoxide dismutase level on the thymus and spleen of adult rats. These changes alter the functioning of thymus and spleen and cause immunotoxic effects. However, these toxic effects are reversible though the normal structure of the spleen and thymus would need time to regain.

Chronic oral administration of MSG at a dose of 4g/ Kg body weight induced oxidative stress with associated increase in lipid peroxidation markers such as malondialdehyde and conjugated diene.

It also exhibits a decline in oxidative enzymes superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase and glutathione-S-transferase in cardiac tissue. Cyto-architectural changes in cardiac muscles, vascular congestion associated increase in cardiac markers such as aspartate transaminase, creatine phosphokinase and lactate dehydrogenase in serum indicate severe cardiac toxicity (Paul et al., 2012b). Sakr, (2004) reported that oral gestational MSG administration cause atrophy and deterioration of myocardial fibers, narrowing of the lung airways, thickening of the alveolar walls, collapsing of the alveoli and damage of type I and II pneumocytesin fetal mice. Okon et al., (2013) reported that administration of MSG in adult increased lipid peroxidation and hypertrophy. The neonatal administration of MSG in Male *Wistar* rats displayed higher Lee obesity index, fat accumulation, dyslipidemia and insulin resistance at its sixteen week of age. Additionally increased norepinephrine (NE), reduced acetylcholine (ACh), reduced prostacyclin/ tromboxane ratio, reduced nitric oxide (NO), increased reactive oxygen species (ROS) generation, increased protein expression of endothelial nitric oxide synthase (eNOS) and cyclooxygenase (COX)-2 in mesenteric arterioles (Lobato et al., 2011).

Glutamate

Glutamate, one of the most common amino acids found in nature, is present in many proteins and peptides and most tissues. It is a non-essential amino acid that can be synthesized in the body through distinct metabolic pathways. Human digestive tract metabolizes added glutamate (food additives) in the same manner as it metabolizes

glutamate found naturally in many foods. Glutamate released from food in the intestine is absorbed from the lumen by the enterocytes, a chief oxidative substance of intestinal mucosa. Inside the cell, this glutamate is utilized for the synthesis of L-proline, L-arginine, α-keto-glutarate and ammonia fixation. It is a major source of energy, carbon (C) and nitrogen (N) for cells. In the GI tract, glutamate enhances gastric exocrine secretion, secretion of mucus from duodenum to protect intestinal wall against gastric acid attack, activates the brain nuclei related to appetite, thermoregulation, memory and gut function *via*vagus afferent pathway. Furthermore, it is an excitatory neurotransmitter in brain and the precursor molecule for both inhibitory neurotransmitter gama-aminobutyric acid (GABA) and antioxidant tripeptide glutathione. Glutamate plays a central role in learning and memory in the CNS through different signaling pathways associated with different glutamate receptors including AMPA and NMDA. Alterations in glutamate metabolism or glutamate metabolizing enzymes associated with Alzheimer's disease, coronary heart disease, hyperinsulinism, hyperammonemia, certain type of seizures and accelerated proliferative changes in cancer cells are also observed (Jinap and Hajeb, 2010; Yelamanchi *et al*., 2016).

Plasma glutamate concentration is about 50–100 µmol/ L. In brain it is about 10,000–12,000 µmol/ L, however it is merely 0.5–2 µmol/ L present in extracellular fluids (ECFs). A low extracellular glutamate concentration is very vital for the functioning of brain and it is maintained by the neurons, astrocytes and the blood-brain barrier (BBB). Blood brain barrier is a highly selective barrier surrounding

the entire nervous tissue and separating it from blood. It is made up of several layers consisting capillary endothelial cells, a basement membrane and astrocyte processes. Blood brain barrier is impermeable to glutamate even at high concentrations, except in some areas where they have fenestrated capillaries called circumventricular organs. The surface close to brain tissue (abluminal) of the BBB contains Na^+-dependent glutamate co-transporters (EAATs) and the surface near blood vessels (luminal) contains facilitative transporters. Na^+-dependent glutamate co-transporters rely on sodium gradient and it is maintained by Na^+/K^+-ATPase. When glutamate concentration increases above the normal level in the brain ECF, the abluminal membrane of the BBB transports glutamate into the endothelial cells and from the endothelial cells to blood through luminal membrane by facilitative transporters (Hawkins, 2009).

Glutamate receptors

Glutamate is one of the most abundant excitatory neurotransmitters of the brain and plays an important role in learning and memory acting through glutamate receptors. Glutamate receptors are numerous and highly complex receptors and mediate fast excitatory synaptic transmission in neuronal and non-neuronal cells of mammalian central nervous system. They are mediated through glutamate in learning and memory through plasticity or modification of channel properties. They are classified into ionotropic receptors and metabotropic receptors. Ionotropic receptors are attached to membrane ion channels and the metabotropic receptors are attached to intermediary compounds, including G protein and intracellular second

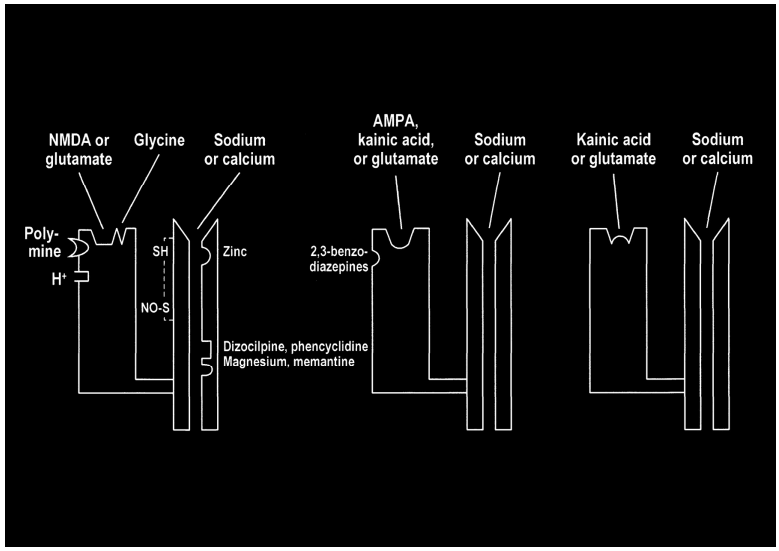
messengers, such as inositol-1,4,5-trisphosphate (IP3), calcium and cyclic nucleotides (Mark *et al*., 2001). Each ionotropic or metabotropic receptor has three types and each type has multiple subtypes summarised in table 1.1

Table 1.1Subunits and properties of Glutamate receptors.

Receptor	Protein Subunit	Receptor Properties
Ionotropic Receptors		
NMDAR	NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B	Heterotetrameric; calcium permeability high; long channel open time
AMPAR	$GluR_1$, $GluR_2$ edited, $GluR_2$, $GluR_3$ and $GluR_4$	Heterotetrameric; calcium permeability low if edited $GluR_2$, otherwise moderate; short channel open time
Kainate receptor	$GluR_5$, $GluR_6$, $GluR_7$, KA1 and KA2	Homotetrameric or heterotetrameric; calcium permeability low; short channel open time
Metabotropic Receptors		
Group 1	$mGluR_1$ and $mGluR_5$	Homodimeric; signals *via* phospholipase C; located post-synaptically
Group 2	$mGluR_2$ and $mGluR_3$	Homodimeric; signals *via* adenylyl cyclase; located mostly pre-synaptically; agonists and antagonists mostly distinct from Group 3
Group 3	$mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$	Homodimeric; signals *via* adenylyl cyclase; located mostly pre-synaptically; agonists and antagonists mostly distinct from Group 2

(Kew and Kemp, 2005; Pleasure, 2008; Altevogt *et al*., 2011)

The ionotropic glutamate receptors are ligand-gated ion channels subdivided into NMDA receptors, AMPA receptors and kainate receptors named according to the agonists that activate them: NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainic acid. After glutamate binds to an ionotropic receptor, the receptor's channel undergoes a conformational change to allow an immediate influx of extracellular sodium and an efflux of potassium ions. This triggers membrane depolarization in the post-synaptic cell sufficient to induce signal transmission (Altevogt *et al*., 2011).



(Mark *et al*., 2001)

Figure 1.2: Schematic representations of NMDA, AMPA, and kainate receptors as receptor–channel complexes.

NMDA receptor, one of the main glutamate ionotropic receptor is permeable to calcium ions in addition to sodium and potassium ions; calcium ions have both beneficial and toxic effects. As a result, EPSPs

(excitatory post synaptic potential) produced by NMDA receptors can increase the concentration of Ca^{2+} within the postsynaptic neuron; the Ca^{2+} concentration change can then act as a second messenger to activate intracellular signaling cascade. The activation of NMDA receptor also requires the presence of a co-agonist (the amino acid glycine) and that extracellular Mg^{2+} blocks the channel at hyperpolarized, but not depolarized, voltages. NMDA receptors are present on both neurons and astrocytes, but they are highly expressed on astrocytes (Purves *et al*., 2001; Traynelis *et al*., 2010).

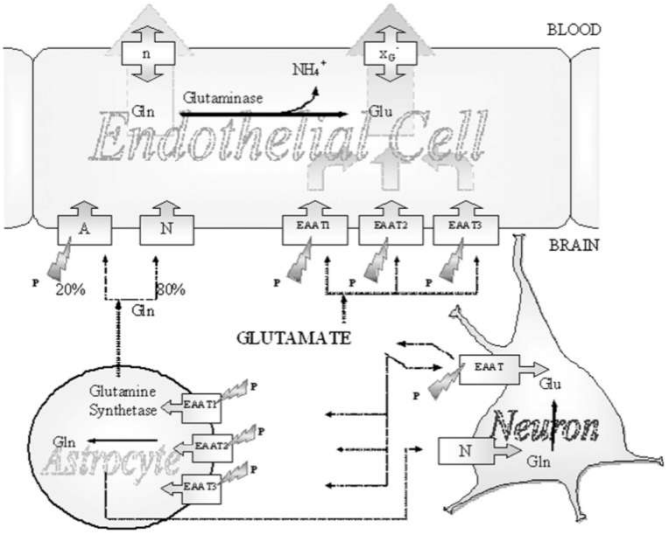
Metabotropic glutamate receptors are slower acting; they exert their effects indirectly, typically through gene expression and protein synthesis. There are three types of metabotropic glutamate receptor and subdivided into a total of eight receptors. Group I metabotropic receptors are mainly in postsynaptic membrane and are associated with addiction, motor regulation, learning and memory. Group II metabotropic receptors are present in both pre and post synaptic cells, possibly to suppress glutamate transmission and implicated in anxiety, schizophrenia and Alzheimer's disease. Group III metabotropic receptors, like group II are found within the hippocampus and hypothalamus and may play a role in Parkinson's disease and anxiety disorders (Swanson *et al*., 2005; Niswender & Conn, 2010; Altevogt *et al*., 2011).

Glutamate transporter

Glutamate transporters are a family of neurotransmitter transporter proteins that move glutamate, the principal excitatory

neurotransmitter, across a membrane. The family of glutamate transporters is composed of two primary subclasses: the excitatory amino acid transporter (EAAT) family and vesicular glutamate transporter (VGLUT) family. In the brain, EAATs remove glutamate from the synaptic cleft and extrasynaptic sites *via* glutamate reuptake into glial cells and neurons, while VGLUTs move glutamate from the cell cytoplasm into synaptic vesicles. The glutamate transporters on glial cells are primarily responsible for maintaining extracellular glutamate concentrations. Five mammalian EAATs have been cloned and characterized: EAAT1, EAAT2, EAAT3, EAAT4 and EAAT5. In the CNS, while EAAT1 and EAAT2 are found primarily in astrocytes, EAAT3 and EAAT4 are found principally in neurons. EAAT5 is expressed primarily in the retina. These receptors depend upon the Na^+ and K^+ concentration and are called Na^+-dependent glutamate transporters. The EAAT2 may be responsible for 80– 90 % of all extracellular glutamate uptake activity in the central nervous system (CNS). Three types of vesicular glutamate transporters are known, VGLUT1, VGLUT2 and VGLUT3 found on neurons. These transporters pack the neurotransmitter into synaptic vesicles and is dependent on the proton gradient (Dingledine and McBain, 1999; Lin *et al*., 2012).

Under normal conditions, glutamate is recycled continuously between neurons and glia known as the glutamate-glutamine cycle. Excess glutamate in the synapse is taken up by glial cells *via* EAAT transporters, where it is converted to glutamine with the help of an enzyme glutamate synthase.



(Hawkins, 2009; Hawkins*et al*., 2006)

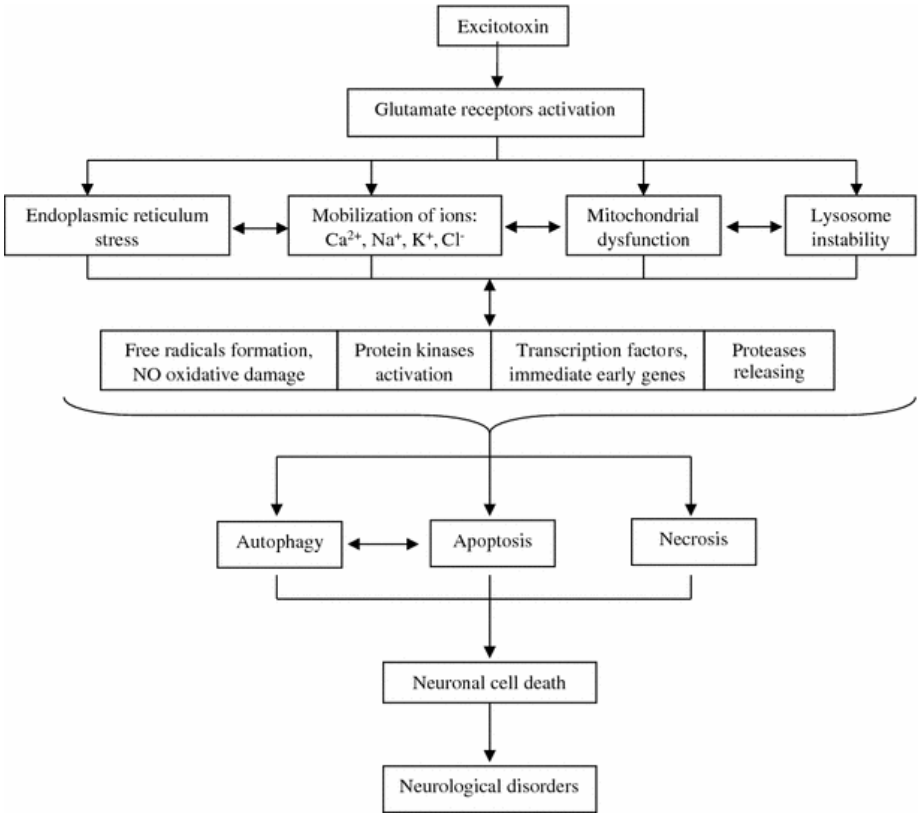
Figure 1.3: Glutamate and glutamine transport between neurons, astrocytes, and endothelial cells of the blood-brain barrier. (EAATs: Na+-dependent glutamate transporters; A and N: facilitative glutamate transporter; n, X_G and the lightning symbols:Na+ dependence and P: stimulation by pyroglutamate).

Glutamine is then transported back into neurons, where it is reconverted to glutamate. However, glial cells, under certain pathological conditions such as brain damage and stroke, or reduced oxygen availability may cause the release of glutamate by reversal of uptake *via* glutamate transporters (Rothman *et al*., 2003; Malarkey and Parpura, 2008; Grewer *et al*., 2008; Altevogt *et al*., 2011).

Excitotoxicity

Glutamate is an excitatory neurotransmitter abundantly presents in the brain and has a wide role in its function. Disturbance in glutamatergic system may lead to impaired cognitive functioning, psychological and neurodegerative disorders. Excessive release of

glutamate into the extracellular fluid from neurons or astrocytes causes excessive stimulation of glutamate receptors (ionotropic and metabotropic receptors) especially NMDA receptors. Excessive stimulation of glutamate receptors causes high influx of calcium ions into the cytosol along with sodium ions. It also causes depolarization of the neuronal membrane and activation of voltage gated Ca^{+} channels and Ca^{+} influx. As a result, a very high calcium load is generated in the cytosol and triggers an excitotoxic event. Apart from this, cytosolic calcium load also trigger calcium release from mitochondria and endoplasmic reticulum (Ezza and Khadrawyb, 2014).



(Wang and Qin, 2010)

Figure 1.4: Mechanisms of excitotoxicity.

This elevated calcium level proceeds to over activate a number of enzymes which cause neuronal death. The enzymes including, protein kinase C, calcium/cadmodulin-dependent protein kinase II, phospholipases, proteases, phosphatases, nitric oxide synthase, endonucleases and ornithine decarboxylase (Wang and Qin, 2010). Activation of phospholipase A would generate platelet-activating factor and arachidonic acid and its metabolites. Platelet-activating factor activates more glutamate release and arachidonic acid inhibit re-uptake of glutamate from ECF, lead to over stimulation of glutamate receptors and excitotoxic effect. Arachidonic acid also generates oxygen free radicals and phospholipase A, cyclooxygenase-2 and lipoxygenases, which activate calcium influx and excitotoxic events. Activation of nitric oxide synthase generate nitric oxide, which produce superoxide ions and peroxynitrite, which is extremely toxic, resulting in neuronal death (Gagliardi, 2000). It also causes DNA damage, inhibits mitochondrial respiration, production of free radical and additional depolarization leads to activation of the neurotoxic cascades and neuronal death. This kind of neuronal death is implicated in many neurodegenerative disorders including Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, AIDS dementia complex and Parkinson's disease (Mark *et al*., 2001; Mattson, 2003; Ezza and Khadrawyb, 2014).

Hippocampus

The hippocampus is a part of archicortex and major part of the brain situated bilaterally below the cerebral cortex, and in humans it is in the temporal lobe of the cerebral cortex. It is a part of the limbic

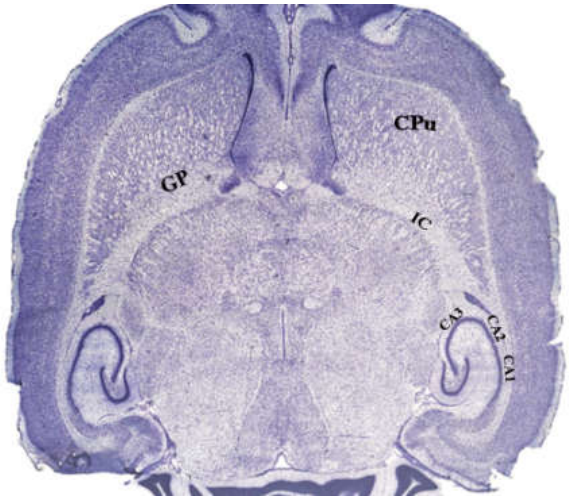
system, structures that line the edge of the cortex, called a primitive brain, concerned with the consolidation of memory, reward and punishment system, mood changes, motivation etc (Wright, 2017). Hippocampus contains two interconnected parts Cornuammonis (CA) or hippocampus proper and dentate gyrus separated by hippocampal sulcus and curve. Hippocampus and some other structures together iscalled hippocampal formation. It includes hippocampus proper (Cornuammonis; CA), dentate gyrus (DG), subiculum and entorhinal cortex (EC) (Anand and Dhikav, 2012). Based on the histoarchitecture, hippocampus proper (Cornuammonis) is divided into CA1, CA2, CA3 and CA4 area, which surrounds the dentate gyrus. Subiculum is placed just opposite to the CA1, which connects hippocampus with entorhinal cortex in the ventricle. The upper surface of hippocampus includes dentate gyrus, subiculum and entorhinal cortex and the lower surface is expanded like a paw called pes hippocampi. Hippocampus consisted of a central layer of pyramidal cells and on either side is covered by plexiform layers (Last, 1999;Standring, 2003). The cytoarchitecture of the hippocampus is more complex and most extensively been studied part in neurophysiology. Long-term potentiation (LTP), the cellular basis of memory storage was first discovered from this region (Amaral and Lavenex, 2007; Anderson *et al*., 2007).

Hippocampus has a lot of sensory and motor connections from different parts of the brain through perforant path. The perforant pathway is a connectional route originating from the entorhinal cortex to CA regions, dentate gyrus and the subiculum responsible for pattern

recognition and encoding of memories. It is originated from II and III layers of entorhinal cortex with few fibers from deep layers V and VI. Axons from layer II and IV project to granule cells and pyramidal cells of CA3. Axons from III and IV project to pyramidal cells of CA1 and subiculum through the temporoammonic (TA-CA1) pathway. TA-CA1 neurons of perforant path play a crucial role in spatial memory consolidation. Lesions or insults of this pathway have been implicated in stress-based animal models of depression and temporal lobe seizures. The retrieval of memory is achieved through the same pathway originating from entorhinal cortex to CA3 layer and CA1 (III of the perforant path) (Amaral and Lavenex, 2007; Anderson *et al*., 2007).

Shaffer's collaterals are axon branches arising from CA3 to CA1 region, a crucial part of the hippocampal tri-synaptic loop, plays an important role in memory formation and emotional networks. Another pathway called Recurrent Collaterals excites CA3, area which is vital for holding memory. Another route connecting dentate gyrus plays a role in the processing of information from entorhinal cortex to CA3 and pattern separation and associative memory (Anand and Dhikav, 2012).

Hippocampus is vital for learning, memory and spatial navigation and approach-avoidance conflict processing. It is connected to ventral striatal loop and participated in motor behavior and regulate the release of adrenocorticotropic hormones by the connections with the hypothalamus (Matsumura *et al*., 1999; Gluck *et al*., 2003; O'Neil *et al*., 2015).



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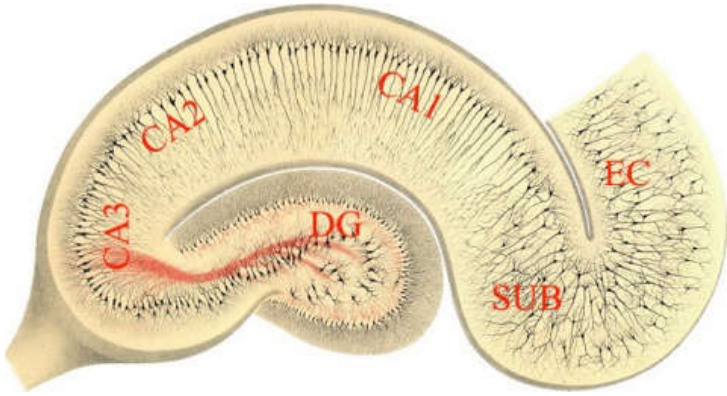

b (Paxinos and Charles, 2007)

Figure 1.5: Diagram of (a)striatum and (b) hippocampal region of brain. (CPu: caudate putamen (striatum), GP:globuspallidus, IC: internal capsule, CA1, CA2 and CA3 regions of hippocampus, DG:dentate gyrus, SUB: subiculum and EC: entorhinal area).

Corpus Striatum

The corpus striatum or striatum is the largest subcortical grey matter complex of basal ganglia responsible for the control of voluntary movements, cognition and reward system of the body (Hikosaka *et al*., 2000; Taylor *et al*., 2013; Yager *et al*., 2015). Anatomically it is a striped or striated grey and white matter structure composed of a ventral and dorsal striatum. The ventral striatum is again divided into nucleus accumbens and the olfactory tubercle and the dorsal striatum divided into caudate nucleus and the putamen by a white matter tract called internal capsule (Ferré *et al*., 2010). But in non-primates, the striatum is divided into caudate nucleus and the lentiform nucleus and the lentiform nucleus is again divided into an outer putamen and an inner globuspallidus(Telford and Vattoth, 2014).

Striatum contains three types of neurons namely medium spiny neurons (MSN), cholinergic interneurons and GABAergic interneurons. Medium spiny neurons are the major inhibitory GABAergic neurons in the striatum (Wilson, 1998; Tepper and Bolam, 2004; Nishi *et al*., 2011; Yager *et al*., 2015). Cholinergic interneurons are acetylcholine releasing neurons which has a strong relationship to the reward and punishment system (Apicella *et al*., 1991; Ravel *et al*., 2003). GABAergic interneurons are divided into many types and among these fast-spiking interneurons are well known for the inhibition of MSN and concerned in learning and reward prediction error coding (Tepper *et al*., 2010; Stalnaker *et al*., 2012; Schulz & Reynolds, 2013; Báez-Mendoza and Schultz, 2013).

The striatum has two main efferent pathways, the direct pathway and indirect pathway. The direct pathway comprises the axons of medium spiny neurons with D1 receptors extended into GABAergic neurons in the substantianigra pars reticulata (SNr) (Parent *et al*., 1984). The indirect pathway is formed by MNS neurons with D2 receptors projected to an external segment of globuspallidus (GPe) and then to internal globuspallidus (GPi). A subpopulation of MNS in the striatum has both D1 and D2 receptors (Parent and Hazrati, 1995; Nishi *et al*., 2011; Yager *et al*., 2015).

Functional and anatomical evidence suggests that the activities of striatum forms a limbic-motor interface in the brain and integrate information about the reward with motor information to guide the behavior (Mogenson *et al*., 1980; Hollerman *et al*., 1998; Schultz and Dickinson, 2000). The ventral striatum is primarily involved in reward cognition, planning, decision making reinforcement and motivation, whereas the dorsal striatum is involved in cognition involving motor function, certain executive functions through inhibitory activities and stimulus-response learning. The interconnections between the striatum and the prefrontal cortex regulate the behaviour particularly in the adolescent development (Hollerman *et al*., 2000; Steinberg, 2010; Yager *et al*., 2015).

Ayurvedic spices

Plant based products have been used for human welfare right from the dawn of history. The traditional remedies of the ancient world were all based on natural products. Herbs and spices have been

used throughout history for flavoring a variety of dishes or folk medicine in different cultures and regions. India is known as the home of spices. From the ancient times India has been a hub of spice cultivation, processing and export. Apart from adding colour, flavor, taste or other culinary art, consumption of spices provide infinite health benefits. In Ayurveda, spices are used in many combinations to balance different kinds of *dosha* and generate digestive fire, which is imperative for proper digestion. It intensifies salivary flow, antimicrobial and prevents dental caries, reduce respiratory ailments, boost immunity, aid in weight loss, reduce pain and inflammation, reduce the chance of stroke and maintain blood pressure. It is also a substitute for costly beauty products, perfumes and even medicines (Marisa, 2016; Dubey, 2017).

***Myristica fragrans*Houtt.**

Myristica fragransHoutt. (Nutmeg tree) is a tropical evergreen dioecious tree, which is a native of Molucca (Banda) island of East Indonesia known as "spice island" and is also now cultivated in Grenada in West Indies, Philippines, Sri Lanka, Africa, India, Fiji and Malaysia. In India, it is mainly cultivated in certain areas of Kerala, Tamil Nadu, Karnataka, Goa, Maharashtra, Andaman Islands,Assam and Meghalaya.It belongs to a small primitive family Myristicaceae with about 18 genera and 300 species. It yields the spices Nutmeg and Mace, highly coveted for their aromatic, culinary and medicinal properties and has a rich diversity of phytochemicals. The vernacular names of the tree are Jatiphala (Sanskrit), Jaiphal (Hindi),

Jadipatri(Malayalam and Tamil) (Krishnamoorthy *et al*., 2000; Khan and Abourashed, 2011).

Taxonomy

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Magnoliidae
Order	:	Magnoliales
Family	:	Myristicaceae
Genus	:	MyristicaGronov.
Species	:	MyristicafragransHoutt.

(USDA, 2018)

Nutmeg and Mace

Nutmegs are the seeds inside the fruits of *M. fragrans*. They are oval, about 25 mm (1 in) in length, lightly wrinkled and dark brown on the outside and lighter brown on the inside. When broken, it presents a yellowish surface, with dark branching veins, in which volatile oil abounds.

Mace is the aril, bright red, fleshy and branched lacy covering of the nutmeg. The bright scarlet covering, in the process of drying, is converted to yellow, orange-yellow, or golden yellow.

The bright scarlet colour of the mace is because of the bioflavonoid lycopene, a dominant carotenoid in human blood. Mace has a pleasant aroma and a warm, bitterish, moderately pungent taste.

Figure 1.6: Nutmeg and mace from *Myristica fragrans*Houtt.

Nutmeg and mace are valued kitchen spice that has been used for centuries all over the world.In India it is used in some exotic meat and poultry dishes, especially Mughali type and in meat, pulaos and biriyanis and also in milk sweets and confectioneries. In other countries, it is also used for flavoring baked foods, cakes, cookies, puddings, pickles, ketchup, cheese, sauces, liquors, canned soups, season cabbage, poached fruits, meat dishes, sea foods etc. Apart from cooking, it is used as a natural preservative in order to extend the shelf life. It is also used in cosmetics and pharmacy (Prajapathi*et al*., 2003; Pimenta, 2003; Nybe *et al*., 2007; Šojić *et al*., 2015).

Chemical composition

The constituents of nutmeg and mace are almost similar but their proportions are different. Nutmeg contains 6 – 16 % volatile oil and 25 - 40 % fixed oil and mace contain 4-15 % essential oil and about 30 % fixed oil. The main constituents of *Myristica fragrans*have

been found to be alkyl benzene derivatives (myristicin, elemicin, safrole etc.), terpenes, alpha-pinene, beta-pinene, myristic acid and trimyristin. Nutmeg essential oil is mostly composed of terpene hydrocarbons (sabinene and pinenes; further more camphene, p-cymene, phellandrene, terpinene, limonene and myrcene), terpene derivatives (linalool, geraniol, terpineol and phenylpropanoids (myristicin, elemicin, safrole, eugenol and eugenol derivatives). Oil of mace contains the same aroma components but the total fraction of terpenoids is very high. Both nutmeg and mace contain lignans (diarylpropanoids), which are nonvolatile dimers of phenylpropanoid constituents of the essential oil (eg. Dehydrodiisoeugenol). The main glycoside is trimyristin having anxiogenic activity (Qiu*et al*., 2004; Asgarpanah and Kazemivash, 2012; Abourashed and El-Alfy, 2016).

Pharmacological studies

M. fragrans is a popular herbal plant and a folk medicine; possess a spectrum of pharmacological activities. It is not native of India but both nutmeg and mace are frequently mentioned in Ayurvedic literature. For a long time, China, Arab and African countries have been using*M. fragrans* as a traditional medicine for the treatment of appetite, diarrhea, rheumatism, muscle spasm, rheumatism, stomach and kidney disorders. Recently it has been investigated for antioxidant, anticonvulsant, analgesic, carminative, insomnia, anti-inflammatory, antipyretic, aphrodisiac, abortifacient, anti-rheumatoid, antiflatulant, antidiabetic, hypolipidemic, hypocholesterolemic, antibacterial and antifungal. It is also used for the management of menorrhagic pains, hemorrhoids, dysentery,

impotence, stomach ache, malaria, bad breath, nausea and vomiting etc. (Somani*et al*., 2008; Asgarpanah and Kazemivash, 2012; Paul *etal*., 2013; Dewi *et al*., 2015; Abourashed and El-Alfy, 2016; Nagja *et al*., 2016; Loizzo *et al*., 2017).

Recent studies show that *M. fragrans* volatile oil has the ability to increase brain monoamine neurotransmitters such as serotonin, norepinephrine and dopamine in the rat hippocampus, which are very crucial for the regulation of cognitive processes such as emotion, arousal and certain types of memory (Plaingam *et al*., 2017). Dhingra *et al*., (2006) revealed that oral administration of 5 mg/ Kg of hexane extract of *M. fragrans* in a Swiss male young albino mouse significantly reduces the acetylcholinesterase (AChE) activity and thereby enhance the learning and memory process by increasing the level of acetylcholine. Mukherjee *et al*., (2007) also showed that hydroalcoholic extract of *M. fragrans*suppressed the AChE activity in a hippocampal culture at an IC_{50} value of 133.28 ± 11.26. Another study reported that nutmeg extract inhibitsAChE activity in penis and thereby increasing the activity of acetylcholine and acetylcholine positive fibers in penis (Odubanjo *et al*., 2018). Wahab *et al*., (2009) observed that the volatile oil of nutmeg may be effective against grandmal and partial seizures, as it acts as an anticonvulsant and prevents seizure spread in a set of established animal seizure models.

Jin *et al*., (2005) reported that lignan isolated from mace treated with murine hippocampal cell line and primary culture of rat microglial cells displayed a down regulation of cyclooxygenase-2 and suppress the production of nitric oxide synthase, inhibit the activity

and synthesis of pro-inflammatory cytokines including tumor necrosis factor-α and interleukin-6. The suppression of such enzymes by mace lignan opens a new therapeutic window to the neurodegenerative diseases associated with oxidative damage and neuro-inflammation. Parle *et al*., (2004) explained that 5 mg/ Kg body weight of *M. fragrans* extract has the capacity to improve learning and memory in young and aged mice and they also showed that this extract has the capacity to reverse the effect of scopolamine and diazepam induced learning and memory impairments in young mice. This memory enhancing activity may be due to the wide range of activity attributed to the plant including antioxidant, anti-inflammatory and procholinergic activity.

The acetone, ethanol, methanol, butanol and water extracts of *M. fragrans*seed possess antioxidant, hypolipidaemic and antimicrobial activity. Among all these extracts, acetone extract has shown the highest antioxidant activity. They are excellent in scavenging radicals, reducing metal ions and inhibiting lipid oxidation (Capasso*et al*., 2000; Kapoor *et al*., 2013; Gupta *et al*., 2013). The nutmeg essential oil also showed anti-angiogenic activity and antioxidant properties (Piaru *et al*., 2012a). It prevents lipid peroxidation in chicken tissue homogenates and egg yolk (Damien *etal*., 1995). It is also reported that antioxidant and hypolipidaemic effects of *M. fragrans* extracts are mainly due to the presence of myristicin (Yadav and Bhatnagar, 2007; Rahman *et al*., 2018).

Ethanol extract of mace at a concentration of 100 µg/ ml has a high potent effect (70 %) on growth inhibition against human cancer

cell line. The methanol extract of *M. fragrans*displayed anti proliferative activity on human tumor cells, suppress the growth of human lymphoid leukemia Molt 4B cells, anti-carcinogenic activity and cell death of jurkat leukemia T cell line by SIRT 1 mRNA down regulation (Jannu *et al*., 1991; Moteki *et al*., 2002; Murcia *et al*., 2004; Lee *et al*., 2006; Dzotam *et al*., 2018). Park *et al*., (1998) reported that *M. fragrans* extract has the capacity to suppress various *in vitro* cancer cells and protected bone marrow from genotoxicity in male *Wistar albino* mice (Kumari, 1992). Thuong *et al*., (2014) explained that lignans isolated from mace has the capacity to suppress tumor growth in allogenic tumor-bearing mice model and cytotoxic activity against different cancer cell lines. The essential oil obtained from the*M. fragrans* seed, especially myristicin, showed a significant inhibitory activity on the growth of human colorectal carcinoma (HCT-116), human breast carcinoma (MCF-7) (Piaru *et al*., 2012b), a Caco-2 colon cancer cell line (Piras *et al*., 2012) and cytotoxic and apoptotic effects in human neuroblastoma SK-N-SH cells (Lee *et al*., 2005). Myristicin also has anti-inflammatory properties by inhibiting the production of calcium, nitric oxide, cytokines, interleukins (Lee & Park, 2011). Zheng *et al*., (1992) reported that Myristicin present in the essential oil of mace is responsible for the potent chemoprotective effect of *M. fragrans*. The mace of *M. fragrans* also significantly protected mice from methylcholanthrene induced carcinogenesis in the cervix (Hussain and Rao, 1991) and dimethylbenzanthracene induced papilloma genesis in the skin of mouse (Jannu *et al*., 1991).

Lignan from mace also reported its potent effect on anti-bacterial, anti-inflammatory, anticancer, anti-diabetes, hepatoprotective and neuroprotective activities (Yang *et al*., 2006; Paul *et al*., 2013). Oseni and Idowu, (2014) showed that water extract of nutmeg enhances GSH, GPx, catalase and SOD level, while lipid peroxidation and blood glucose level is declined in alloxan induced diabetic rats. Patil *et al*., (2011) reported that regular use of nutmeg and mace enhances insulin secretion and thereby maintain the blood glucose level. It also prevents elevation in glucose levels by inhibiting intestinal alpha-glucosidase. Nguyen *et al*., (2010) reported that nutmeg and its active constituents stimulate AMP-activated protein kinase (AMPK), a metabolic enzyme enhances glucose intake by cells and insulin secretion from pancreatic beta cells. Therefore, it is considered as a therapeutic target for metabolic disorders such as diabetes and obesity. Anti-diabetes factors isolated from methanolic extract of *M. fragrans*, named meso-dihydroguaiaretic acid and otobaphenol, are very effective to prevent protein tyrosine phosphatase 1B and up regulation of insulin receptor, a potent target for treating type-2 diabetes and obesity (Yang *et al*., 2006). The essential oil of mace exhibits a strong anti-inflammatory, anti-pyretic, antidiarrheal, sedative and analgesic effect in rats and mice. The pharmacological mechanisms behind the anti-inflammatory effects are similar to those of non-steroidal anti-inflammatory drugs (NSAIDs) (Olajide*et al*., 2000; Grover *et al*., 2002).

Myristicin present in the volatile oil of mace exhibited significant hepatoprotective effects (Schenk and Lamparsky, 1981;

Morita *et al*., 2003; Yimam *et al*., 2016). Chhabra and Rao, 1994 reported that the principal compound present in the water extract of mace, administrated through the trans-mammary route, enhanced the hepatic glutathione peroxidase and other hepatic xenobiotic metabolizing enzymes in the liver of mouse pups and potentiate the hepatic activity. Mace is also reported to modulate glutathione-s-transferase activity, control the development of *in vitro* aflatoxins induced DNA adducts and influenced the hepatic detoxification system in mouse liver (Shin *et al*., 1988; Kumari and Rao, 1989; Hashim *etal*., 1994). Essential oil as well as chloroform extract of the seed of *M. fragrans* is reported of its anti-thrombotic effects by inhibiting platelet aggregation and anti-inflammatory effects similar to NSAID indomethacin (Janssens *et al*., 1990). This pharmacological properties was due to the presence of myristicin in the extract (Ozaki *et al*., 1989). Essential oil of nutmeg is also used externally for rheumatism, internally as a carminative (Oliver-Bever, 1986), relaxant effect on rat ileum, potentiated hexobarbital induced hypnosis like behaviour in rats (Bhagwat and Saifi, 1980) and inhibited prostaglandin synthesis in rat kidney (Misra *et al*., 1978). Extract also improve sexual activity in male rats, used for the treatment of erectile dysfunction, enhances libido and potency collectively called aphrodisiac activity. The aphrodisiac activity of mace or nutmeg extract is due to the inhibitory effect on phosphodiesterase-5, arginase, AChE, angiotensin-I converting enzyme activity (ACE) and oxidative damage in reproductive structure (Tajuddin *et al*., 2003; Tajuddin *et al*., 2005; Odubanjo *et al*., 2018).

Nutmeg and mace extracts of*M. fragrans* showed a wide spectrum of antimicrobial activity due to the presence of essential oil. It is very effective against *S. aureus*, *S. epidermis*,*S. dysenteriae*, *S. typhi* and *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*,*Brevibacterium linens*, *Enterobacteraerogenes*, *Klebsiella pneumonia*, *Micrococcus luteus*, *Proteus vulgaris*, *Yersinia enterocolitica*(Dorman & Deans, 2004; Lima *et al*., 2012; Nurjanah *et al*., 2017). *M. fragrans* extracts also showed inhibition against human Rotavirus, a major agent for diarrhea (Gonçalves *et al*., 2005) and very effective againstmultidrug-resistant (MDR) *Salmonella entericaserovarTyphi* (Rani and Khullar, 2004; Balakrishnan *et al*., 2017). Dawidowicz and co-worker (2013) reported that 3′,4′,7-trihydroxyflavone, a potent compound isolated from nutmeg is the principal compound against MDR and bactericidal activity. Methanolic extract of mace was reported to inhibit the growth of gram negative bacteria and *Helicobacter pylori*, which is a human carcinogen (Bhamarapravati *et al*., 2003; Mahady *et al*., 2005). Mace essential oil inhibits the growth of *Listeria monocytogenes* by inhibiting bacterial extracellular protein listeriolysin and bacterial enzyme phospholipase (Smith-Palmer *et al*., 2002), inhibitory effect on the fluid accumulation capabilities of enterotoxigenic*E.coli* in the ligated gut (Rashid and Misra, 1984) and nematicidal activity (Gotke *et al*., 1990). Dehydro-di-isoeugenol and 5-methoxy eugenol from mace prevent the growth of *Streptococcusmutants* in mouth, which cause dental caries (Hattori *et al*., 1993). It is also used in the food industry as a food preservative by inhibiting and killing bacterial spores and

specific meat spoilage bacteria (Valero and Salmeron, 2003; Arief and Velly, 2018).

In addition to the pharmacological effects, researchers also reported that certain active compounds present in the *M. fragrans* have toxicological effects, especially myristicin and elemicin (Sonavane *etal*., 2001). It causes the formation of DNA adducts in liver of fetal and adult mice, hypothermia, vertigo, delirium, weak pulse etc (Randerath *et al*., 1993; Hallström and Thuvander, 1997). Hallström and Thuvander, (1997) also reported the psycho pharmacological effects of nutmeg in humans, when consumed 6 -7 mg / Kg body weight or 1 -2 mg myristicin / Kg body weight, while it vary between 5 and 30 g of nutmeg in other animals (Dawidowicz and Dybowski, 2013). The psychoactive and hallucinogenic effects of *M. fragrans* are because of the metabolic conversion of myristicin and elemicin into amphetamine-like compounds (Quin *et al*., 1998; Gupta *et al*., 2013; Ehrenpreis *et al*., 2014). Because of its hallucinogenic effects, nutmeg abuse was observed in prisoners, college students and adolescents as a substitute of narcotic drugs (Stein *et al*., 2001). Amphetamines are non-catecholamine sympathomimetic amines with CNS stimulant activity. It mimics the structures of the catecholamine neurotransmitters, noradrenaline and dopamine and modulates monoamine release, reuptake and signaling within the brain (Drugbank, 2018). Currently it is used for the treatment of attention deficit hyperactivity disorder (ADHD), narcolepsy and obesity. It had been used to treat depression, stress, concentration improvement, athletic performance enhancer, cognitive enhancer, aphrodisiac and

euphoriant. Clinical studies revealed that, usage of low dose of amphetamines improve cognition including working memory, long-term episodic memory and attention in normal healthy adults. However, high doses of amphetamines may impair cognitive functioning (Amphetamine, 2018).

AIM AND OBJECTIVES OF THE STUDY

Monosodium glutamate is a sodium salt of L-glutamate, a well-known flavor enhancer and a source for the unique flavor called "*umami*". The presence of MSG in food increases the palatability or alters and magnifies the desirable taste, thus it is an essential part of human diet and commonly found in most of the Asian Western diets. Body can never discriminate the glutamate coming from the monosodium glutamate or natural foods, which are absorbed into the enterocytes from the intestinal lumen and performs various physiological functions of the body. Even though glutamate of MSG has a wide range of physiological functions, the dose dependent elevation of glutamate may be a concern to be toxic on brain cells and resultant overall lethality in the physiological systems.

So, the present study investigates the effects of ingestion of food additive monosodium glutamate on neurobehavioural, neurochemical and histoarchitectural changes in striato-hippocampal areas of the brain in male *Wistar albino* rats and its recovery. Also we examine the neuroprotective, antioxidant, euphoric and memory enhancing effects of decoction of mace of *Myristica fragrans*, a house hold spice.

The main objectives of the present study include:

1. Investigate the dose depended effects of monosodium glutamate on the neurobehavioural alterations of male *Wistar albino* rats by using the following behavioural paradigm.

- Anxiety like behaviour: Open field test, elevated plus maze test, light/dark transition test, free-exploratory paradigm and marble burying test.
- Depression like behaviour: Forced swim test and tail suspension test.
- Memory and learning: T-maze test and novel object recognition test.
- Aggression: Resident intruder paradigm.
- Social interaction: Sociability and social novelty test.
- Sensory examination: Hot plate test.
- Motor examination: Rotarod test.

2. Investigate the effects of monosodium glutamate on lipid peroxidation level, oxidative status, neurotransmitter level and histoarchitecture of striatum and hippocampal areas of the brain in male *Wistar albino* rats.
    - Lipid peroxidation: TBRSA assay and conjugated dienes.
    - Antioxidant status: Catalase, superoxide dismutase, reduced glutathione and glutathione-s-transferase.
    - Na^{+}/k^{+}-ATPase.

- Neurotransmitters: Acetylcholinesterase, Glutamate and GABA.
- Histoarchitecture by Hematoxylin and Eosin staining.

3. To determine the possible protective role of house hold spice - mace against the monosodium glutamate induced neurobehavioural alteration and oxidative damage of striatum and hippocampal areas of the brain in male *Wistar albino* rats.
   - Anxiety like behaviour: The open field test, elevated plus maze test, light/dark transition test.
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   - Antioxidant status: Catalase, superoxide dismutase and reduced glutathione.
   - Neurotransmitters: Acetylcholinesterase, Glutamate and GABA.

MATERIALS AND METHODS

Chemicals and reagents

Monosodium glutamate (MSG), Oxidized glutathione (GSSG), Nicotinamide adenine dinucleotide phosphate (NADPH), Phenazinemethosulfate (PMS), Nitrobluetetrazolium (NBT), Nicotinamide adenine nucleotide (NADH), Thiobarbituric acid (TBARS),Sodium pyrophosphate, Hematoxylinand Eosin were purchased from HiMedia Laboratories, India. 1, 1- diphenyl 2-picrylhydrazyl (DPPH), Reduced glutathione (GSH), Glutamic acid (Glu), Gamma-aminobutyric acid (GABA), Acetylthiocholine iodide, Adenosine triphosphate (ATP), were purchased from Sigma-Aldrich, India. 5, 5'-dithio-bis [2-nitrobenzoic acid] (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), 1-Amino-2-naphthol-4-sulphonic acid (ANSA) and Potassium dihydrogen orthophosphate were purchased from Sisco Research Laboratories, India. All other chemicals and reagents used were of analytical reagent grade.

Experimental animals

Male *Wistar albino* rats, weighing about 30 – 50 gm and approximately 4 weeks old, were used for the study. The rats were maintained under laboratory conditions in the animal house of the Department of Life Sciences (Reg. No: 426/ 2 CPCSEA). Rats were accommodated in polypropylene cages of dimensions 43 cm x 27 cm x 15 cm with wire mesh lids. Paper strips were used as bedding

material, which was changed every alternate day. Rats were fed with laboratory animal feed and water *ad lib*. The rooms were controlled with a 12 hour light and 12 hour dark cycle. All experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethics Committee (IAEC) of the University of Calicut, Malappuram.

Experimental design

Sub Groups		**Treatment and dosages**
Control	:	Treated with tap water
100 mg MSG	:	Treated with 100 mg MSG/ kg body weight
400 mg MSG	:	Treated with 400 mg MSG/ kg body weight
2 g MSG	:	Treated with 2 g MSG/ kg body weight
4 g MSG	:	Treated with 4 g MSG/ kg body weight

Male *Wistar albino* rats were randomly distributed into two groups namely group I and group II, which is again subdivided into control, 100 mg MSG fed group, 400 mg MSG fed group, 2 g MSG fed group and 4 g MSG fed group. MSG was dissolved in water and given by oral gavage for 60 days. Control rats were fed with tap water through oral gavage. Food and water consumption, body weight, physical and behavioural changes of all the groups were observed every day. Immediately after sixty days of MSG administration, group I rats were used for behavioural, neurochemical and histological studies while group II rats were allowed to have normal diet without

MSG for further 60 more days and later was used for the above experiments. The experimental design is summarised below:

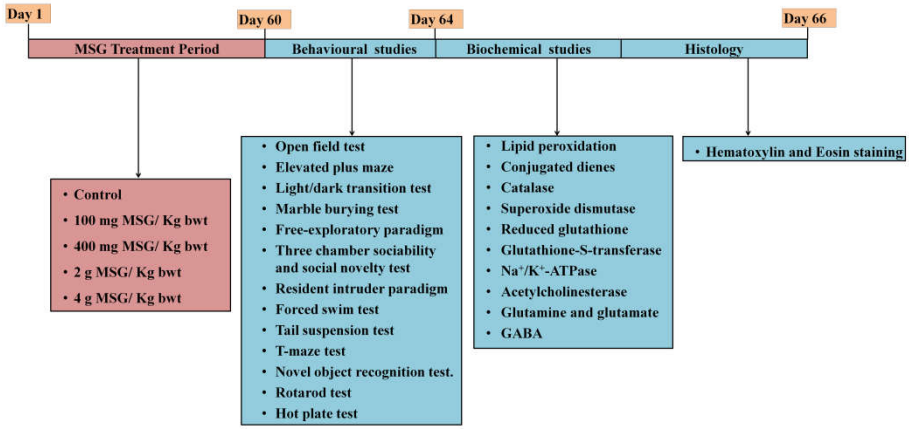


Figure 3.1: Experimental design of MSG treated rats of group I.

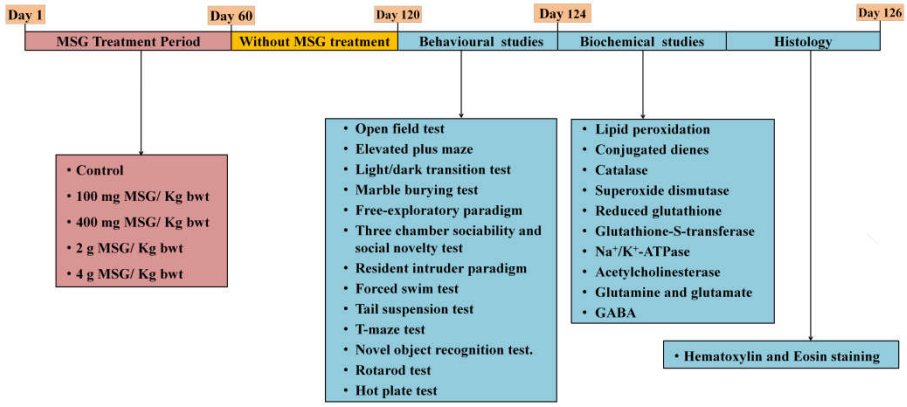


Figure 3.2: Experimental design of MSG treated rats of group II.

From the results of group I, it is observed that the 4 g MSG/ kg body weight showed a significant alteration in neurobehaviour and a deleterious effect on striatal and hippocampal region of the brain. Hence, this dose was selected to study the attenuating effect of mace.

After the treatment of 60 days, the rats were used for behavioural and biochemical studies. The experimental design is as summarised below:

Groups		**Treatment and dosages**
Control	:	Control rat fed with tap water
4g MSG	:	Treated with MSG 4 g/ kg body weight
Mace	:	Treated with mace 100 mg/ Kg body weight
4 g MSG + Mace	:	Treated with MSG 4 g/ kg body weight along with mace 200 mg/ Kg body weight

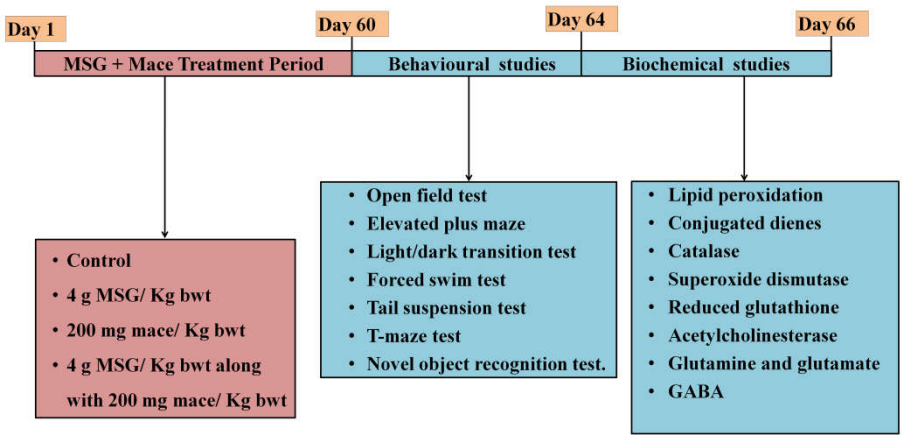


Figure 3.3: Experimental design of MSG and Mace treated groups.

Collection and authentication of plant materials

Mace is a reddish yellow outer covering of the seed nutmeg from the tree *Myristica fragrans*Houtt. It was collected in the month of April and May from Engapuzha, Calicut. The collected mace was identified and authenticated by a taxonomist of the Department of Botany, University of Calicut with Accession no. 6913.

Preparation of mace decoction

Mace of *Myristica fragrans* collected was washed and shade dried at room temperature and then coarsely powdered. About 10 g of coarse powder was weighed and taken in a flat bottom flask with 200 ml water. The mixture was heated carefully in a boiling water bath until the volume was reduced to half of its original volume. This crude mixture was subjected to filtration using a filter paper using a suction pump and used for feeding.

Some of the filtrate was transferred into a round bottom flask and lyophilized to get in a powdered form and used for preliminary phytochemical analysis, DPPH radical scavenging assay and GC-MS analysis (Asika*et al*., 2016).

Preliminary phytochemical screening

The phytochemical analysis of the decoction of mace for phenols, flavonoids, tannins, sterols, alkaloids, glycosides, saponins, phlobatannins, terpenoids, amino acids and carbohydrates were carried out using the methods described by (Harborne, 1973; Trease and Evans, 1989).

- **Test for Flavanoids**: About 5 ml of dilute ammonia solution was added to a portion of the decoction followed by the addition of concentrated H_2SO_4. Appearance of yellow colour indicated the presence of flavonoids, which disappeared on standing.

- **Test for Phenols**: The decoction was spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spot and was exposed to ammonia vapours. Blue colouration of the spot indicated the presence of phenols.

- **Test for Tannins**: About 0.5 g of dried powdered samples were boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1 % ferric chloride was added and observed for brownish green or blue-black colouration.

- **Test for Saponins**: To 0.5 ml of the decoction, 0.5 ml of distilled water was added and vortexed for 10 minutes. The formation of foams indicated the presence of saponins.

- **Test for Alkaloids**: Wagner's test: A fraction of the decoction was treated with Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of distilled water) and observed for the formation of a reddish brown precipitate.

- **Test for Phlobatannins**: The decoction was heated with 1 % aqueous HCl. Formation of red precipitate indicated the presence of phlobatannins.

- **Test for Terpenoids**: About 5 ml of the decoction was mixed with 2 ml of chloroform and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration at the interface indicated the presence of terpenoids.

- **Test for Glycosides**: About 5 ml of each the decoction was added with 2 ml of glacial acetic acid which was followed by the addition of a few drops of ferric chloride solution and 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface confirms the presence of glycosides.

- **Test for Coumarins**: About 5 ml of the decoction was treated with 2 ml of 10 % sodium hydroxide. The appearance of an yellow colour indicated the presence of coumarins.

- **Test for Steroids**: To 1 ml of the decoction, equal volume of chloroform and few drops of concentrated H_2SO_4 were added. Formation of brown ring indicated the presence of steroids.

- **Test for Carbohydrates**: To 2 ml of the decoction, 1 ml of Molisch's reagent and few drops of concetrated H_2SO_4 were added. Formation of purple or reddish ring indicated the presence of carbohydrate.

- **Test for Aminoacids**: To 2 ml of the decoction, few drops of 0.2 % Ninhydrin was added and heated for 5 minutes. Formation of blue colour indicated the presence of amino acids.

DPPH radical scavenging Assay

The antioxidant activity of the mace decoction was evaluated by DPPH (1, 1- diphenyl 2-picrylhydrazyl) radical scavenging assay described by Brand-Williams *et al.,* 1995 with slight modifications. DPPH is considered as a stable free radical with a deep violet colour in absolute ethanolic solution and an absorbance band at 520 nm. If free radicals have been scavenged by the antioxidants, DPPH solution becomes pale violet. Different concentrations (0.2, 0.4, 0.6, 0.8, 1 mg/ ml) of 1 ml of decoction was added to an equal volume of 0.1 mMethanolic solution of DPPH. The mixture was shaken well and incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm against a blank containing 1 ml of distilled water and an equal volume of ethanol using Jasco UV spectrophotometer. Ascorbic acid was used as a positive control. The inhibition % was calculated using the following formula.

$$\%\ \boldsymbol{inhibition} = \frac{\boldsymbol{OD\ control - OD\ test}}{\boldsymbol{OD\ control}} \boldsymbol{X\ 100}$$

GC-MS Fingerprint of mace extract

GC-MS analysis was performed on an Agilent Gas chromatograph series 6850 system fitted with a HP-5 MS fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 µm) coupled with an Agilent 5975C VL-MSD with triple-axis detector under the following conditions: oneµL of diluted samples (1 mg mace extract/ ml in Hexanes, w/v) were injected automatically and in a split mode (1:25); Helium as carrier gas at 1 ml/ min constant flow mode, injector

temperature 230 °C, oven temperature 60 °C to 180 °C at 2.5 °C /min, hold at 180 °C for 5 min followed by 180 °C to 250 °C at 10 °C/ min. Mass spectra: electron impact (EI+) mode, 70 eV and ion source temperature 230 °C. Mass spectra were recorded over 50-500 amu range; electron multiplier 1460 eV; scan rate, 2.96 scan/ second. The retention indices were used for identifying the chemical constituents based on homologous series C8-C20 n-alkanes. Further identification of the components was made by the comparison of Mass spectra with the literature or by the comparison of their mass spectra with those stored in NIST-8 library. Component relative percentages were calculated based on GC-MS peak areas (Varughese*et al*., 2016).

Docking studies

Molecular docking is a computational procedure that attempts to determine the non-covalent binding of active compounds or small molecule (ligand) with macromolecule (receptor) and to find the best orientation of ligand and the binding affinity which would form a complex with overall minimum energy (Trott and Olson, 2010).

Preparation of receptors and ligands

The active compounds in the decoction of mace identified by GCMS (Table 4) were used for ligand receptor interaction studies by molecular docking. The active compounds in the decoction were docked with NMDA receptor subunits such as GluN1/GluN2A, GluN1A/GluN2B and GluN1B/GluN2Bcompared with a well known NMDA receptor antagonist Memantine. The three dimensional X-ray crystal structure of NMDA receptor subunits were retrieved from the

Protein Data Bank (PDB) (http://www.rcsb.org). Before docking the ligands into the receptor active site, the needed chains of the PDB format of the receptors and active sites was selected, a number of missing atoms and Gasteiger charges were supplemented, multiple ligands and water molecules were removed and hydrogen atoms to these target proteins was added for correct ionization and tautomeric states of amino acid residues by using Auto Dock 4.2.6 (http://autodock.scripps.edu).The ligands were retrieved from PubChem Database (https://pubchem.ncbi.nlm.nih.gov) in the SDF format and were first converted to the PDB format using OpenBabel GUI version 2.3.2a (http://openbabel.org). The Gasteiger charges and rotatable bonds were then assigned to the PDB ligands using Auto Dock 4.2.6 and saved in the PDBQT file format. These modified receptors and ligands were used for all flexible docking studies (Alhazmi, 2015).

Receptor - Ligand Docking

The grid parameter file of each protein was generated using Auto Dock Tool. A grid-box was created that was large enough to cover the entire protein binding site and accommodate all ligands to move freely in it. The number of grid points in x, y and z-axes were set to 40×40×40. The distance between the two connecting grid points was 1.0 A. The grid center x, y and z were also specified and it varied from one receptor to another. This prepared receptor was saved in the pdbqt file format. The exhaustiveness was set to the default value of eight for all the docking runs. The binding energy/affinity of the ligand to the protein was computed using the AutoDockVina version

1.1.2 software package by search Algorithm. After the successful completion of the docking runs, different conformations of the ligands known as binding modes were obtained with their respective binding energy/affinity and the stable one which happens to be the one with the lowest binding energy/affinity was picked as the best binding pose. The receptors in the PDBQT file format as well as the different binding modes of the ligands obtained after running AutoDockvina, which were also in PDBQT file format were opened with EduPymol version 1.7.4.4 to check the best binding mode that fitted well with the binding site cavity (Adejoro *et al.,* 2016).

Behavioural experiments

All the behavioural experiments were conducted on alternate days during the first half of the dark phase of their light/dark cycle under red light. All the experimental apparatus were cleaned with 70 % ethanol and permitted to dry between tests to prevent olfactory cue bias and to ensure proper disinfection.

Open field test

The open field test, developed by Calvin S Hall, is the most widely used method to determine anxiety related behaviour, general activity levels, gross locomotor activity and exploration habits in rodents. The open field is an empty test arena, usually a square, in which the animal's activity is measured. The open field apparatus consisted of a square wooden box, measured 100 cm X 100 cm with a wall height of 40 cm. The floor was divided into 25 squares with each

20 cm X 20 cm sides. The experiment was video recorded for analysis.

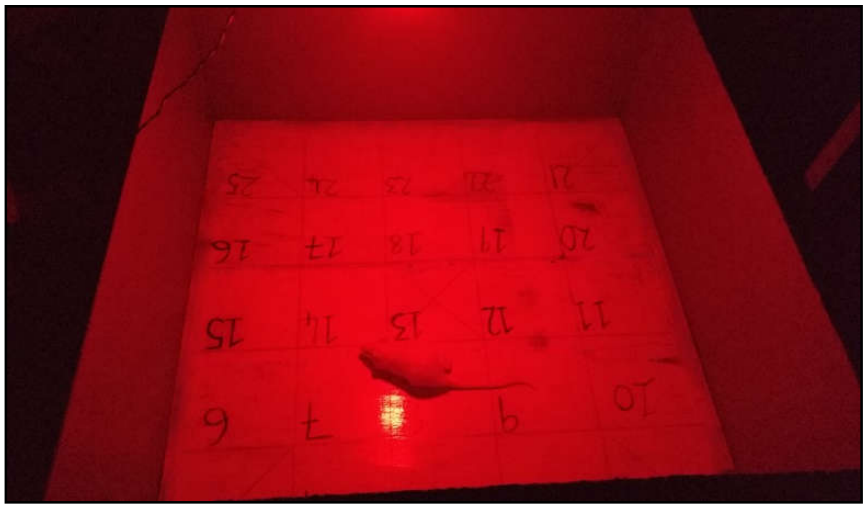


Figure 3.4: Open field apparatus.

The experimental rat was exposed to the open field, two days before the test for habituation of the novel environment. On the experimental day, the experimental rat was placed in the testing room in their home cages for approximately 30 minutes to acclimatize. After 30 minutes, the rat was placed into the center of the open field and allowed to explore the apparatus for 6 minutes. After the 6 minute test, the rats were returned to their home cage and the apparatus was cleaned with 70 % ethanol. The following parameters were observed during each test: the number of total squares crossed, number of central square entries, central square duration,self-grooming duration, rearing frequency and duration of immobility. An entry was defined as the presence of all four paws in the square (Buccafusco, 2000; Sestakova *et al.,* 2013).

Elevated plus maze test

The elevated plus maze is a tool to assess anxiety and anxiety like behaviors in rodents by their proclivity towards dark, enclosed spaces and an unconditioned fear of heights or open spaces. Elevated plus maze was introduced by File and co-workers in 1985 (figure 3.5). The elevated plus maze is a plus shaped wooden apparatus with four arms perpendicular to each other, in which two are open arms and two are closed arms.

Figure 3.5: Elevated plus maze apparatus.

The two open arms lie across from each other measuring 50 cm X 5 cm and perpendicular to two closed arm measuring 50 cm x 5 cm with a center platform (5 cm X 5 cm). The closed arms have a side and end high wall measuring 40 cm to enclose the arms, whereas the open arms have no side wall. The apparatus was placed 40 cm above

the floor level by a support. The experiment was video recorded for analysis.

The experimental rat was exposed to the EPM 2 days before the test for habituation of novel environment. On the experimental day, the experimental rat was placed in the testing room in their home cages for approximately 30 minutes to acclimate. After 30 minutes, the experimental rat was placed in the central platform facing the closed arm and allowed to explore for 6 minutes. After the 6 minute test, the rats were returned to their home cages and the apparatus was cleaned with 70 % ethanol. The following parameters were observed during test: open and closed arm entries, time spent in open and closed arms. An entry was defined as the presence of all four paws in the arm (Pellow *et al.,* 1985; Walf and Frye, 2007; Bourin *et al.,* 2007).

Dark/light transition test

The Dark/light transition test is a characteristic tool used in the assessment of anxiety developed by Crawley and Goodwin in 1980. The test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stress, such as novel environment and light. The Dark/light transition test apparatus consisted of a wooden box measuring 60 x 30 x 40 cm divided into two chambers that were of the same size but with different colors. The first chamber measuring 30 X 30 X 40 cm was painted black called dark box and the other chamber 30 X 30 X 40 cm was painted white called light box. An opening measuring 7 X 7 cm was located at the floor level in the wall between

the two chambers. Upper part of the dark box was covered with a wooden lid and white box is covered with a clear Plexiglass lid. Bright illumination was provided by red bulb located 40 cm above the center of the white box.

Figure 3.6: Dark/light transition test apparatus.

The experimental animals were brought into the behavioural room at least 30 minutes before beginning the experiment. The video recorder was set in front of the box. The animal was very carefully placed at the centre of the light box and allowed to explore for 6 minutes. After a 6 minute test, the rat was returned to their home cage and the light dark chamber was cleaned with ethanol and permitted to dry between each trial. The following parameters were observed during test: time spent in dark and light boxes, number of transitions between dark and light boxes (Crawley and Goodwin, 1980; Bourin and Hascoët, 2003; Bourin *et al.,* 2007)**.**

Marble Burying Test

Marble burying is a proven behavioural model for anxiety, depression, neophobia, obsessive compulsive disorders and their pharmacological manipulations. Digging and burying are typical natural behaviour of rodents for searching food, store food, create shelter from enemies etc. Laboratory animals also dig and bury harmful or harmless substances in their beddings. Serotonin reuptake inhibitors normally reduce anxiety and depression like behaviour, also reduce marble burying. Therefore, on the basis of this pharmacological evidence, we used these studies to identify anxiety and depression like behavioural changes (Deacon, 2006; Angoa-Pérez *et al.,* 2013).

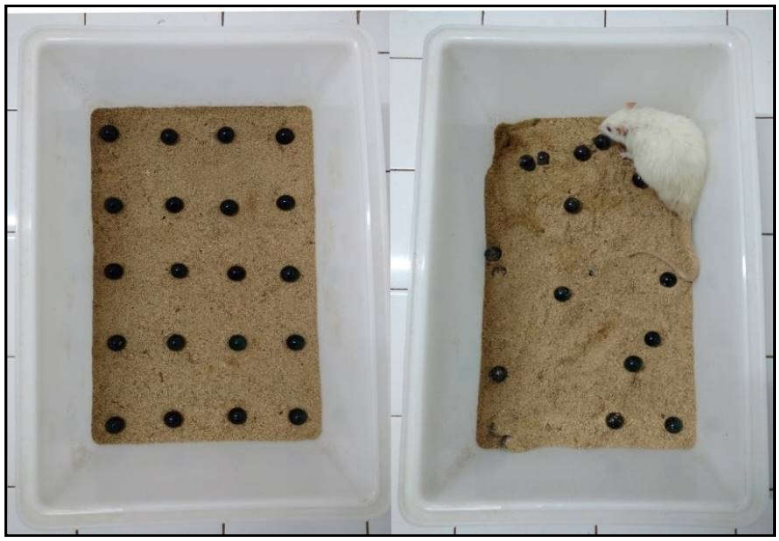


Figure 3.7: Marble burying apparatus.

Marble burying apparatus consisted of a large polypropylene cage measuring 43 X 27 X 15 cm filled approximately 5 cm deep with

saw dust bedding, lightly tapped down to make a flat and even surface. The bedding substrate can be reused; reuse of bedding does not seem to affect the burying performance of subsequently tested rats. Experimental rat was placed individually in the Marble burying apparatus for 30 min (habituation period) and then returned to their home cage. Place a regular pattern of 20 glass marbles (15 mm diameter) on the surface of the bedding in a 4 X 5 arrangement, each about 4 cm apart. Experimental rat was reintroduced into the same cage in which they had been habituated. After 30 min, the marble burying period was terminated by removing the rat and the number of marbles that were more than two-thirds covered under the bedding material was counted (Kedia and Chattarji, 2014).

Free-exploratory paradigm

The free-exploratory paradigm is a unique animal model for neophobia and trait anxiety in rodents. Rodents exhibit a preference for familiar places and a marked number of attempts at entry into the novel compartment by avoidance response. These avoidance responses are increased in stressed and anxious rodents, which are tested here (Griebeii *et al.,* 1993; Teixeira-Silva *et al.,* 2009; Antunes *et al.,* 2011; de Oliveira *et al.,* 2014).

Figure 3.8: Free-exploratory paradigm apparatus.

Free-exploratory paradigm apparatus consisted of a wooden box, divided into two compartments, with each compartment further subdivided into three exploratory units (20 X 20 cm), interconnected by small openings. The two compartments were separated by a removable partition. The box was placed on a table in the rat house. Approximately 24 h before testing, the partition was installed and an animal was put into one half of the apparatus and left there until the test time, in order to acclimatize with it. This familiar half had fresh paper bedding covering the floor and the animal had free access to food and water. On the test day the partition, between the familiar and the novel compartments was removed and the animal was allowed to explore the apparatus for 10 minutes. A video camera was used to record the whole experiment and to estimate the percentage of time spent in the novel side.

Three chamber sociability and social novelty

Social interactions are a fundamental and adaptive component of numerous species. Social recognition is critical for the structure and

stability of the networks and relationships that define societies. Disruptions in social behavior and social recognition are exhibited in a variety of neuropsychiatric disorders such as depression, autism spectrum disorders, bipolar disorders, obsessive-compulsive disorders and schizophrenia. Three chamber paradigm or Crawley's sociability and preference for social novelty protocol was used to study the social interaction behaviour in rodents (Moy *et al.,* 2004; Nadler *et al.,* 2004; Kaidanovich-Beilin *et al.,* 2011; Reilly *et al.,* 2015).

The apparatus consists of a rectangular, three-chamber box. Each chamber is 25 X 50 X 45 cm and the dividing walls of the chambers are removable and made of plywood, allowing free access to each chamber. Two identical, perforated containers with removable lids that were large enough to hold a single rat was used. These containers were placed vertically inside the apparatus, one on each side of the chamber.

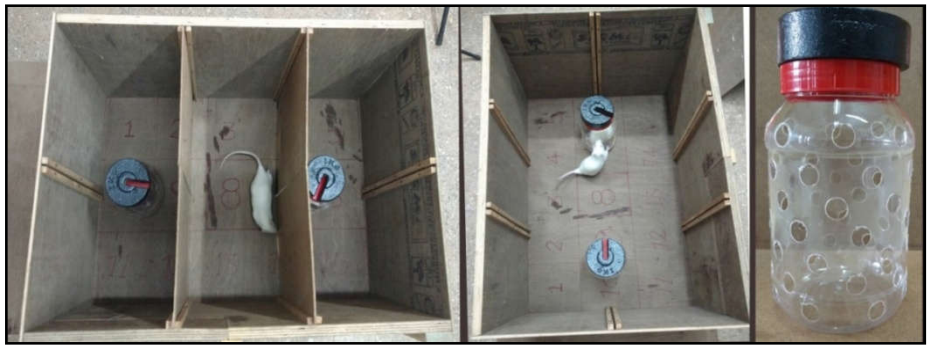


Figure 3.9: Three chamber Sociability and social novelty test apparatus.

Habituation: An empty perforated container was placed within each of the side chamber and a weight was placed on top of it to prevent the

experimental rat from climbing over the perforated containers. The test rat was placed in the middle chamber and the right and left removable dividers were removed to allow free exploration of the entire apparatus for 10 minutes. The sociability test was immediately conducted following the habituation phase.

Sociability test: Shortly after the habituation period, the right and left chamber doors were closed and the test rat was enclosed in the center compartment of the social test box. An unfamiliar rat (stranger 1, same sex) was enclosed in one of the perforated container and placed in one of the side chambers. The compartment dividing walls were removed and the test rat was again allowed to explore the entire social test box for another 10 minutes. A video camera was used to record the experiment and the duration of sniffing and contact in each perforated containers was noted.

Social novelty test: Following the sociability test, each rat was again tested in a third 10 minutes session to measure preference in spending time with a new stranger animal. The test rat was again enclosed in the center compartment by reinstalling the dividing walls. A new unfamiliar rat (stranger 2, same sex) was placed in the perforated containers that had been empty during the previous 10 minutes session (sociability test). The test rat was given a choice between the first, already investigated, now familiar rat (stranger 1) and the novel unfamiliar rat (stranger 2). A video camera was used to record the experiment and the duration of sniffing and contact in each perforated container was noted. Using the recorded data the following parameters were analyzed: the total duration of contacts between experimental rat and empty container Vs container housing stranger 1 (sociability test),

or between experimental rat and the container housing stranger 1 Vs stranger 2 (social novelty test).

Resident-intruder paradigm

The Resident-intruder paradigm is a widely accepted test to study aggression and violence in rodents. In this test, the male rat establishes a territory in its home cage, which is beefed up by the presence of females or a sexual experience. As a result of territoriality, the resident rat shows aggressive violence over the strange intruder animal in its home cage. Thus, the resident animal of this study is used as a model of aggression and violence in rodents (Rammal *et al.,* 2010; Koolhaas *et al.,* 2013).

Figure 3.10: Resident-intruder paradigm test apparatus.

The Resident-intruder paradigm apparatus consists of a plastic cage with a size of 43 X 27 X 15 cm. The experimental male rat was housed with a female for at least one week before the start of the experiments, which will facilitate the development of territoriality. The bedding of the cage during that week was not cleaned, because territoriality is strongly based on the presence of olfactory cues. The companion female was removed from the residential cage one hour before the test. A strange male was introduced into the home cage of the resident at the start of the test. Preferably, the intruder should be slightly smaller than the resident and should not have been used in previous interactions with the same resident. The behavior of the resident was recorded for 10 minutes, using a video camera. After completion of the test, the intruder male was removed from the cage and reunite the resident male with its companion female. Medical care was to be provided to the intruder, if they have any wounds or bite marks received from the resident.

The duration of the following behavioral parameters of the resident male was determined: attack latency, move towards, social exploration, anogenital sniffing, rearing, lateral threat, upright posture, clinch attack, keep down, chase, non-social exploration including rest or inactivity.

Calculating scores of different behavioral categories, particularly:

- Total offense score: sum of lateral threat, upright, clinch, keep down and chase.

- Social exploration score: sum of social explore, anogenital sniffing and social groom.
- Nonsocial exploration

Data can be expressed as percentages of the total observation time.

Forced swim test

Forced swim test is a behavioural test used to study depressive behaviour in rodents which was developed by Porsolt and colleague in 1977. The swim test involves the scoring of active (swimming and climbing) or passive (immobility) behaviour when rodents are forced to swim in a cylinder from which there is no escape. Increase in passive behaviour is interpreted as a depression like behaviour. Free swim apparatus consisted of a glass cylinder with 20 cm in diameter and 70 cm height, filled with clean warm water (25 + 3 °C).

Figure 3.11: Forced swim test apparatus.

The water level was 50 cm from the bottom and should be marked on the tank to ensure that the volume of water consistent all through the experiment. The dimensions of the tanks should be selected in a way that the rat will not be able to touch the bottom of the tank, either with their feet or their tails, during the swimming test. The height of the tank should be high enough to prevent the rat from escaping from the tank. On the day of the experiment, the rat was brought into the testing room at least 30 minutes prior to the test. The cylindrical tank should be filled with clean warm water (25 + 3 °C) to a depth that the rat's feet or tail cannot touch the bottom. Hold the animal carefully by its tail and gently and slowly place at the centre of water filled cylinder for 6 minutes and record the movements. After the 6 minutes session, the rat was taken out of the water and was towel dried and returned to a dry paper towel lined cage under a warm light for 15 minutes then was returned to their home cages. The water was changed frequently to eliminate fur, urine and excrement after each test was done. The duration of immobility was observed during the test. When the rat making only minimum movements of its limbs necessary to keep its head above the water surface were considered to be immobile (Porsolt *et al.,* 1977; Can *et al.,* 2012; Slattery and Cryan, 2012).

Tail suspension test

The tail suspension test is a most widely used model for assessing depression like behaviour in rodents. It is based on the observation that if a rat is subjected to short term inescapable stress, first it struggles to escape and eventually stops trying and become

immobile. Longer periods of immobility are characteristic of a depression like state. Tail suspension apparatus consisted of a simple setup of suspension hooks, which was suspended from a bar approximately 30 cm above the floor. On the experiment day, the rat was brought into the testing room at least 30 minutes prior to test. The animal was carefully wrapped with an adhesive tape around the animal's tail. The animal was suspended on the suspension hook using an adhesive tape as proximal as possible to the tail, to ensure that the animal hangs with its tail in a straight line. All the movements of the rat were recorded for 6 minutes. At the end of the experiment, carefully the animal was removed from the hook and the tape was removed gently pulling it off, before returning the animal to its home cage. The duration of immobility was observed during the test. Rat was considered immobile only when they hung passively and completely motionless (Steru *et al.,* 1985; Can *et al.,* 2012a; Villarinho *et al.,* 2012).

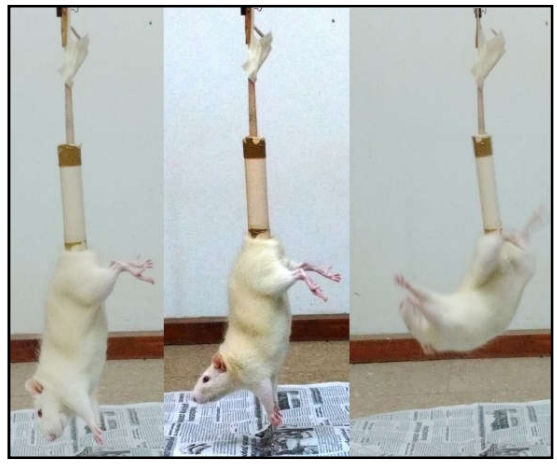


Figure 3.12:Tail suspension test apparatus.

T-Maze test

The T-maze testis used for assessing spatial working memory in rodents, especially for delayed alternation tasks. The T-maze is an elevated or enclosed apparatus in the form of the letter T placed horizontally. The animal was allowed to find out food kept inside, at one end of the short arm of the T-maze by exploration. If two trials are given in quick succession, on the second trial the rodent tends to choose the arm not visited before, reflecting memory of the first choice. This is called 'spontaneous alternation'. This tendency can be reinforced by making the animal hungry and rewarding it with a preferred food if it alternates. Both spontaneous and rewarded alternation is very sensitive to dysfunction of the hippocampus, but the other brain structures are also involved.

Figure 3.13: T-maze apparatus.

T-maze apparatus was made of wood. It is a T shaped hollow wooden box with 40 cm high walls. It has a long tail of 60 cm length and 2 short arms with 50 cm each. The hollow space inside the apparatus has a width of 10 cm, just enough for the animal to move

straight and will not allow it to turn. The experimental rat was exposed to the T-maze 2 days before the test for habitation to a novel environment. On the experimental day, the experimental rat was placed in the testing room in their home cages for approximately 30 minutes to acclimatize. After 30 minutes, the rat was carefully taken and gently placed at the starting end of the long tail of the T-maze and allowed to explore the apparatus. In one of the short arm of the T-maze, animal feed was placed in such a way that the rat is not able to see it, as soon as it reaches the end of the long arm. The animal has to move forward through the long arm and turn left or right short arm of the T-maze. When the animal reaches the food, that move was taken as a positive response. When it moves to the other end, that move was taken as a negative response. Ten trials were given for a set with a gap of 5 minutes per set. The trials were continued till a set gets at least 90 % positive response. Then the total number of positive responses was added in all the sets and divided by the total number of trials given in all the sets to get the percentage of positive response. Before doing this experiment, the animals are left starving for 36 hours with only water to drink. This makes the animals more active to find food during the experiment. A few drops of diluted vidaylin, sweet multivitamin syrup, was allowed to be sipped to whet appetite just before starting the experiment. If an animal fails to run in the T-maze within 90 seconds, the rat was removed, re-habituated and tried again in the T-maze later. After each trial, the apparatus was cleaned well using water and 70 % ethanol and permitted to dry (Deacon & Rawlins, 2006).

Novel object recognition test

Novel object recognition test is a non-force driving (without external stimuli such as electrical shock, hunger) and spontaneous test for recognition memory, introduced by Ennanceur and Delacour in 1988. The NOR apparatus consisted of a wooden box with a size of 60 X 50 X 45 cm and with different shaped and coloured objects. The objects consisted of a set of blue coloured rubber balls (familiar) and red coloured cylindrical caps (novel).

Novel object recognition has 3 phases including a habituation phase (30 min for one day), a training phase (10 min for one day) and a test phase (6 min for one day) in each rat. Before all these experimental phases, the experimental rat was placed in the testing room in their home cages for approximately 30 minutes to acclimatize. During the habituation phase, rat was placed in the NOR apparatus and allowed to adapt for 30 min without any objects. The next day, during the training phase, two identical objects were presented to the rat and allowed to explore the objects for 10 minutes. Twenty-four hours after the training phase, during the test phase, one of the old objects was replaced with a novel object and presented to each rat and allowed to explore the objects for 6 minutes.

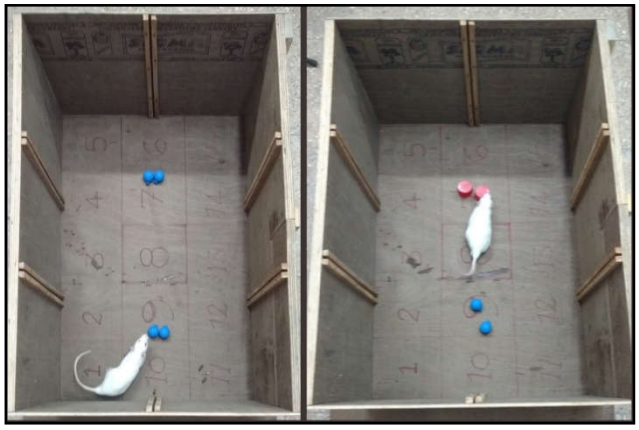


Figure 3.14: Novel object recognition test apparatus.

Record the object exploration time and find out discrimination between two objects (familiar and novel) by using a discrimination index (DI). DI = novel object exploration time/ total exploration time of both objects) − (familial object exploration time/ total exploration time of both objects) × 100. Increase in the DI indicates increased exploration of the novel object and successful retention of memory for the familiar object. Discrimination index of "zero" indicate equal exploration of both objects (Ennaceur and Delacour, 1988; Win-Shwe and Fujimaki, 2012).

Rotarod test

The rotarod test is used to assess motor coordination, muscle strength and balance in rodents. The rotarod apparatus consisted of a rotating rubber cylinder with a diameter of 2 cm placed in a horizontal plane. The rotation of the rod is controlled by an electrical motor with a speed of 12 rpm. The cylinder was mounted above a platform at a

height of 30 cm from the floor, in order to discourage the animals from jumping off the cylinder.

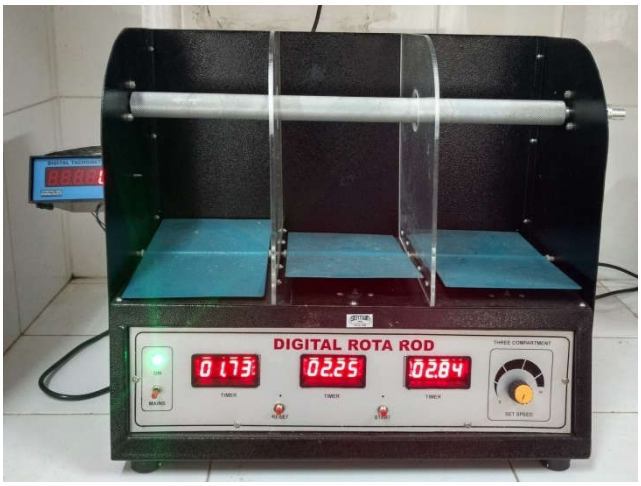


Figure 3.15: Rotarod apparatus.

On the experimental day, the experimental rat was placed in the testing room in their home cages for approximately 30 minutes and then trained on the rotarod for habituation and make it able to walk forward on a rotating rod of novel environment. After completion of the training, the rat was returned back to the home cage with food and water *ad lib.* After 1 hour, the rat was used for the test. Rat was kept in the rotating rod of the rotarod at a speed of 12 rpm for a maximum of 3 minutes and recorded the time the rat could stay on the rotating rotarod by using a stopwatch. Procedure was repeated for a total of three trials separated by 15 min intervals (Dunham and Miya, 1957).

Hot-plate test

Hot plate test is used to measure the thermal nociception of rodents. The experimental apparatus consisted of an electrically

heated metal hot plate with a glass beaker of diameter 10 cm, which was placed on top of a hot plate to hold the rat.

Figure 3.16: Hot plate test apparatus.

On the experimental day, the rat was placed in a glass beaker on a heated hot plate maintained at a temperature at 55±1 °C. Closely observed the rat and recorded the latency of nociceptive responses such as licking or shaking of hind limb or jumping in seconds. In order to prevent tissue damage, the animal when standing on the plate was limited to 60 seconds (Muhammad *et al.,* 2012; Rosa *et al.,* 2015).

Biochemical analysis

After behavioural experiments, all animal groups were sacrificed by using CO_2 euthanasia chamber, brain was quickly dissected out and cleaned with 0.85 % ice cold saline. The tissues from the hippocampal region was weighed, homogenized (10 % w/v) with ice cold 0.1 M phosphate buffer, pH-7.4 using a mortar and pestle. The homogenate was centrifuged at 12000 rpm for 20 minutes

at 4°C using REMI C-24 plus cooling centrifuge. The supernatant was separated and used for various biochemical analyses.

Estimation of lipid peroxidation marker MDA

Lipid peroxidation was estimated according to the method of thiobarbituric acid reactive substances (TBARS) assay described by Okhawa *et al.,* 1979. Lipid peroxidation is a degradation of polyunsaturated lipids by free radicals. The primary products of such degradation are a complex mixture peroxides which then breakdown to from malondialdehyde. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid (TBA) to from a MDA-TBA Chromophore, the absorbance of which is read at 535 nm.

Reagent

- TBA-TCA-HCl reagent.

Procedure

The reaction mixture consisted of 0.25 ml tissue homogenate and 0.75 ml of TBA-TCA-HCl reagent, mixed well and incubated for 15 minutes in a boiling water bath. After incubation, the mixture was rapidly cooled and centrifuged at 2000 rpm for 10 minutes. The absorbance of the supernatant was read at 540 nm against the blank that contain no tissue homogenate. The amount of MDA was calculated as nanomoles of MDA/ mg of tissue using a molar extinction coefficient of MDA-TBA adducts, which is 1.56×10^5 $m^{-1}cm^{-1}$.

Estimation of conjugated dienes

The amount of conjugated dienein the tissues was estimated according to the method described by Recknagel and Ghoshal, 1966. In the initial stage of lipid peroxidation, pro-oxidants abstract hydrogen from unsaturated lipids to form the carbon-centered lipid radical; the carbon -centered lipid radical tends to be stabilized by a molecular rearrangement to form a conjugated diene(Ayala *et al.,* 2014).

Reagents

- Chloroform: methanol mixture (2:1)
- Cyclohexane.

Procedure

The reaction mixture consisted of 0.5 ml tissue homogenate and 3 ml of chloroform: methanol mixture (2:1), mix well and centrifuge at 2000 rpm for 10 minutes and the upper layer was removed by aspiration. The conjugated dienes containing, 2 ml of the lower chloroform layer was taken in a test tube and evaporated to dry at 45 ºC in water bath. The residue was dissolved in 1 ml cyclohexane and absorbance read at 233 nm against a cyclohexane blank. The concentration of conjugated dienes was calculated as nanomoles of conjugated dienes/ mg tissue using a molar extinction coefficient 2.52 X 10^4 M^{-1} cm^{-1}.

Estimation of catalase activity (EC 1.11.1.6)

Catalase activity was determined by the method of Claiborne, 1985.Catalase is a metalloenzyme present in almost all living cells. It catalyzes the decomposition of hydrogen peroxide (H_2O_2), a harmful byproduct of many normal metabolic processes, to water and oxygen and thereby protecting the cell from oxidative damage.

$$H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

In this assay catalase present in the tissue homogenate decompose H_2O_2, followed by direct decrease in the absorbance at 240 nm.

Reagents

- 50 mM Sodium phosphate buffer, pH 7.4.
- 19 mM Hydrogen peroxide.

Procedure

The reaction mixture consisted of 0.95ml of 19 mMhydrogen peroxide and 0.05 ml of tissue homogenate in a 1 ml cuvette. The disappearance of H_2O_2 was monitored spectrophotometrically at 240 nm at 1 minute interval for 3 minutes against a control cuvette containing enzyme solution in H_2O_2 free phosphate buffer. Catalase activity was expressed as μmoles of H_2O_2 consumed/ minute/ mg of protein using a molar extinction coefficient of $0.71M^{-1}$ cm^{-1}.

Estimation of superoxide dismutase activity (EC 1.15.1.1)

Superoxide dismutase activity was determined by the method of Kakkar *et al.,* 1984. Superoxide dismutases are metalloenzymes that catalyze the dismutation of the superoxide anion (O_2^-) to molecular oxygen and hydrogen peroxide and thus form a vital part of the cellular antioxidant defence mechanism.

$$Cu^{2+}\text{-SOD} + O_2^- \longrightarrow Cu^{+}\text{-SOD} + O_2$$

$$Cu^{+}\text{-SOD} + O_2^- + 2H^+ \longrightarrow Cu^{2+}\text{-SOD} + H_2O_2$$

Superoxide dismutase activity is measured as the inhibition of the rate of reduction of nitrobluetetrazolium by superoxide radicals produced in PMS-NADH systems by oxidation of NADH. The blue colourdformazan formed at the end of the reduction reaction of Nitrobluetetrazoliumcan be extracted into butanol and measured at 560 nm.

Reagents

- 0.052 M Sodium pyrophosphate buffer, pH 8.3.
- 186 µM Phenazinemethosulphate (PMS).
- 300 µM Nitrobluetetrazolium (NBT).
- 780 µM NADH.
- Glacial acetic acid.
- N-butanol.

Procedure

The reaction mixture consisted of 1.2 ml 0.052 M sodium pyrophosphate buffer, pH 8.3, 0.1 ml of 186 μM phenazinemethosulphate, 0.3 ml of 300 μM nitrobluetetrazolium, 0.2 ml of tissue homogenate and water into a total volume of 3 ml. The reaction was initiated by the addition of 0.2 ml of NADH. After incubation at 30^{o}C for 90 seconds the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was taken. Color intensity of butanol layer was measured at 560 nm against a butanol blank. A system devoid of enzyme served as a control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula. Percentage inhibition = $[(A_o-A_1) /A_o] X 100$, where A_o was the absorbance of the control and A_1 was the absorbance of test. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50 % under the specified conditions and expressed as units/mg protein.

Estimation of reduced glutathione activity

Reduced glutathione activity was determined by the method of Sedlak and Lindsay, 1968 and Jollow *et al.,* 1974. Reduced glutathione, a tripeptide (g-glutamylcysteinyl glycine), is the major free thiol and electron donor in most living cells and is involved in

many biological processes such as detoxification of xenobiotics, removal of hydroperoxides and maintenance of the oxidation state of proteins sulfhydryls. It is the key antioxidant in animal tissues. The thiol reagent 5, 5'-dithio-bis [2-nitrobenzoic acid] (DTNB, Ellman's reagent) react with GSH to form the 412 nm chromophore, 5-thionitro benzoic acid (TNB) and GS-TNB.

$$\mathbf{GSH + DTNB \longrightarrow TNB_{(412\ nm)} + GS\text{-}TNB}$$

Reagen

- 10 % Trichloro acetic acid (TCA).
- 0.6 mM 5, 5'-dithio-bis [2-nitrobenzoic acid] (DTNB).

Procedure

The tissue homogenate, 0.25 ml, was mixed with equal volume of ice cold 10 % TCA to precipitate the protein present in the tissue. The precipitate was removed by centrifugation at 4000 rpm for 10 minutes. To 1 ml cuvette, 0.25 ml aliquot of supernatant, 0.25 ml sodium phosphate buffer of pH 8.0 and 0.5 ml DTNB was added and mixed well. The absorbance was read at 412 nm for 5 minutes at an interval of 1 minute. The GSH content was calculated as μmoles of TNB formed/ mg protein using molar extinction coefficient of $13.6\times10^3\ M^{-1}\ cm^{-1}$.

Estimation of glutathione-S-transferase activity (EC 2.5.1.18)

Glutathione-S-transferase activity was determined by the method of Habig*et al.,* 1974. GST catalyse the conjugation of

thiolgroup of GSH with many xenobiotics and their reactive metabolites to form more water soluble compounds (glutathione S-conjugate). In this assay, Glutathione and 1-chloro-2,4-dinitrobenzene conjugated by using glutathione –S- transferase present in the tissue homogenate. The reaction is as follows:

$$\text{CDNB + GSH} \xrightarrow{\text{GST}} \text{CDNB-GSH conjugate}$$

Reagents

- 0.1 M Sodium phosphate buffer, pH 6.4.
- 30 mMReduced glutathione.
- 30 mM 1-chloro-2,4-dinitrobenzene (CDNB).

Procedure

The reaction mixture consisted of 1 ml 0.1 mM phosphate buffer of pH 6.4, 0.1 ml CDNB, 0.2 ml tissue homogenate and 0.2 ml water to a total of 1.5 ml. The reaction mixture was pre-incubated at 37 ºC for 5 minutes. After incubation, 0.1 ml 30 mM GSH was added and the change in the absorbance was recorded at 340 nm for 5 minutes at 30 seconds interval. The enzyme activity was calculated as µmoles of CDNB-GSH conjugate formed/ minute/ mg protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$.

Estimation of Na^+/K^+-ATPase activity (EC 3.6.3.9)

Na^+/K^+-ATPase was determined by the method of Fiske and Subbarow, 1925 and Sweadner, 2016. Glutamate transporters in the

brain are sodium-dependent proteins that putatively rely indirectly on Na^+/K^+-ATPase to generate ion gradients that drive transmitter uptake. The liberation of organic phosphorous on incubation of tissue extract in medium containing TrisHCl buffer, NaCl, KCl and ATP was measured spectrophotometrically. Inorganic phosphate was treated with molybdic acid reagent and phosphomolybdatewas formed. This was then reduced to molybdenum blue, a blue solution by the reagent 1-amino-2-naphthol-4-sulphonic acid (ANSA). The intensity of this blue solution was the measure of inorganic phosphate present. The reaction can be summarized as follows:

Phosphorous + Ammonium molybdate ⟶ **Ammoniumphosphomolybdate**

Ammonium phosphomolybdate + ANSA ⟶ **Heteropoly-molybdenum (blue colour)**

Reagents

- 75 mMTrisHCl, pH 7.5.
- 50 mM $MgSO_4$.
- 50 mMKCl.
- 600 mMNaCl.
- 10 % TCA.
- 5 N H_2SO_4.

- 1 mMEthylenediaminetetraacetic acid (EDTA).
- 3 mMAdenosine triphosphate (ATP).
- 2.5 % Amoniummolybdate.
- 0.1 % 1-amino-2-naphthol-4-sulphonic acid (ANSA).
- 35.1mg % of potassium dihydrogen orthophosphate.

Procedure

The reaction mixture consisted of 100 µl of tissue homogenate, 1.5 ml TrisHCl buffer, 0.1ml each of NaCl, KCl, $MgSO_4$, EDTA and ATP taken in a centrifuge tube and incubated for 30 minutes at 37 °C. The reaction was arrested by the addition of 1 ml of TCA. The control tissue was taken separately, containing the TrisHCl buffer, NaCl, KCl, $MgSO_4$, EDTA and ATP was incubated on par with the test. About 100µl of homogenate was added in this tube after addition of 1 ml of TCA. The precipitate formed after addition of TCA in both test and tissue control tubes were removed by centrifugation and their supernatants were transferred to fresh tubes. The reagent blank contained 1.8 ml of TrisHCl buffer. The standard tubes containing Pi (potassium dihydrogen orthophosphate) taken at a concentration range of 2 to 10µg/ ml were placed in distilled water and were made up to 1.8 ml with Tris-HCl buffer.

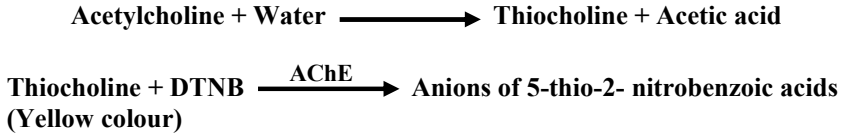
About 0.5 ml of ammonium molybdate and 0.2 ml of ANSA was added to each tube and left for 20 minutes for the blue colour to develop, which was read at 620 nm against reagent blank using a

spectrophotometer. The results were expressed in nano moles of inorganic phosphorus liberated/ min/ mg of protein.

Estimation of acetylcholinesterase activity (EC 3.1.1.7)

Acetylcholinesterase activity was determined by the method of Ellman *et al.,* 1961. AChE is a carboxylesterase family of enzyme, located at the neuromuscular junction and function to cease synaptic transmission by catalyzing the breakdown of ACh and allowing activated cholinergic neurons to a resting state.

The enzyme acetylcholinesterase catalyses the hydrolysis of acetylcholine into thiocholine and acetic acid.Thiocholine reacts with DTNB (5, 5′-dithiobis (2-nitrobenzoic acid) to form yellow coloured anions of 5-thio-2- nitrobenzoic acids, which is measured spectrophotometrically at 412 nm (Srikumar*et al.,* 2004). The rate of colour production is directly proportional to the activity of AChE present in the homogenate.



Reagents

- 0.1 M Sodium Phosphate buffer, pH 7 and 8.
- 75 mMAcetylthiocholineiodide.
- 10 mM DTNB reagent.

Procedure

The reaction mixture consisted of 2.33 ml of 0.1 M phosphate buffer of pH 8, 0.1 ml of DTNB reagent and 0.05 ml of tissue homogenate. The mixture was allowed to equilibrate for 20 minutes and then the absorbance was read at 412 nm on a spectrophotometer, whichwas recorded as a basal reading. The substrate acetylcholine iodide, 0.02 ml, was then added to the reaction mixture to make a total volume of 2.5 ml. The change in the absorbance was recorded exactly after 2 minutes for a period of 10 minutes at intervals of 1 minute. The enzyme activity was expressed as nano moles of conjugate formed/ min/ mg of protein using a molar extinction coefficient 2.52 X 10^4 M^{-1} cm^{-1}.

Plasma glutamate concentration

Plasma glutamate concentration was assessed by using glutamate colorimetric assay kit by Sigma-Aldrich, India. Hundred µl of working reaction mix containing the reaction enzyme buffer mix and sample or standard is added to the wells,mixed well and incubated at 37°C for 30 minutes. The absorbance was read at 450 nm and expressed as ng/ dl.

Protein estimation

About 0.1 ml of the homogenate was precipitated with equal volume of 10 % TCA and kept in ice for 10 minutes, centrifuged and discarded the supernatant to get the protein at the bottom, which was dissolved in 1 ml of 0.1 N NaOH and used for the estimation of protein by Lowry's method (Lowry *et al.,* 1951)

Determination of glutamate, glutamine and Gamma-Aminobutyric Acid (GABA) using HPTLC

Glutamate, glutamine and Gamma-Aminobutyric Acid contents were estimated by the method of Sanchet*i et al.,* 2013. Animals were euthanized and their brains were dissected out immediately and washed with ice cold normal saline to remove blood and adhering tissues. Brain regions such as striatum and hippocampus were dissected and homogenized in 0.1NHCl in 80 % ethanol (for every 10mg tissue/ 100µL). The homogenates were centrifuged at 4500rpm for 20min at room temperature. This clear supernatant was used for the spot application for the estimation of glutamate, glutamine and GABA. Chromatographic Conditions: Pre-coated silica gel GF254 plates as stationary phase, n-butanol: glacial acetic acid: water (65:15:25v/v/v) as mobile phase, Chamber saturation time: 3h, Instrument: HPTLC (Camag-version 1.3.4), Applicator: Linomat V, Scanner: Camag TLC scanner III, Developing chamber: Twin trough glass chamber (20×10), Developing mode: Ascending mode (multiple development), Detection reagent: 0.2 % w/v ninhydrin in acetone, Scanning wavelength: 486nm, Experimental condition: Room temperature, Relative humidity: 40–65 %. Freshly prepared L-glutamic acid, glutamine and GABA was used as a standard. Component relative percentages were calculated based on the peak areas(Sanchet*i et al*., 2013).

Histology of striatum and hippocampal region of brain using Hematoxylin and Eosin (H&E) staining

Hematoxylin and Eosin staining is an ordinary stain used for the staining of nuclei in a cell. Animals were euthanized and

transcardially perfused with 0.9 % saline followed by infusion of 10 % formalin. Brain regions striatum and hippocampus were dissected out and post fixed with 10 % formalin for 24 hours. This tissue was dehydrated with alcohol, clearedwith xylene, embedded inparaffinand a wax block was prepared. Blocks were cut into sections at 4- 5 microns thick with a microtome. These sections were rehydrated and stained by Hematoxylin and EosinWith the help of a microscope, the changes in the striatum and hippocampal region of brain was examined (Powers and Clark, 1955; Humason, 1979).

Statistical analysis

Results were expressed as mean ± SD of six animals. Statistical analysis of the data was carried out by applying the analysis of variance (ANOVA), followed by Tukey's test with the help of SPSS software, version 21. The values were considered statistically significant when $p < 0.05$.

The key focus of the present study is to identify the dose dependent ingestion of MSG on the neurochemical, antioxidant and histological alterations in striatum and hippocampal regions of the brain and its implications on neuro-behavioural changes such as anxiety, depression, memory, aggression, social behaviour etc. It is also to investigate the neuroprotective, anxiolytic, antidepressant and memory enhancing effects of decoction of the mace from *Myristica fragrans*Houtt.

A. Effect of MSG on body weight, neuro-behaviour, neurochemical and histological alterations in the striato-hippocampal region of the brain.

Body weight

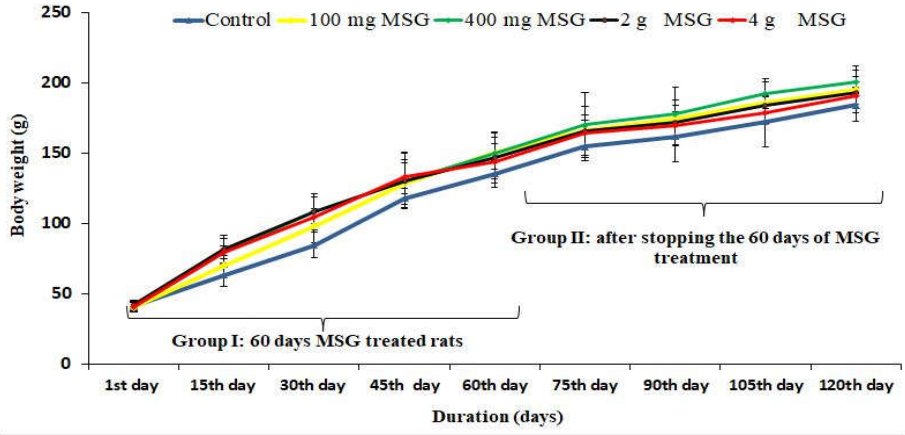
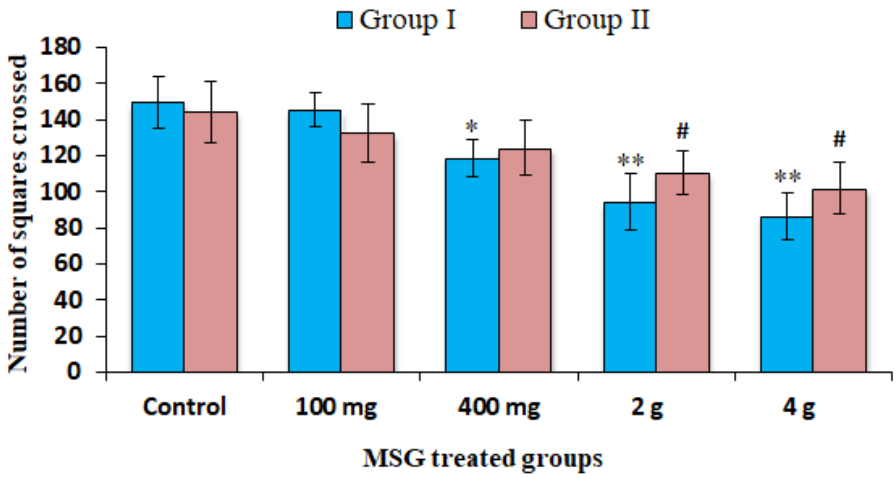


Figure 4.1: Evaluation of body weight of MSG fed rats in group I and group II (n=12).

Figure 4.1 shows the comparison of the body weight of the control rats and MSG fed rats in Group I and Group II. Both group I and II showed a non-significant increase in the body weight when compared to the control group.

Behavioural experiments

Figure 4.2: Number of squares crossed during the six minutes of open field exploration (n = 6).

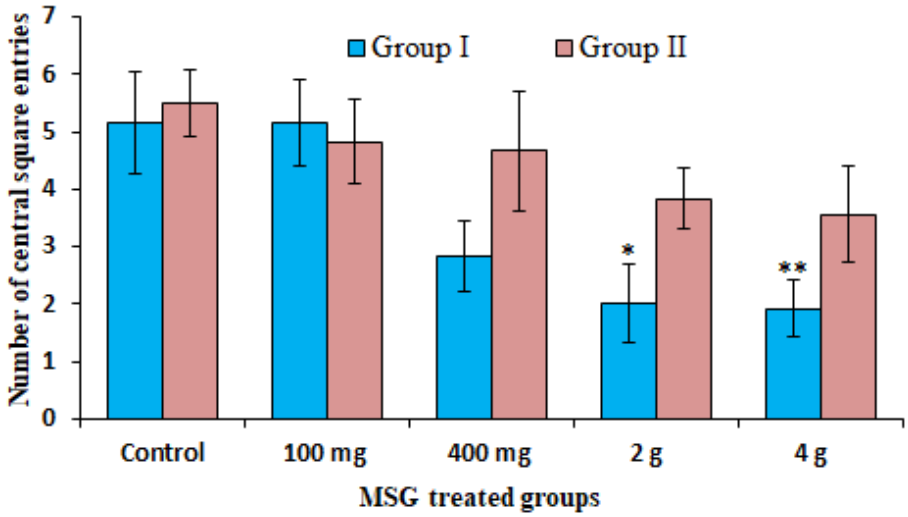


*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.2 shows the effect of MSG on the number of squares crossed during the six minutes of open field exploration. In group I, the rats fed with 400 mg MSG showed a significant ($p<0.05$) reduction in the total number of squares crossed. There was a highly significant ($p<0.01$) reduction in the total number of squares crossed by the 2 g and 4 g MSG fed rats when compared with the control group. However, in group II, the rats fed with 2 g and 4 g MSG, showed a

significant ($p<0.05$) reduction in the total number of squares crossed, while there was no significant difference observed in other groups treated with various doses of MSG when compared with the control group. The number of squares crossed in the open field was considered as the total exploratory activity of the rats.

Figure 4.3: Number of center square entries during the six minutes of open field exploration (n = 6).

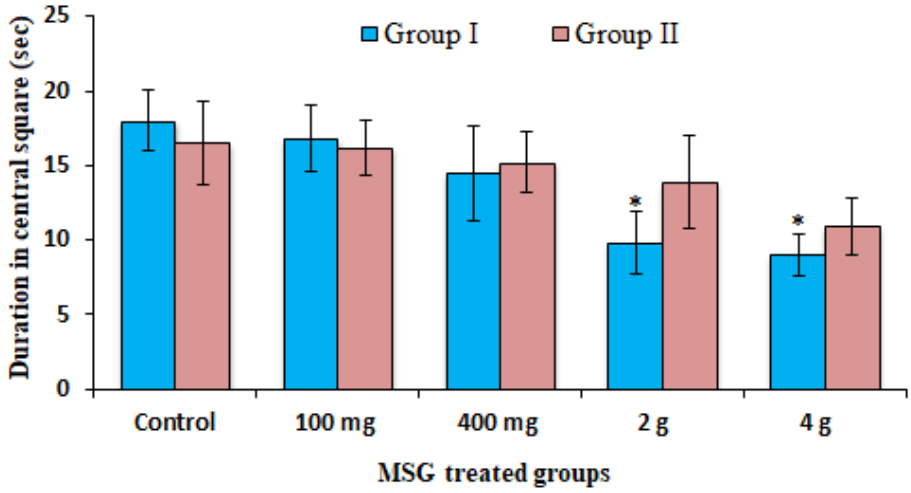


*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.

Figure 4.3 shows the effect of MSG on the number of center square entries during the six minutes of open field exploration. In group I, the rats fed with 2 g MSG showed a significant ($p<0.05$) reduction in the number of center square entries. There was a highly significant reduction ($p<0.01$) in the number of center square entries in the 4 g MSG fed rats when compared with the control group. However, in group II, though there was a decline in the central square

entry, which depicts a reduced exploratory behavior, the rats fed with various doses of MSG did not show any statistical significance when compared to the control rats.

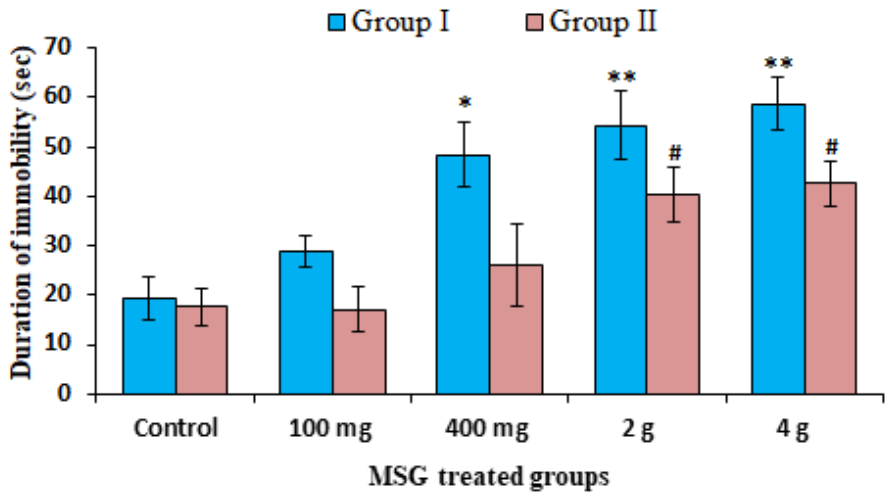
Figure 4.4: Time spent in the central square during the six minutes of open field exploration (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.4 shows the effect of MSG on the duration of time spent in the central square during the six minutes of open field exploration. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) reduction in the total time spent in the central square when compared to the control group. However, in group II, the rats fed with various doses of MSG did not show any statistical significance when compared to the control group.

Figure 4.5: Duration of immobility in the six minutes of open field exploration (n = 6).

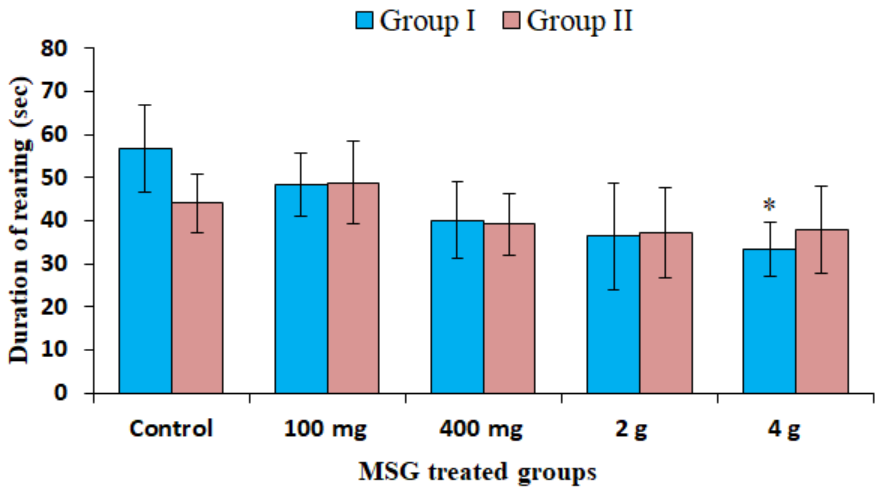


*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.

##p ≤ 0.01 compared with the control rats of group II.

Figure 4.5 shows the effect of MSG on the duration of immobility in the six minutes of open field exploration. In group I, the rats fed with 400 mg MSG showed a significant ($p<0.05$) elevation in the duration of immobility and a highly significant ($p<0.01$) elevation in the duration of immobility in the 2 g and 4 g MSG fed rats when compared with the control group. In group II, the rats fed with 2 g and 4 g MSG also showed a significant ($p<0.05$) elevation in the duration of immobility when compared with the control group. This was found to be lower than the group I animals.

Figure 4.6: Duration of rearing during the six minutes of open field exploration (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.6 shows the effect of MSG on the duration of rearing during the six minutes of open field exploration. In group I, the rats fed with 4 g MSG showed a significant ($p<0.05$) reduction in the duration of rearing and there was no any statistical significance observed in the other MSG fed categories, when compared to the control group.

Figure 4.7: Duration of grooming in the six minutes of open field exploration (n = 6).

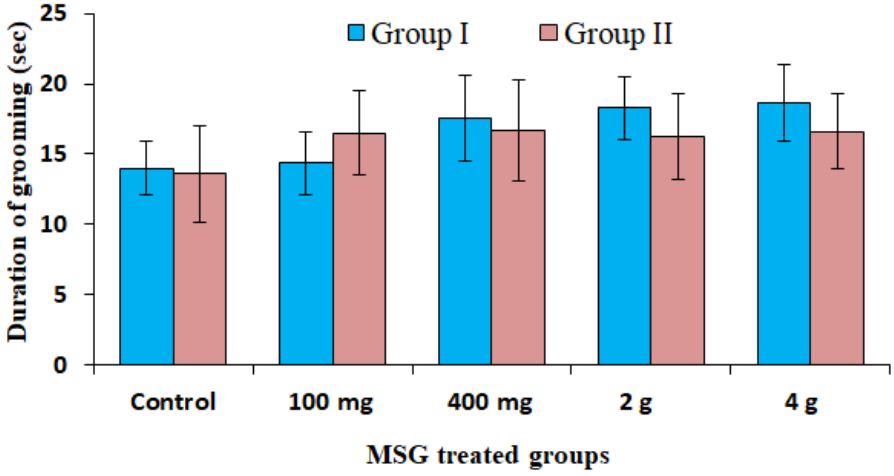
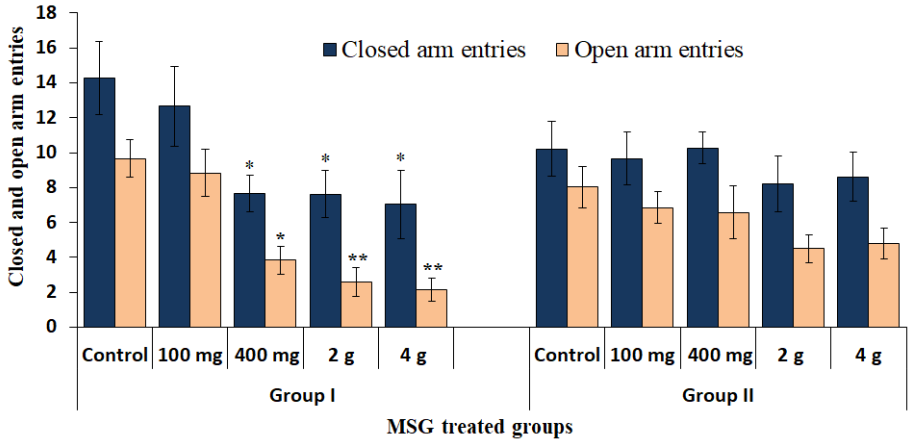


Figure 4.7 shows the effect of MSG on the duration of grooming in the six minutes of open field exploration. The rats fed with various doses of MSG in group I and II does not show any significant difference in the grooming behaviour when compared to the corresponding control group.

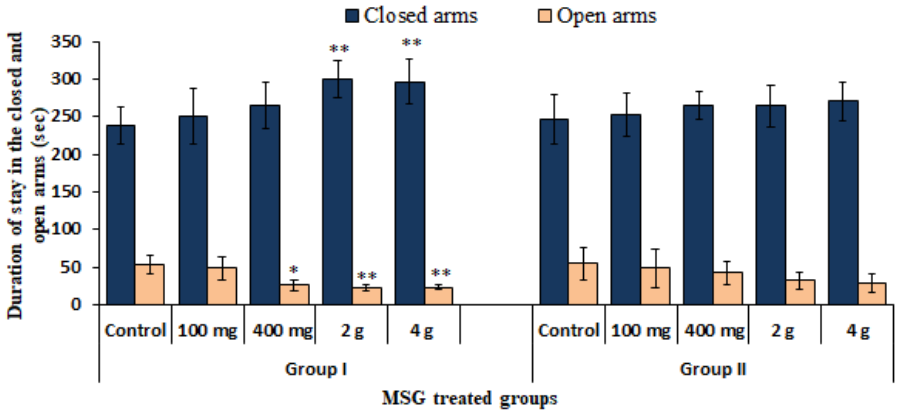
Figure 4.8: Number of closed and open arm entries during the six minutes of exploration in elevated plus maze (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.

Figure 4.8 shows the effect of MSG on the number of entries into the open and closed arms during the six minutes of exploration in the elevated plus maze. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decline in the number of closed arms entry and a very high significant ($p<0.01$) decline in the number of open arms entry, whereas in the 400 mg MSG fed group, there showed a significant ($p<0.05$) decline in the closed and open arm entries when compared to the control group. However in group II, the rats fed with various doses of MSG does not show any statistical significance in the number of entries into the closed and open arms when compare to the control group.

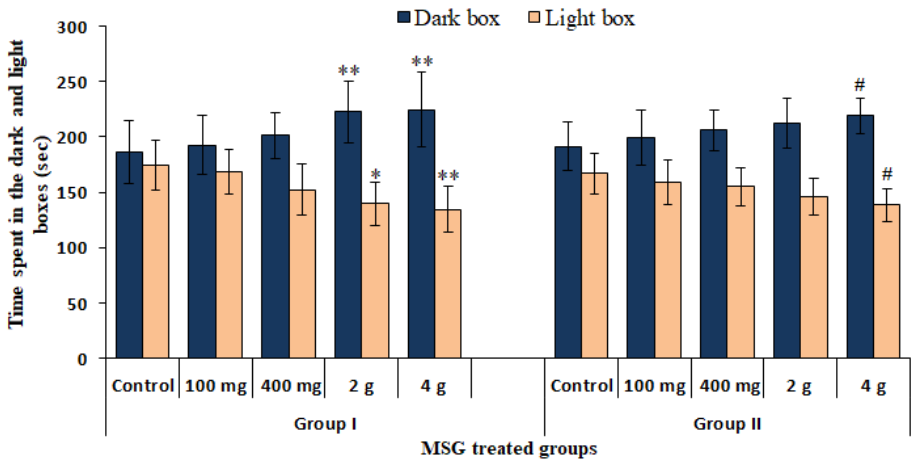
Figure 4.9: Duration of stay in the closed and open arms during the six minutes of exploration in elevated plus maze (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.

Figure 4.9 shows the effect of MSG on the duration of stay in the closed and open arms during the six minutes of exploration in the elevated plus maze. In group I, the rats fed with 2 g and 4 g MSG, there showed a highly significant ($p<0.01$) increase in the duration of stay in the closed arms and decreased duration of stay in the open arms, whereas, the rats treated with 400 mg MSG showed a significant ($p<0.05$) decline in the duration of stay in the open arms, but does not show any significant difference in the closed arms when compared to the control group. However in group II, the rats fed with various doses of MSG does not show any statistical significance in the duration of stay in the open and closed arms when compare to the control group.

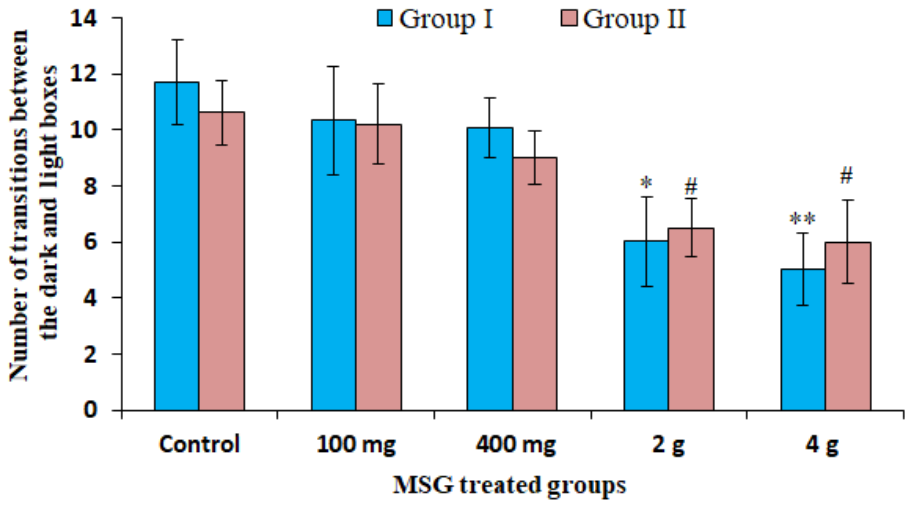
Figure 4.10: Duration of stay in the dark and light box during the six minutes of exploration in the dark/ light transition box (n =6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.10 shows the effect of MSG on the duration of stay in the dark and light box during the six minutes of exploration in the dark/ light transition box. In group I, the rats fed with 2 g and 4 g MSG showed a very high significant ($p<0.01$) increase in the duration of the time spent in the dark box when compared to the control group. A very high significant ($p<0.01$) decline in the duration of the time spent in the light box was observed in 4 g MSG fed rats and a significant ($p<0.05$) decline was observed in 2 g MSG fed rats when compared to the control group. In group II, the rats fed with 4 g MSG showed a significant ($p<0.05$) increase in the duration of the time spent in the dark box and significant ($p<0.05$) decline in the duration of the time spent in the light box when compared to the control group. All other groups fed with various doses of MSG did not show any significant difference when compared to the control group.

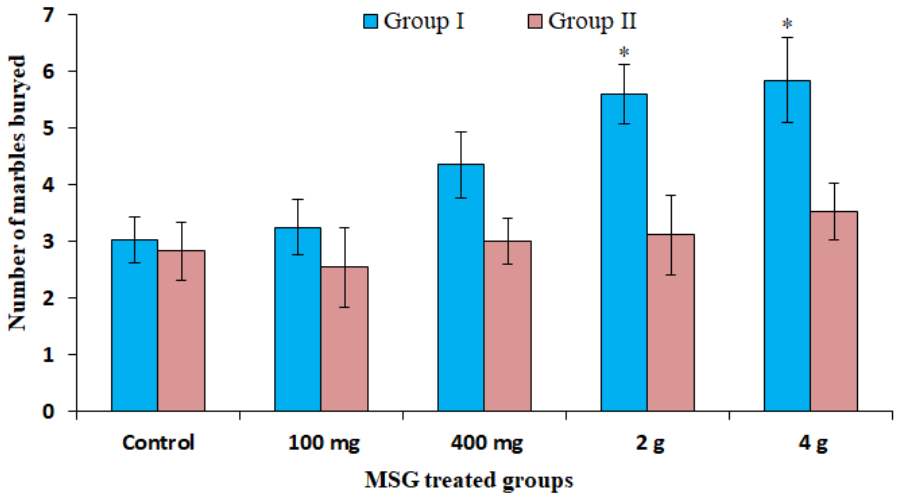
Figure 4.11: Number of transitions between the dark and light boxes during the six minutes of dark/ light transition boxexploration (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.11 shows the effects of MSG on the number of transitions between the dark and light boxes during the six minutes of exploration in dark/ light transition box. In group I, the rats fed with 2 g MSG showed a significant ($p<0.05$) decline in the number of transitions between the dark and light boxes, while the rats fed with 4 g MSG showed a very high significant ($p<0.01$) decline in the number of transitions made between the dark and light boxes when compared with the control group. In group II, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decline in the number of transitions between the dark and light boxes, while the rats treated with 100 mg and 400 mg MSG did not show any significant difference when compared with the control group.

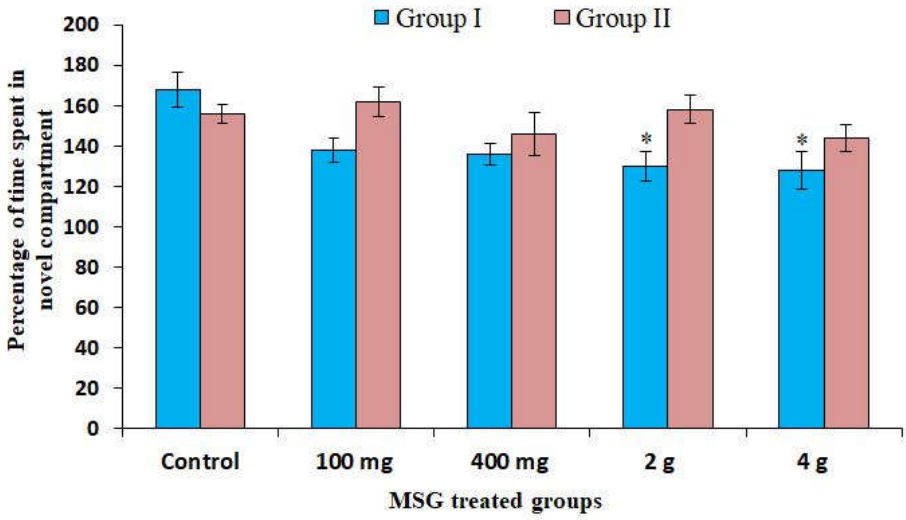
Figure 4.12: Number of marbles buried during the thirty minutes of exploration in the marble burying apparatus (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.12 shows the effects of MSG on the number of marbles buried during thirty minutes of stay in the marble burying apparatus. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decrease in the number of marbles buried, while the other rats in group I and II fed with various doses of MSG did not show any statistical significance in the number of marbles buried when compared to the control group.

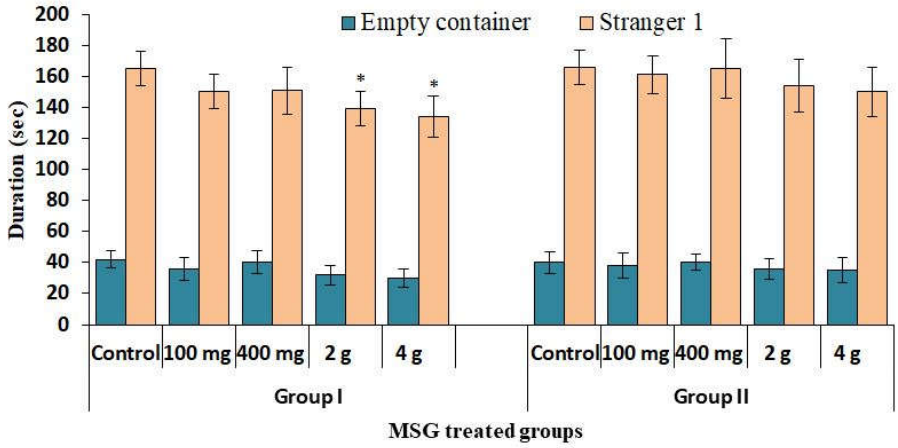
Figure 4.13: Percentage of time spent in novel compartment during the ten minutes of exploration in free-exploratory paradigm apparatus (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.13 shows the effects of MSG on the percentage of time spent in novel compartment during the ten minutes of exploration in Free-exploratory paradigm apparatus. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decrease in the percentage of time spent in the novel compartment, while the other rats in group I and II fed with various doses of MSG did not show any statistical significance when compared to the control group.

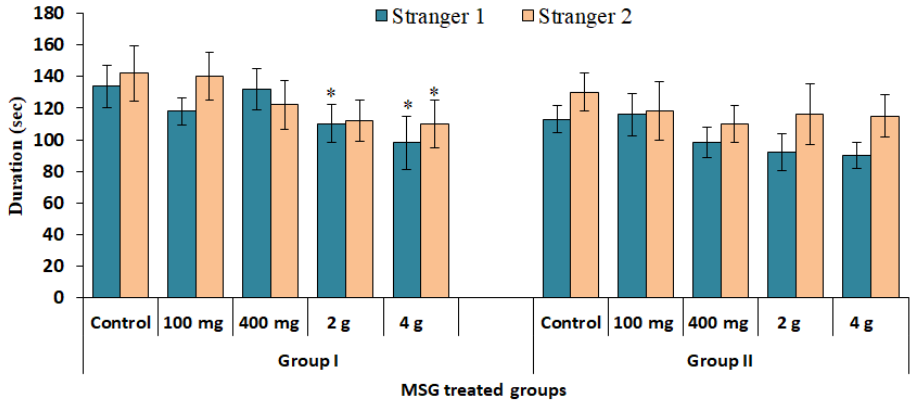
Figure 4.14: Duration of contact between empty container and stranger rat 1 in the thirty minutes of three-chamber sociability test (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.14 shows the effects of MSG on the duration of contact between the empty container Vs stranger animal 1 (sociability test). In group I, the rats fed with 2 g and 4g MSG showed a significant decline in the duration of contact with stranger rat 1, while the other MSG fed groups did not show any significance when compared to the control group. It is also observed that, there is no statistical significance in the duration of contact between the empty container Vs stranger animal 1.

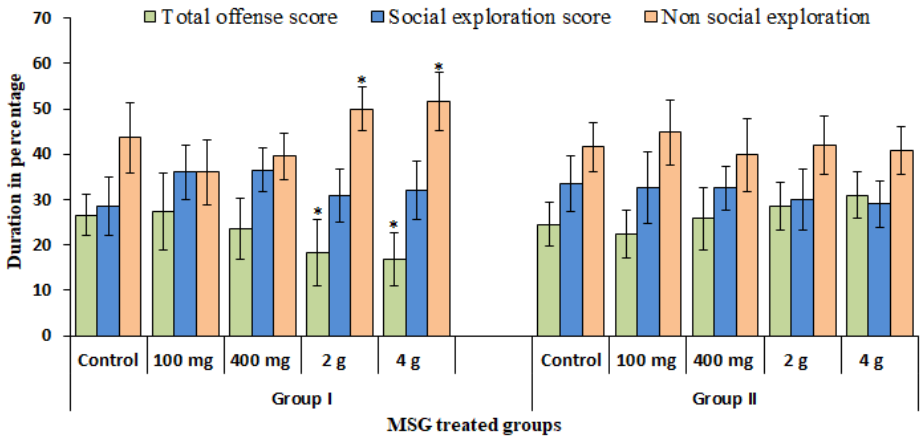
Figure 4.15: Duration of contact between the stranger rat 1 and the stranger rat 2 in the thirty minutes of three-chamber social novelty test (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.15 shows the effects of MSG on the duration of contact between stranger animal 1 Vs stranger animal 2 (socialnovelty test). In group I, the rats fed with 2 g and 4g MSG showed a significant decline in the duration of contact with stranger rat 1 and stranger rat 2, while the other MSG fed rats in group I and group II did not show any statistical significance when compared to the control group. It is also observed that, there is no statistical significance in the duration of contact between stranger rat 1 Vs stranger animal 2.

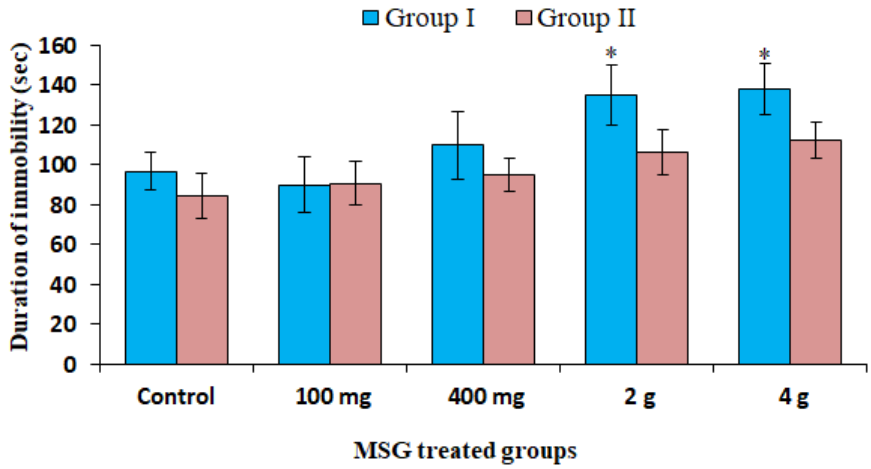
Figure 4.16: Behavioural profile of resident rats during the ten minutes of resident intruder paradigm(n = 6)**.**



*p ≤ 0.05compared with the control rats of group I.

Figure 4.16 shows the effects of MSG on the total offense, social exploration and nonsocial exploration during the ten minutes of interaction between the resident and the intruder. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decline in the total offense score and an increase in the nonsocial exploration but did not alter the social exploration when compared to the control group. However, in group II, the rats fed with various doses of MSG did not show any statistical significance in the total offense score, social exploration score and nonsocial exploration when compared to the control group.

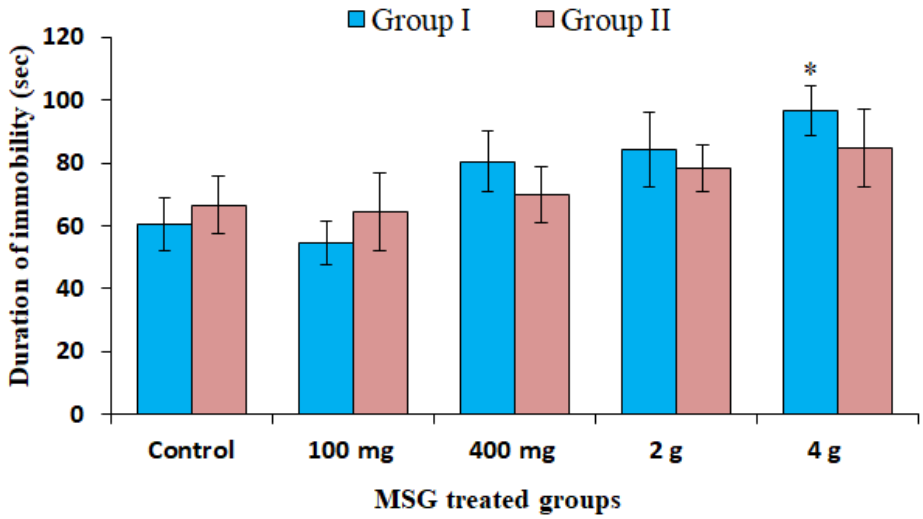
Figure 4.17: Immobility time during the six minutes of forcedswim test (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.17 shows the effect of MSG on the immobility time during the six minutes of forced swim in a water filled cylinder. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) increase in the immobility time, while the other MSG fed rats in group I and group II did not show any statistical significance when compared to the control group.

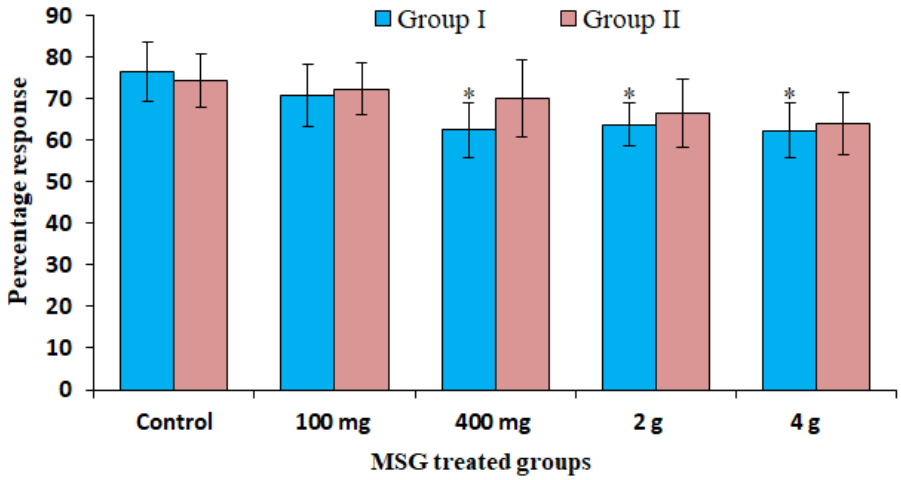
Figure 4.18: Immobility time during the six minutes of tail suspension test (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.18 shows the effect of MSG on the immobility time during the six minutes of tail suspension test. In group I, the rats fed with 4 g MSG showed a significant ($p<0.05$) increase in the immobility time, while the other rats in group I and II fed with various doses of MSG did not show any statistical significance in the immobility time when compared to the control group.

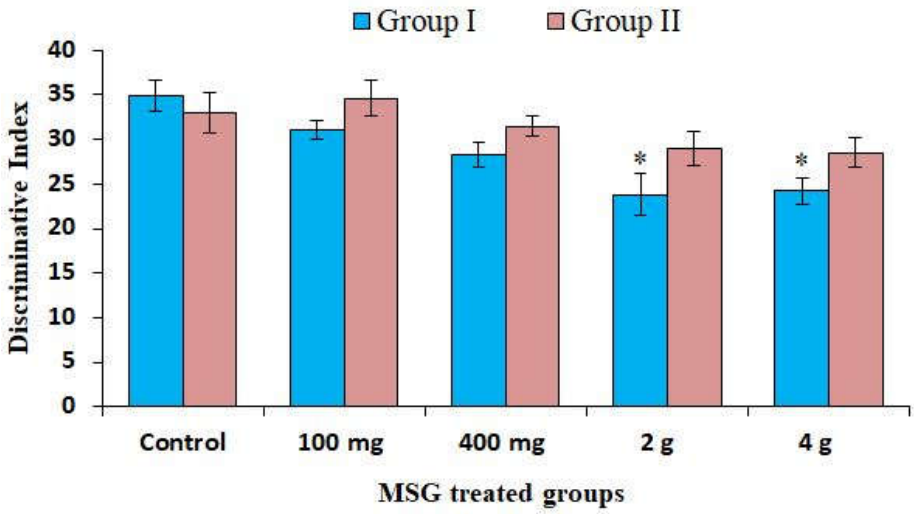
Figure 4.19: Percentage response in the T maze exploration (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.19 shows the effect of MSG on the percentage response in the T maze experiment. In group I, the rats fed with 400 mg, 2 g and 4 g MSG showed a significant ($p<0.05$) decline in the percentage response, whereas in group II there was no any statistical significance when compared to the control group.

Figure 4.20: Discrimination Index during the six minutes of novel object recognition test (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.20 shows the effect of MSG on the discrimination index during the six minutes of exploration in the novel object recognition test apparatus. In group I, the rats fed with 2 g and 4 g MSG showed a significant ($p<0.05$) decline in the discrimination index, while the other MSG fed rats in group I and II did not show any statistical significance in the discrimination index when compared to the control group.

Figure 4.21: Latency to fall off from the rotarod (n = 6).

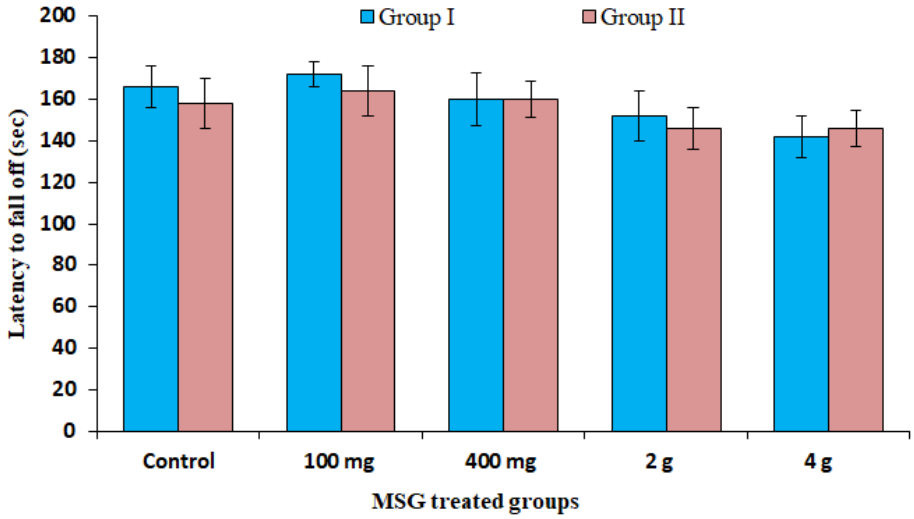


Figure 4.21 shows the effect of MSG on the latency to fall off from the rotarod apparatus during the three minutes test. The rats treated with various doses of MSG in group I and II did not show any significant difference in the duration of stay in the rotarod when compared to the control group.

Figure 4.22: Paw licking time in a hot plate (n = 6).

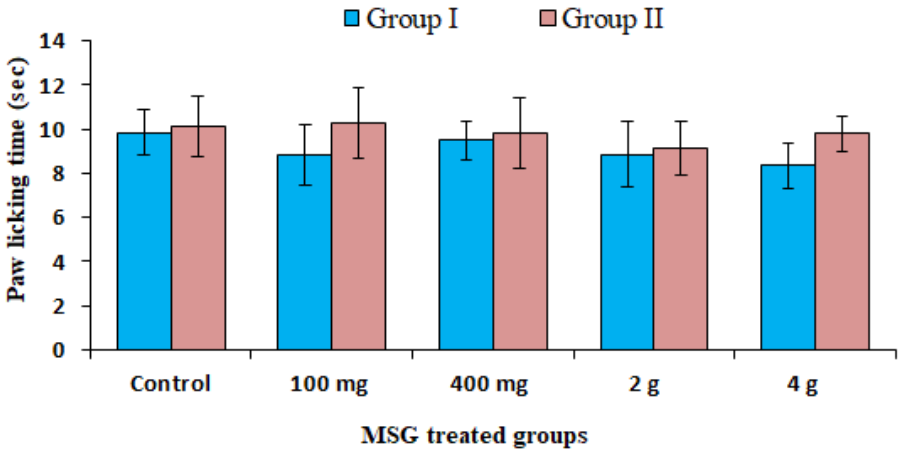
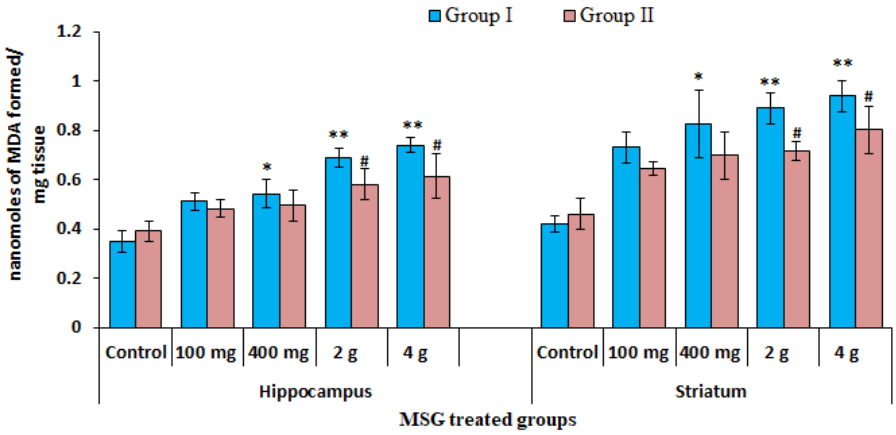


Figure 4.22 shows the effect of MSG on paw licking time in the hot plate. The rats treated with various doses of MSG in group I and II did not show any significant difference in paw licking time when compared to the control group.

Biochemical analysis

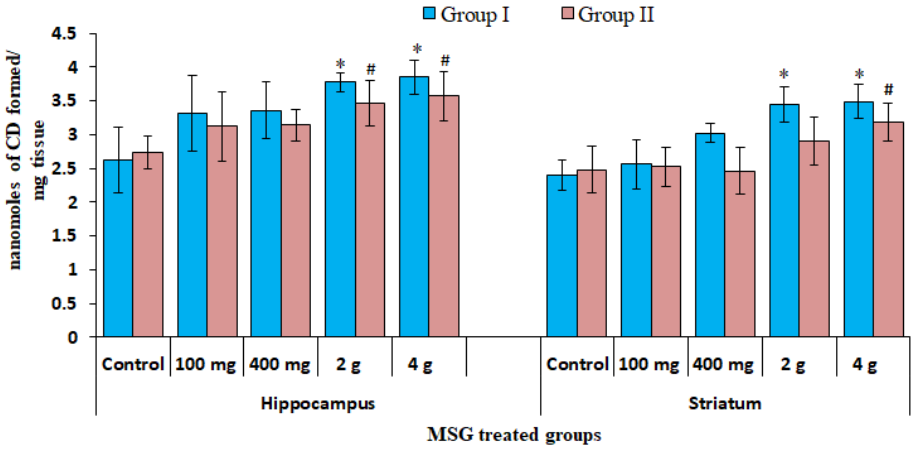
Figure 4.23: Lipid peroxidation marker malondialdehyde level in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.23 shows the effect of MSG on MDAlevel in the striato-hippocampal region of the brain. In group I, hippocampus and striatum region showed a significant ($p<0.05$) MDA activity in 400 mg MSG fed rats and a very high significant ($p<0.01$) MDA activity was observed in 2 g and 4 g MSG treated rats when compared with the control group. However, in group II, striatum and hippocampal region showed a significant ($p<0.05$) MDA activity in 2 g and 4 g MSG fed rats when compared with the control group.

Figure 4.24: Lipid peroxidation marker conjugated dienes level in the striato-hippocampal region of the brain (n = 6).

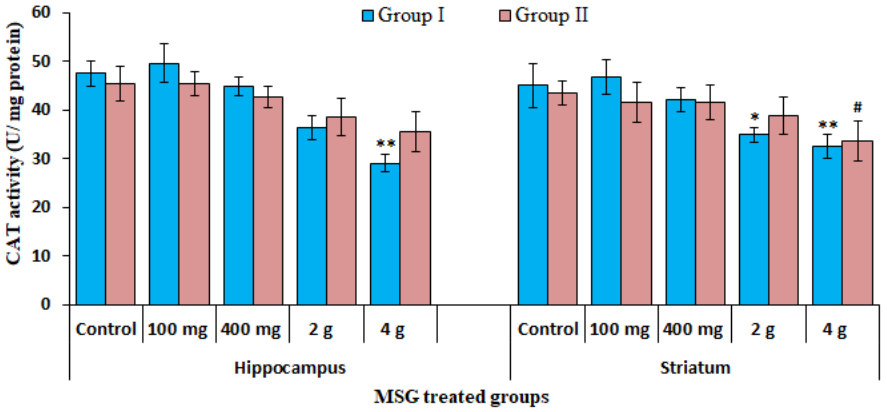


*p ≤ 0.05 compared with the control rats of group I.

#p ≤ 0.05 compared with the control rats of group II.

Figure 4.24 shows the effect of MSG on CDlevel in the striato-hippocampal region of the brain. In group I, both hippocampal and striatal region showed a significant ($p<0.05$) CD activity in 2 g and 4 g MSG fed rats. However, in group II, hippocampal region showed a significant ($p<0.05$) CD activity in 2 g and 4 g MSG fed rats while in striatum it showed a significant ($p<0.05$) increase in CD level in 4 g MSG fed rats when compared with the control group.

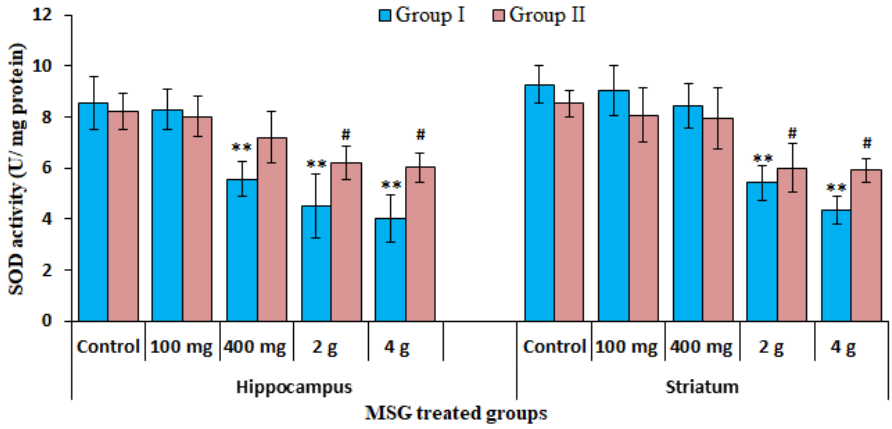
Figure 4.25: Antioxidant enzyme catalase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats in group II.

Figure 4.25 shows the effect of MSG on CATactivity in the striato-hippocampal region of the brain. In group I, hippocampal region showed a very high significant ($p<0.01$) CAT activity in 4 g MSG fed rats, whereas in striatum, it showed a significant ($p<0.05$) increase in CAT activity in 2 g MSG fed rats and a very high significant ($p<0.01$) CAT activity was observed in 4 g MSG fed rats when compared with the control group. However, in group II, hippocampal region did not show any significant CAT activity while in striatum, it showed a significant ($p<0.05$) increase in CAT activity in 4 g MSG fed rats when compared with the control group.

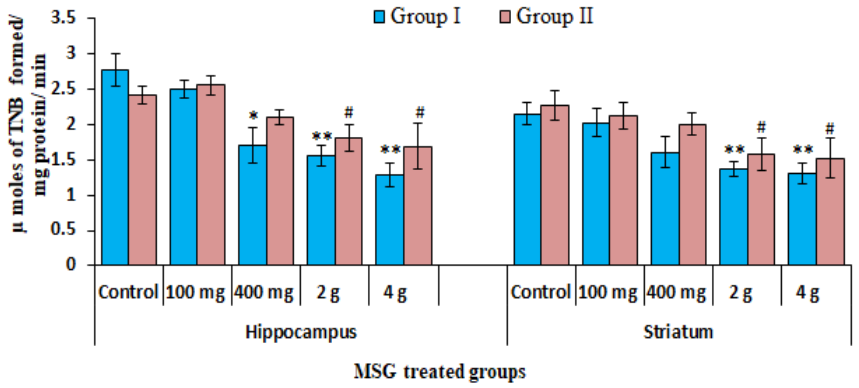
Figure 4.26: Antioxidant enzyme superoxide dismutase activity in the striato-hippocampal region of the brain (n = 6).



**p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.26 shows the effect of MSG on SODactivity in the striato-hippocampal region of the brain. In group I, hippocampal region showed a very high significant ($p<0.01$) SOD activity in 400 mg, 2 g and 4 g MSG fed rats, whereas in striatum, showed a very high significant ($p<0.01$) increase in SOD activity in 2 g and 4 g MSG fed rats when compared with the control group. However, in group II, hippocampus and striatum region showed a significant ($p<0.05$) SOD activity in 2 g and 4 g MSG fed rats when compared with the control group.

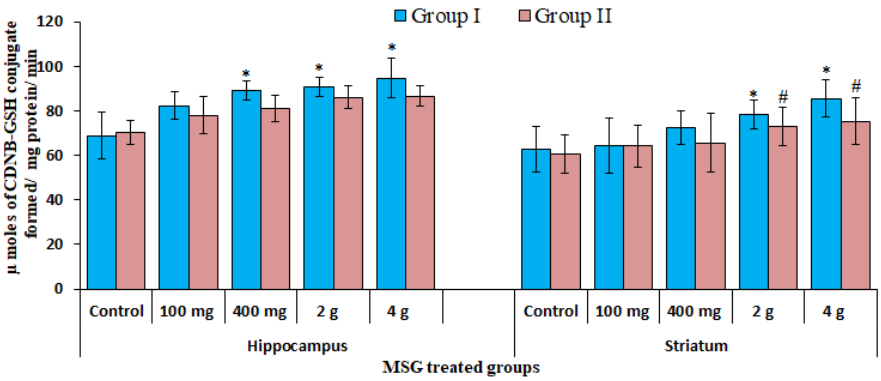
Figure 4.27: Antioxidant glutathione level in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.27 shows the effect of MSG on GSHactivity in the striato-hippocampal region of the brain. In group I, hippocampus region showed a significant ($p<0.05$) GSH level in 400 mg MSG fed rats and a very high significant ($p<0.01$) GSH level was observed in 2 g and 4 g MSG fed rats, whereas in the striatum, it showed a very high significant ($p<0.01$) increase in GSH level in 2 g and 4 g MSG fed rats when compared with the control group. However, in group II, striatum and hippocampal region showed a significant increase in ($p<0.05$) GSH level in 2 g and 4 g MSG fed rats when compared with the control group.

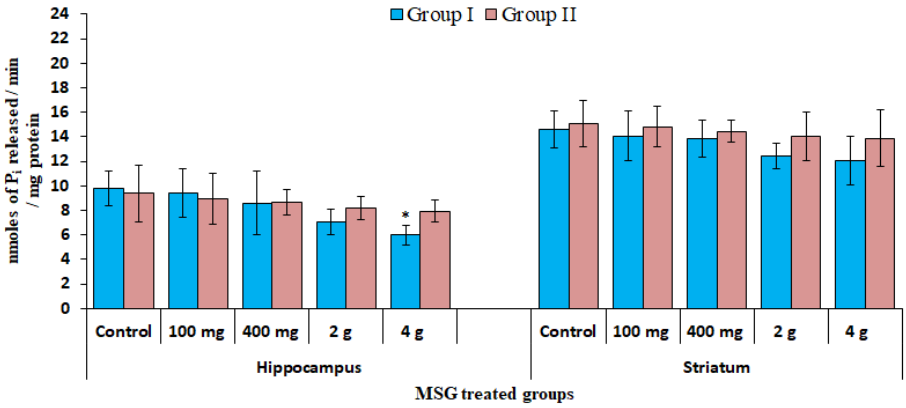
Figure 4.28: Antioxidant enzyme glutathione-S-transferase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 compared with the control rats of group I.
#p ≤ 0.05 compared with the control rats of group II.

Figure 4.28 shows the effect of MSG on GSTactivity in the striato-hippocampal region of the brain. In group I, hippocampus region showed a significant increase in ($p<0.05$) GST activity in 400 mg, 2 g and 4 g MSG fed rats whereas, in striatum, it showed a significant ($p<0.05$) increase in GST activity in 2 g and 4 g fed rats when compared with the control group. However, in group II, hippocampal region did not show any significant increase in GST activity, while in striatum, there showed a significant ($p<0.05$) increase in GST activity in 2 g and 4 g MSG fed rats when compared with the control group.

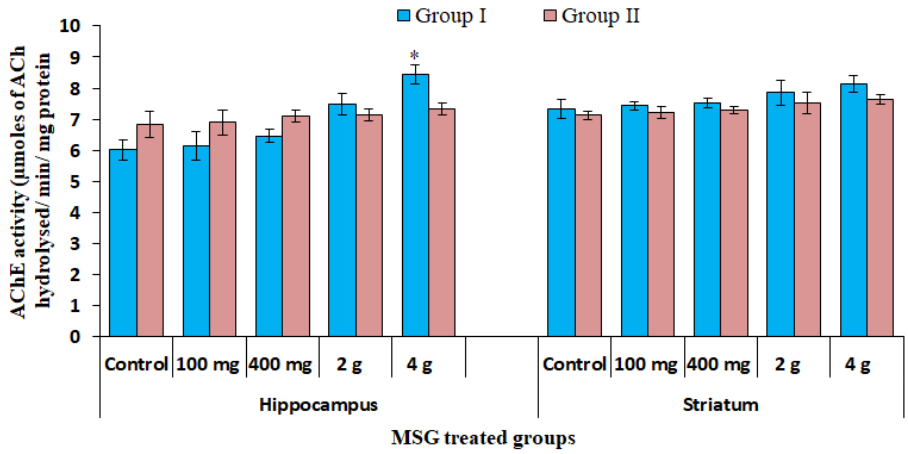
Figure 4.29: Enzyme Na^+/K^+-ATPase activity in the striato-hippocampal region of the brain (n = 6).



*$p \leq 0.05$ compared with the control rats of group I.

Figure 4.29 shows the effects of MSG on Na^+/K^+-ATPase activityin the striato-hippocampal region of the brain. In group I, hippocampus region showed a significant ($p<0.05$) increase in Na^+/K^+-ATPase activity in 4 g MSG fed rats, however, all other MSG fed rats in group I and group II did not show any statistical significance when compared to the control group.

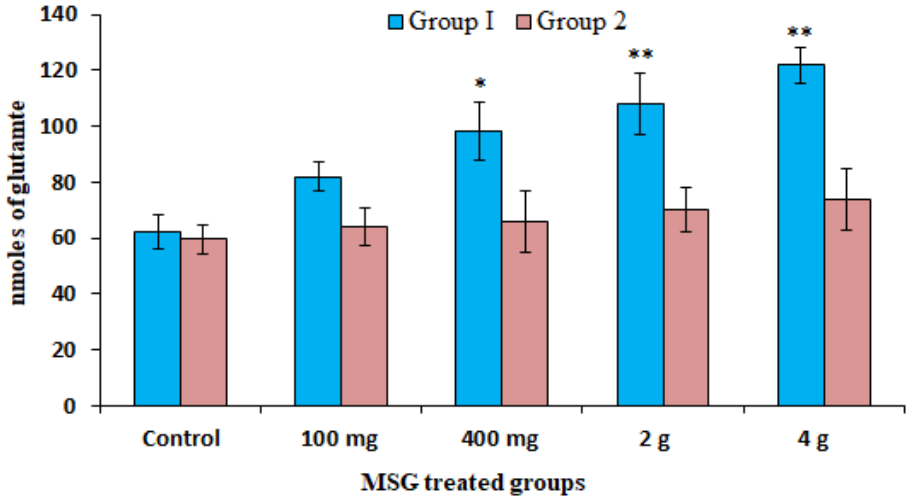
Figure 4.30: Enzyme acetylcholinesterase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 compared with the control rats of group I.

Figure 4.30 shows the effects of MSG on AChEactivity in the striato-hippocampal region of the brain. In group I, hippocampus region showed a significant ($p<0.05$) increase in AChEactivity in 4 g MSG fed rats, however, all other MSG fed rats in group I and group II did not show any statistical significance when compared to the control group.

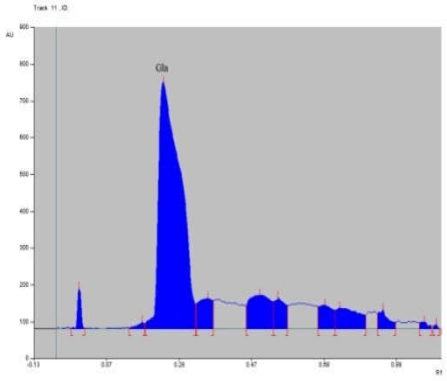
Figure 4.31: Plasma glutamate concentration (n = 6).



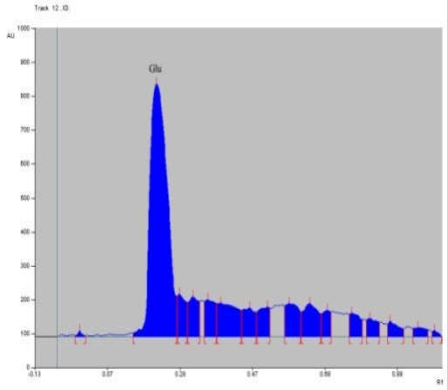
*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats of group I.

Figure 4.31 shows the effect of MSG on plasma glutamate concentration. In group I, 400 mg , 2 g and 4 g MSG fed rats showed a significant ($p<0.05$) increase in the plasma glutamate concentration while the other MSG fed groups did not show any statistical significance when compared to the control group.

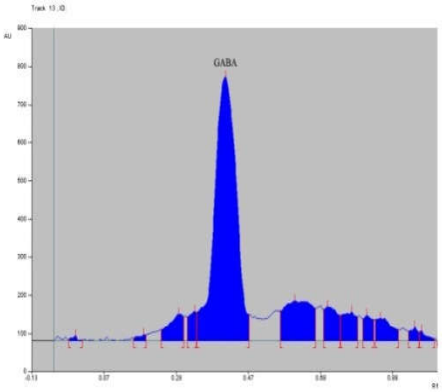
Figure 4.32: Estimation of glutamine, glutamate, and GABA level in striato-hippocampal region of the brain using HPTLC.



Chromatogram of standard glutamine



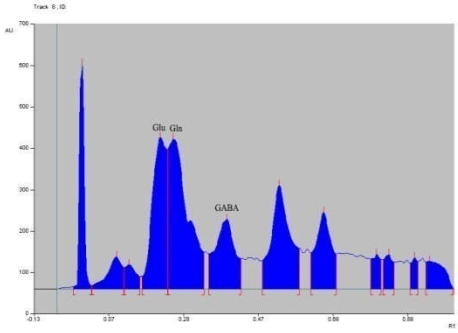
Chromatogram of standard glutamate



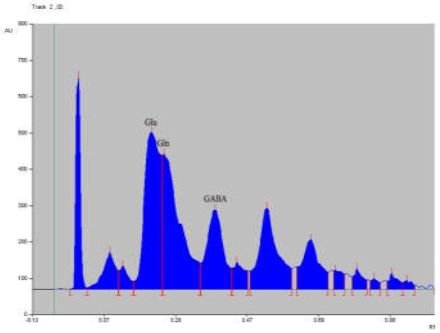
Chromatogram of standard GABA

Group I

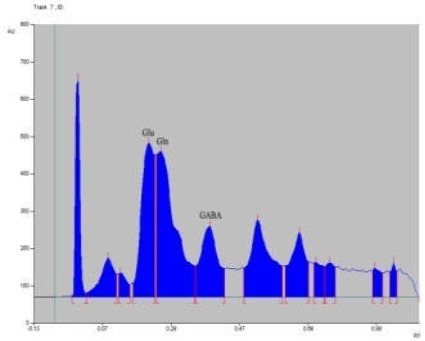
Hippocampus



Control

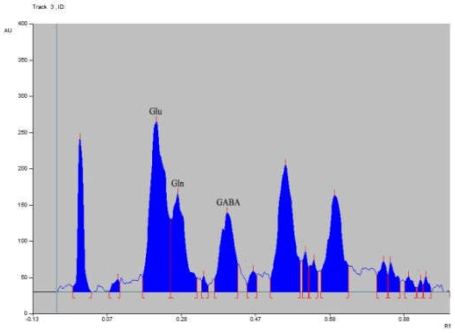


2 g MSG

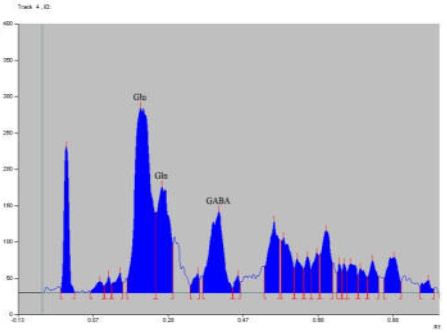


4 g MSG

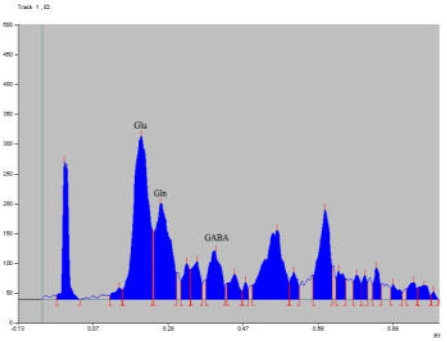
Striatum



Control



2 g MSG



4 g MSG

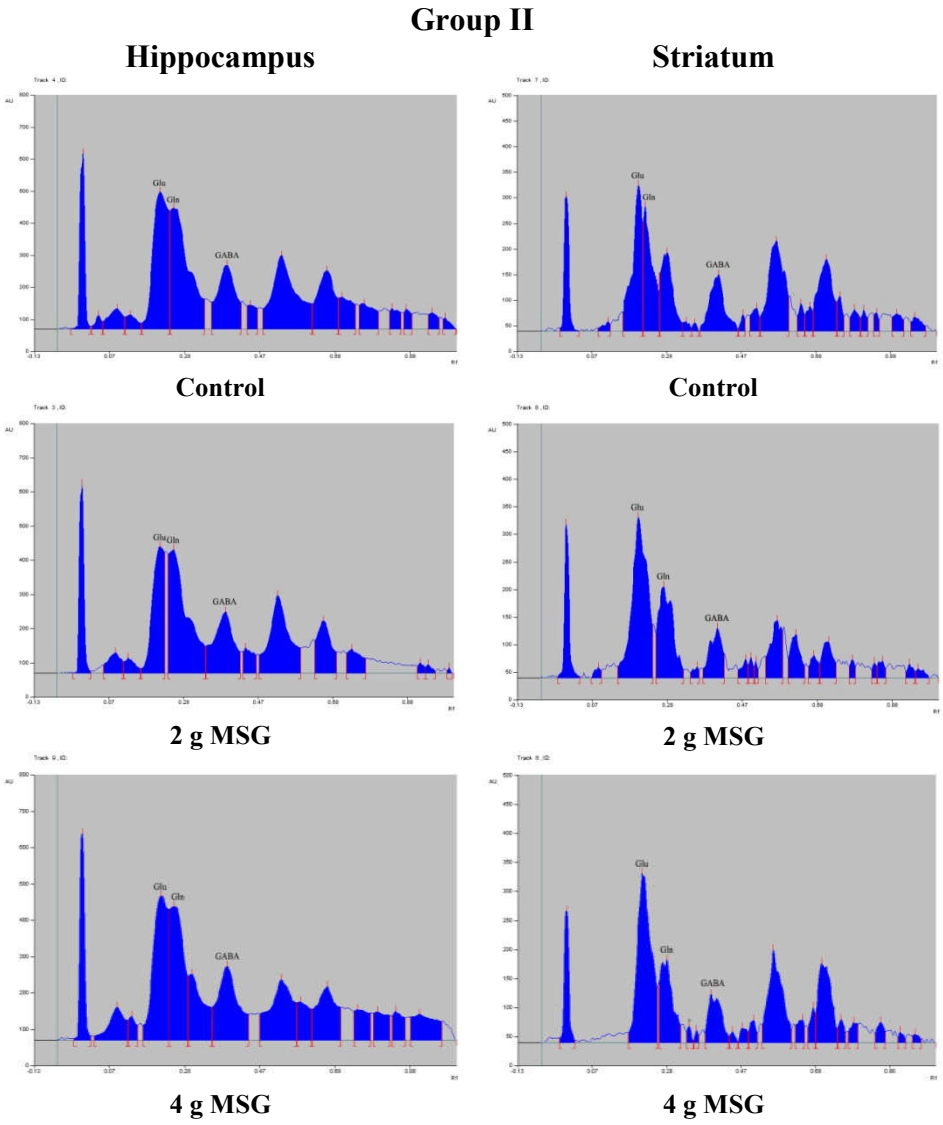


Figure 4.32 shows the effects of MSG on glutamine, glutamate and GABA levelin the striato-hippocampal region of the brain. The glutamine, glutamate and GABA levelin hippocampus and striatum region of group I and II did not show any statistical significance when compared with the control group.

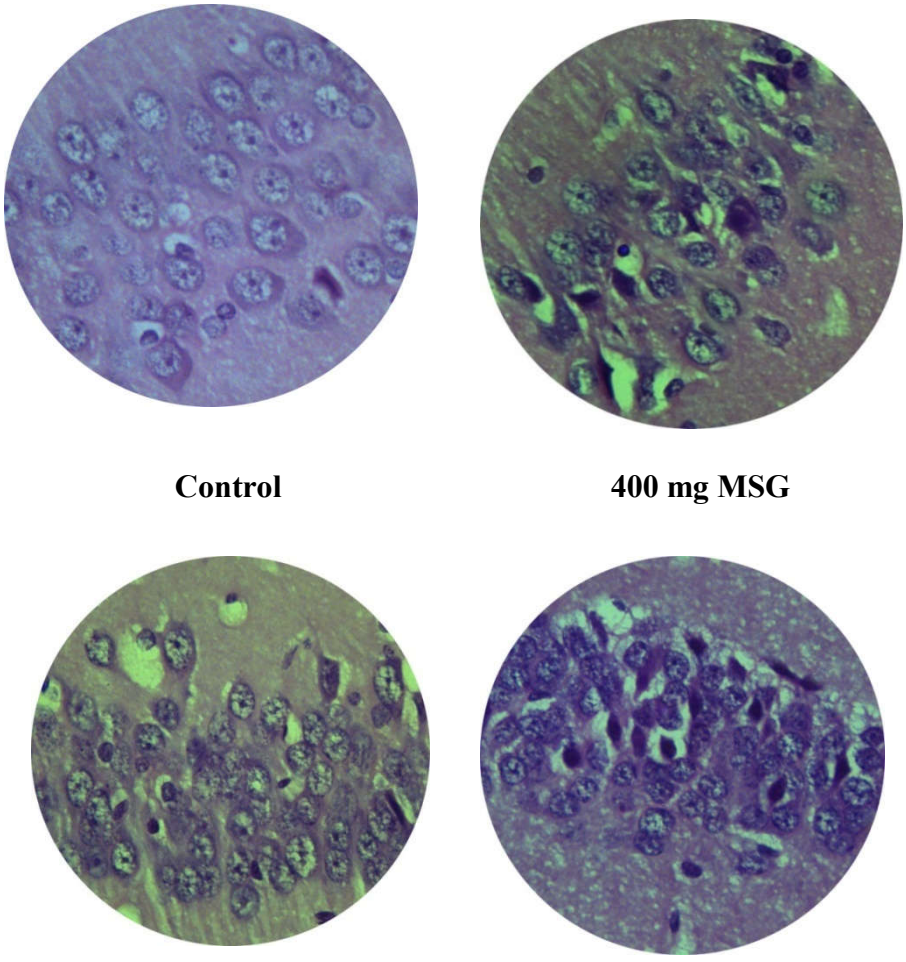
Table 4.1: Retention time and peak area of glutamine, glutamate and GABA level in striato-hippocampal region of the brain using HPTLC.

MSG treated groups		**Hippocampus**					
		Glu		Gln		GABA	
		Rf	A.%	Rf	A.%	Rf	A.%
Group I	Control	16	15.7+0.4	23	16.6+1.2	34	8.2+0.6
	2 g MSG	15	18.08+1.6	23	17.6+1.5	35	8.8+1.4
	4 g MSG	17	14.04+2.2	26	17.26+2.2	37	6.9+1.2
Group II	Control	17	18.76+2.6	23	17.8+0.8	36	7.45+3.4
	2 g MSG	16	14.76+1.2	26	17.2+1.8	37	7.82+2.1
	4 g MSG	17	17.06+3.4	23	16.9+2.5	34	8.03+1.4
MSG treated groups		**Striatum**					
		Glu		Gln		GABA	
		Rf	A.%	Rf	A.%	Rf	A.%
Group I	Control	16	15.82+0.8	26	7.8+2.2	37	7.26+0.8
	2 g MSG	16	15.1+1.8	23	6.52+1.8	34	7.49+2.4
	4 g MSG	16	14.28+2.4	26	6.64+2.8	34	4.62+3.2
Group II	Control	17	13.4+2.1	26	10.02+1	34	6.2+2.1
	2 g MSG	16	17.6+2.1	24	8.8+3.2	37	4.7+1.8
	4 g MSG	17	16.02+1.1	23	7.9+2.8	36	4.64+2.5

Histological studies

Figure 4.33: Histology of hippocampal region of brain of group I rats using Hematoxylin and Eosin staining.

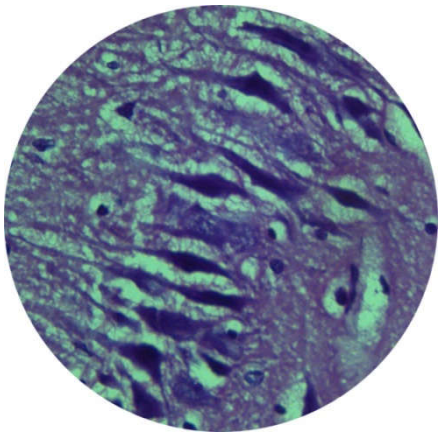
Figure 4.33a: CA1 neurons in the hippocampal region of brain (40X).



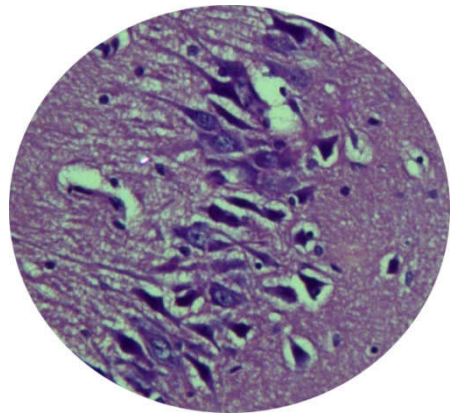
Control **400 mg MSG**

2g MSG **4g MSG**

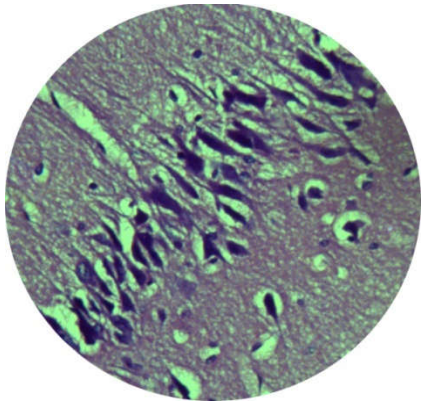
Figure 4.33b: CA2 neurons in the hippocampal region of brain (40X).



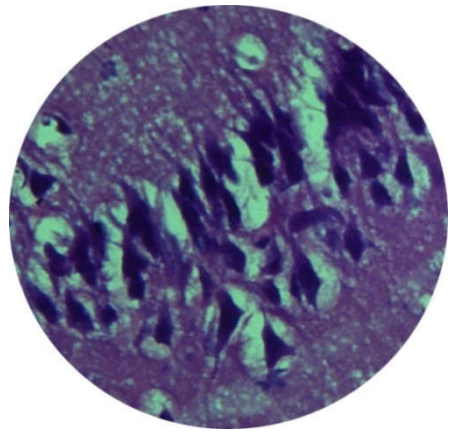
Control



400 mg MSG

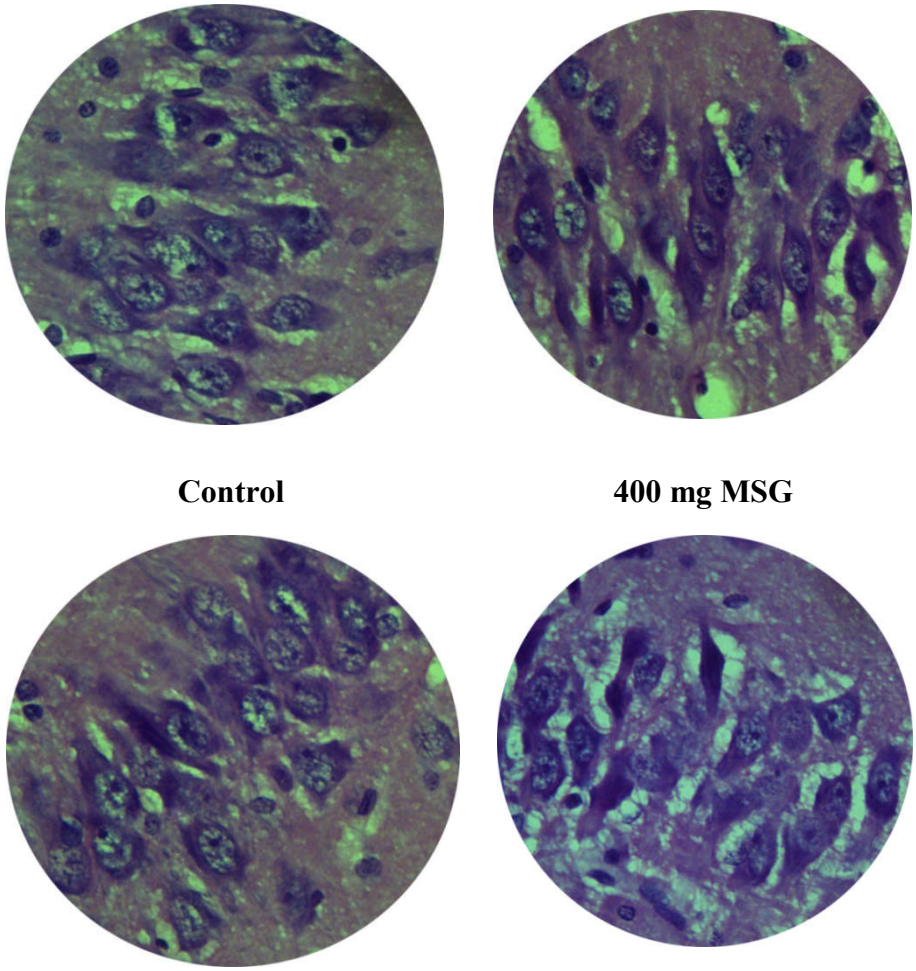


2g MSG



4g MSG

Figure 4.33c: CA3 neurons in the hippocampal region of brain (40X).

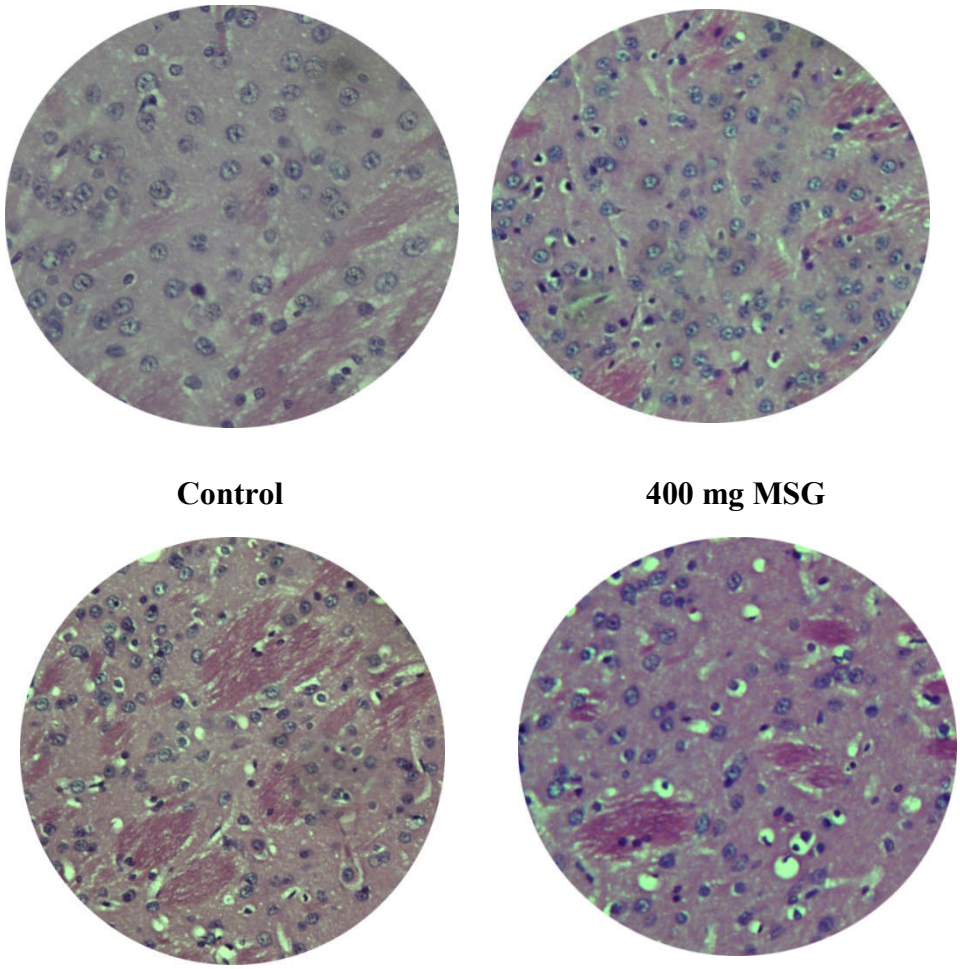


Control **400 mg MSG**

2g MSG **4g MSG**

Figure 4.33 shows the effects of MSG on cytoarchitecture of the hippocampal neuronsin thebrain. CA1, CA2 and CA3 regions did not show any morphological or numerical changes when compared with the control group.

Figure 4.34: Histology of striatal region of brain of group I rats using Hematoxylin and Eosin staining (40X).



Control **400 mg MSG**

2g MSG **4g MSG**

Figure 4.34 shows the effects of MSG on cytoarchitecture of the striatal neurons in thebrain. The neurons in the striatal region did not show any numerical or morphological changes when compared to the control group.

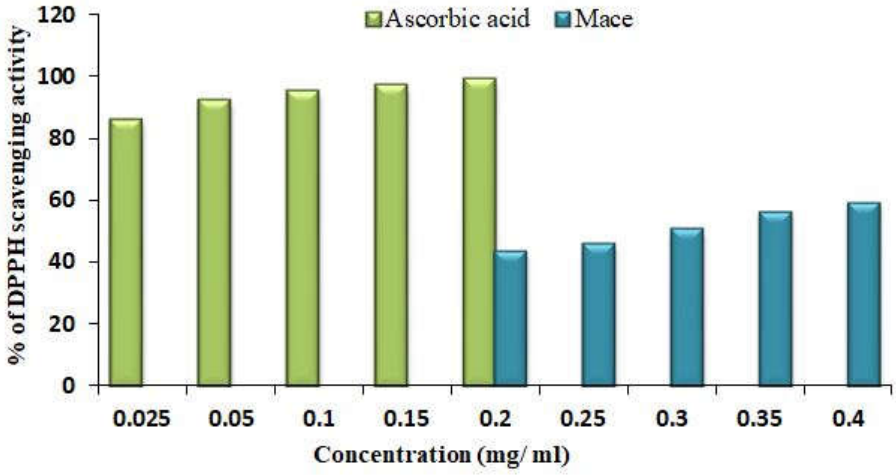
Preliminary Phytochemical Screening

Table 4.2: Preliminary phytochemical screening of the decoction of mace.

Phytochemical components	**Results**
Flavanoids	-
Phenols	+
Tannins	-
Saponins	+
Alkaloids	++
Phlobatannins	-
Terpenoids	+
Glycosides	-
Coumarins	+
Steroids	+
Carbohydrates	++
protein	+
+ : Trace amount, ++ : Moderate amount, +++ : High amount, - : absent	

DPPH radical scavenging assay

Figure 4.35: Antioxidant activity of the mace by DPPH radical scavenging assay.



The antioxidant activity of the decoction of mace was analyzed by DPPH radical scavenging assay and is shown in figure 4.35. The DPPH free radical scavenging activity was expressed as IC_{50}. This is the concentration of the extract (mg/ ml) at which 50 % of DPPH radicals are scavenged. The decoction of mace showed a good DPPH radical scavenging activity with an IC_{50} of 0.3 mg/ ml. The Ascorbic acid is used as a positive control and showed very high DPPH radical scavenging activity with a very low IC_{50} value of less than 0.025 mg/ ml.

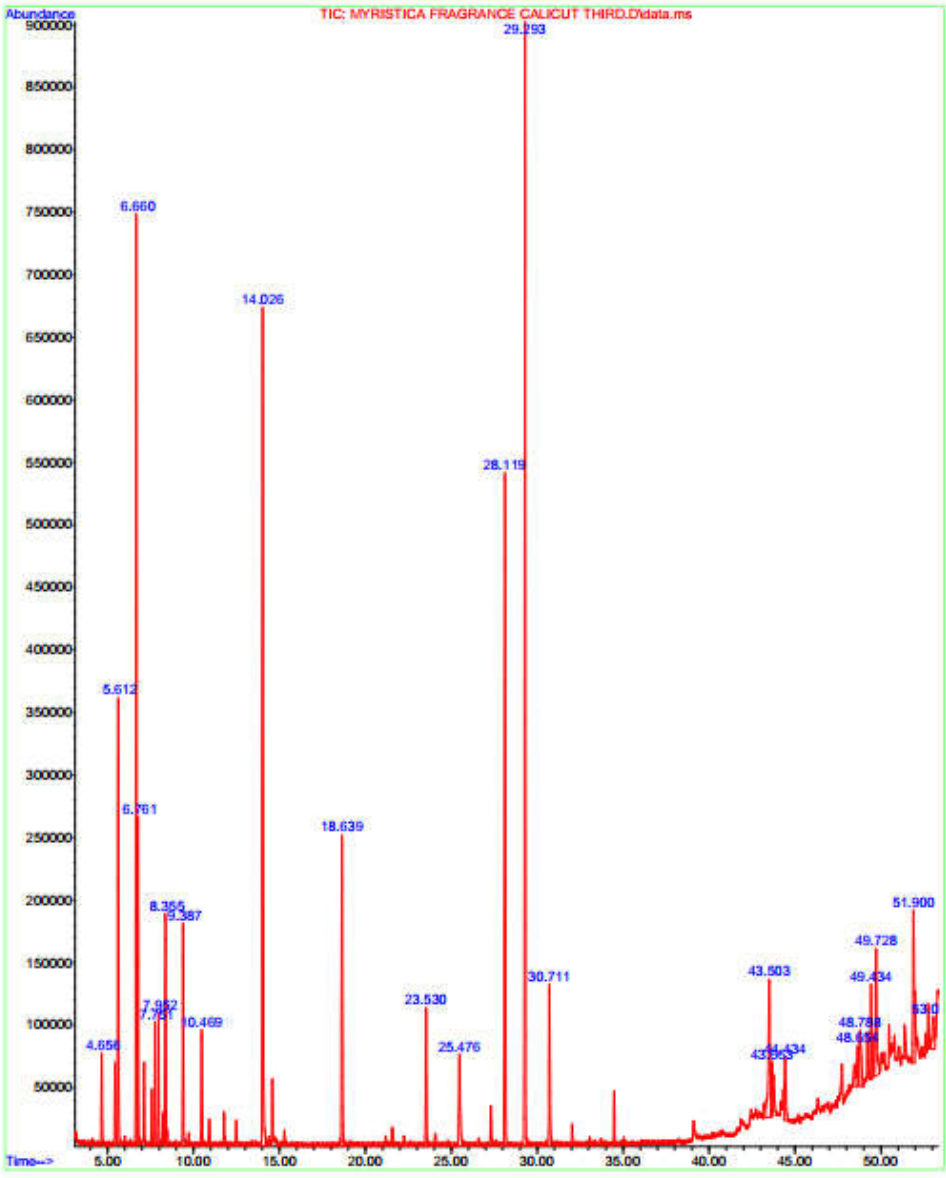
GC-MS fingerprint of the decoction of mace

GC-MS fingerprint of decoction of mace was done and 15 active compounds were identifyed by comparing with the mass spectra of identified compounds and NIST-8 library. The active compounds with their retention time, molecular formula and peak area percentage are summarised in table 4.3 and figure 4.36. The prevailing compounds of the decoction of mace were Terpinen-4-ol (12.6354 %), Elemicin (14.7436 %), Myristicin (10.101 %) and Safrole (4.9104 %).

Table 4.3: Active compounds with their retention time, molecular formula and peak area percentage of GC-MS chromatogram of the decoction of mace.

Sl. No	Name of the compound	Molecular formula	Retention time	Area %
1	α- Pinene	$C_{10}H_{16}$	5.18400	5.18400
2	Sabinene	$C_{10}H_{16}$	6.66040	10.9573
3	β-Pinene	$C_{10}H_{16}$	6.76110	3.91250
4	3-carene	$C_{10}H_{16}$	7.75090	1.44630
5	α-Terpinene	$C_{10}H_{16}$	7.95220	1.63100
6	Limonene	$C_{10}H_{16}$	8.35490	3.43510
7	γ-Terpinene	$C_{10}H_{16}$	9.38670	2.81980
8	Terpinolene	$C_{10}H_{16}$	10.4688	1.59490
9	Terpinen-4-ol	$C_{10}H_{18}O$	14.0256	12.6354
10	Safrole	$C_{10}H_{10}O_2$	18.6394	4.91040
11	Methyleugenol	$C_{11}H_{14}O_2$	23.5299	2.32310
12	Trans-Isoeugenol	$C_{10}H_{12}O_2$	25.4761	2.20990
13	Myristicin	$C_{11}H_{12}O_3$	28.1185	10.1010
14	Elemicin	$C_{12}H_{16}O_3$	29.2929	14.7436
15	Methoxyeugenol	$C_{11}H_{14}O_3$	30.7106	2.60210

Figure 4.36: GC-MS chromatogram of the decoction of mace.



Molecular docking

The active compounds in the decoction of mace identified by GC-MS were docked with NMDA receptor subunits such as GluN1/GluN2A, GluN1A/GluN2B and GluN1B/GluN2B and compared with a well-known NMDA receptor antagonist Memantine. Based on the interaction studies, Myristicin, Elemicin and Safrole are the best ligands against the receptor with best binding energy. The ligand-receptor interactions between selected receptors and best docked ligands by molecular docking is summarised in table 4.4. Figure 4.37 – 4.48 showsthe best binding pose between receptor and ligand was performed by molecular docking using AutoDockVina. The green coloured stick is the ligand and surrounding labeled residues are the amino acids present in the binding cavity. Hydrogen bond is displayed by green coloured thick stick.

Figure 4.37: Best binding pose between GluN1/GluN2A and Myristicin.

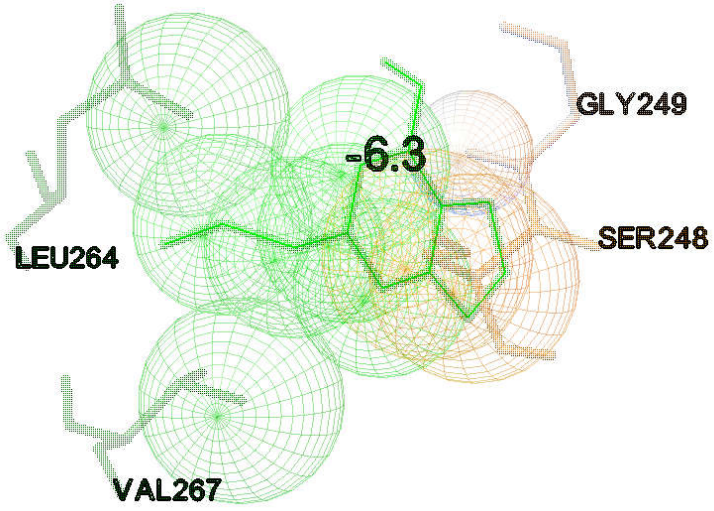


Table 4.4: Results of the ligand-receptor interactions between selected receptors and ligands studied by molecular docking

SL No.	Receptor (PDBID)	Ligand (PubChem ID)	Binding energy (kcal/mol)	Amino acids present in the binding cavity	Number of hydrogen bonds formed	Best Binding pose
1.	GluN1/GluN2A (5VIH)	Myristicin (4276)	-6.3	A chain LEU264, VAL267, GLY249, SER248	Nil	Fig. 4.37
		Elemicin (10248)	-5.6	A chain TYR143, SER248, PHE246, PRO130, VAL267, LEU264	Nil	Fig. 4.38
		Safrole (5144)	-6.5	B chain ANS177, SER173, THR116, ILE136, GLY135	1(ligand)	Fig. 4.39
		Memantine (4054)	-6.0	A chain GLU273, LEU191, TYR184, ILE159, PHE245	Nil	Fig. 4.40
2.	GluN1A/GluN2B(4PE5)	Myristicin (4276)	-6.1	TYR389, LYS454, CYS436, VAL390, TYR164, TRP391	1 (Tyr389)	Fig. 4.41
		Elemicin (10248)	5.9	TRP391, VAL390, TYR164, LYS454, CYS436	1 (ligand)	Fig. 4.42
		Safrole (5144)	-6.1	PRO142, PHE348, ALA349, TYR351, GLY369, TYR367 VAL150	1 (Tyr351)	Fig. 4.43
		Memantine (4054)	-6.0	PRO290, ASP46, ALA100, VAL39, THR44	Nil	Fig. 4.44
	GluN1B/GluN2B(5FXG)	Myristicin (4276)	-6.2	GLY511, GLU412, VAL417, CYS461, CYS467,	1 (Gly 511)	Fig. 34.45
		Elemicin (10248)	-5.9	ALA263, LYS372, PRO270	Nil	Fig. 4.46
		Safrole (5144)	-5.9	VAL419, CYS436, GLU412, THR410	Nil	Fig. 4.47
		Memantine (4054)	6.5	TYR282, GLU284, SER260, GLY264, GLY128, HIS127	1(SER260)	Fig. 4.48

Figure 4.38: Best binding pose between GluN1/GluN2A and Elemicin.

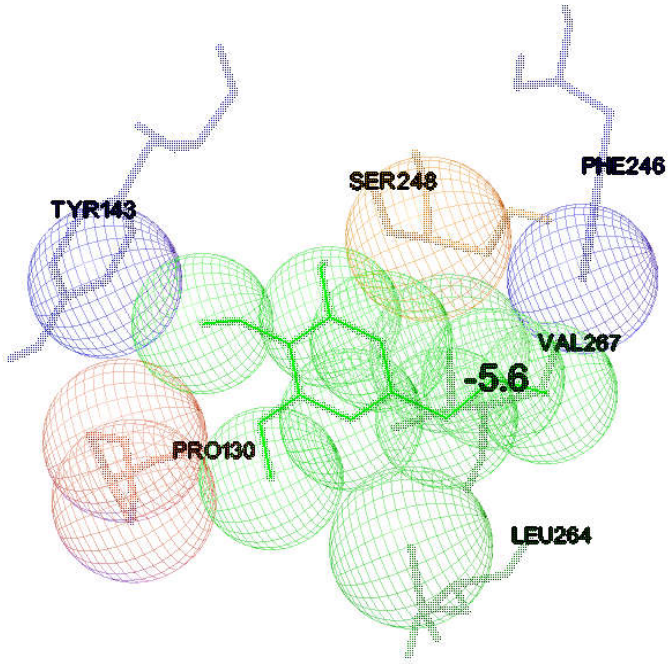


Figure 4.39: Best binding pose between GluN1/GluN2A and Safrole.

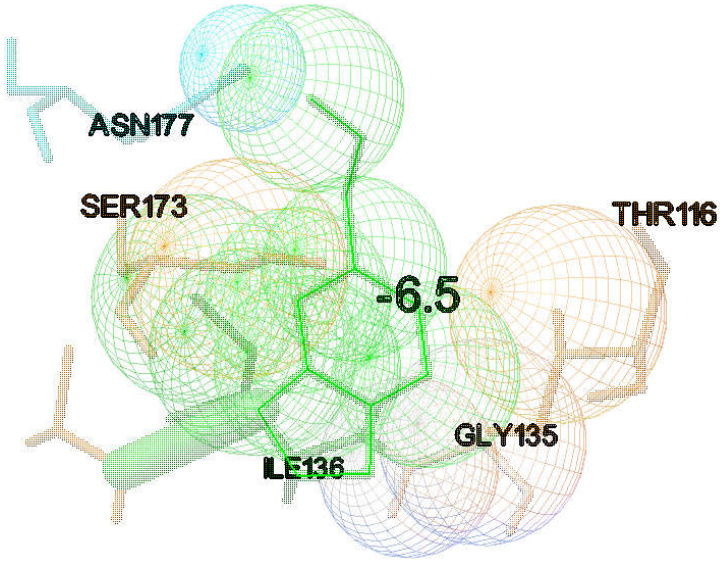


Figure 4.40: Best binding pose between GluN1/GluN2A and Memantine.

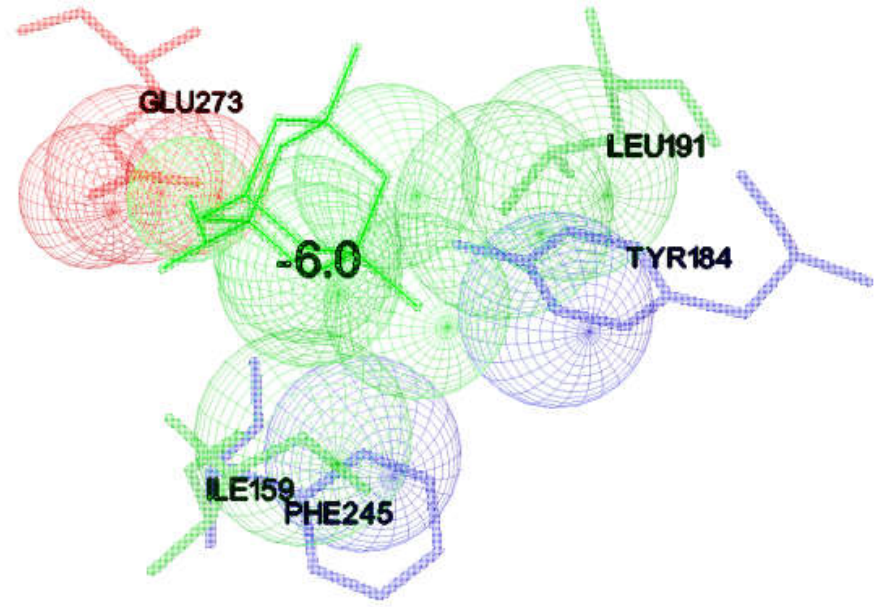


Figure 4.41: Best binding pose between GluN1A/GluN2B and Myristicin.

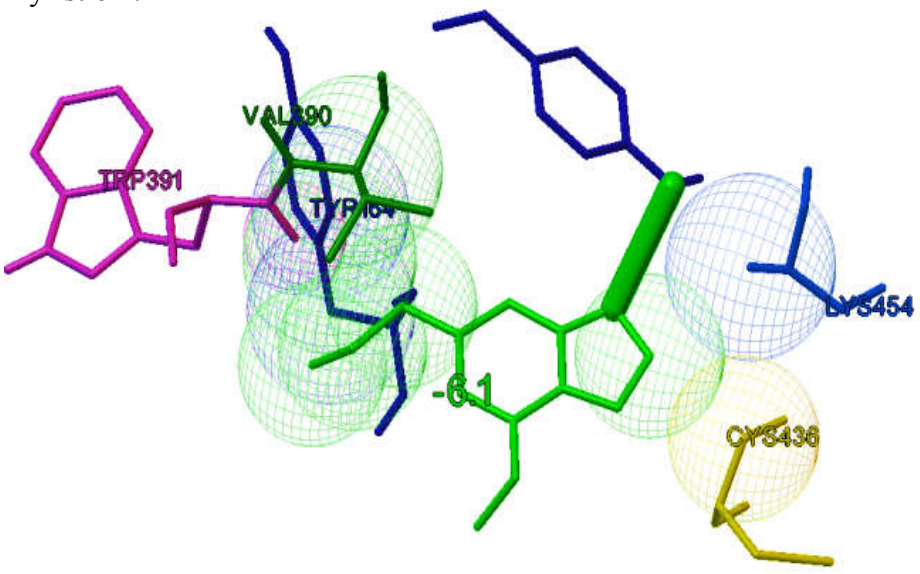


Figure 4.42: Best binding pose between GluN1A/GluN2B and Elemicin.

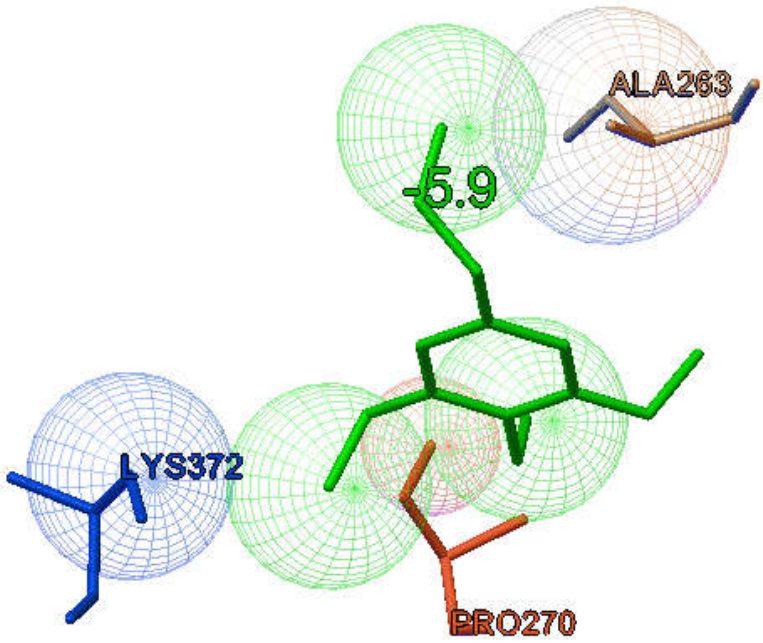


Figure 4.43: Best binding pose between GluN1A/GluN2B and Safrole.

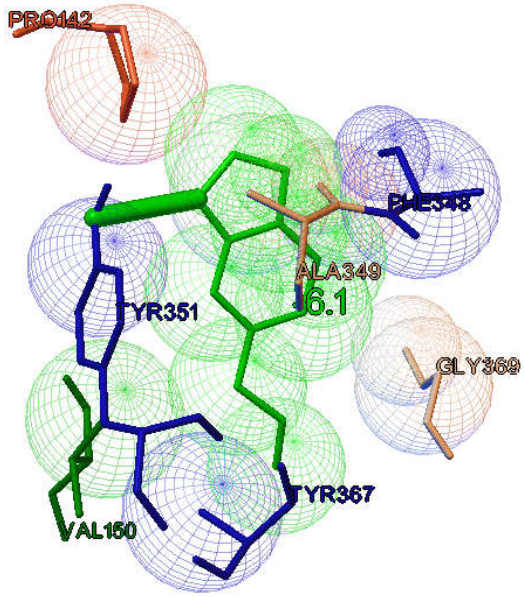


Figure 4.44: Best binding pose between GluN1A/GluN2B and Memantine.

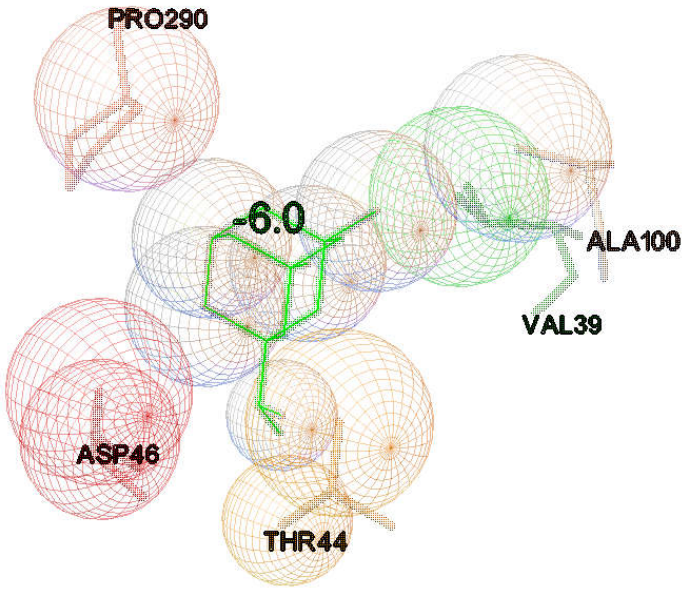


Figure 4.45: Best binding pose between GluN1B/GluN2B and Myristicin.

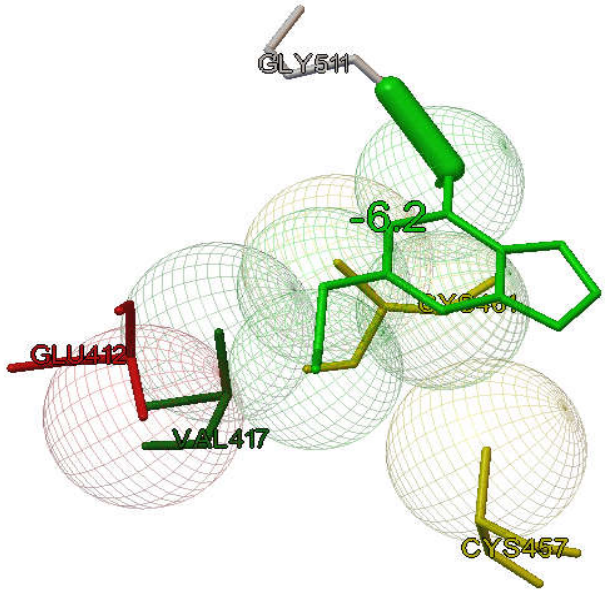


Figure 4.46: Best binding pose between GluN1B/GluN2B and Elemicin.

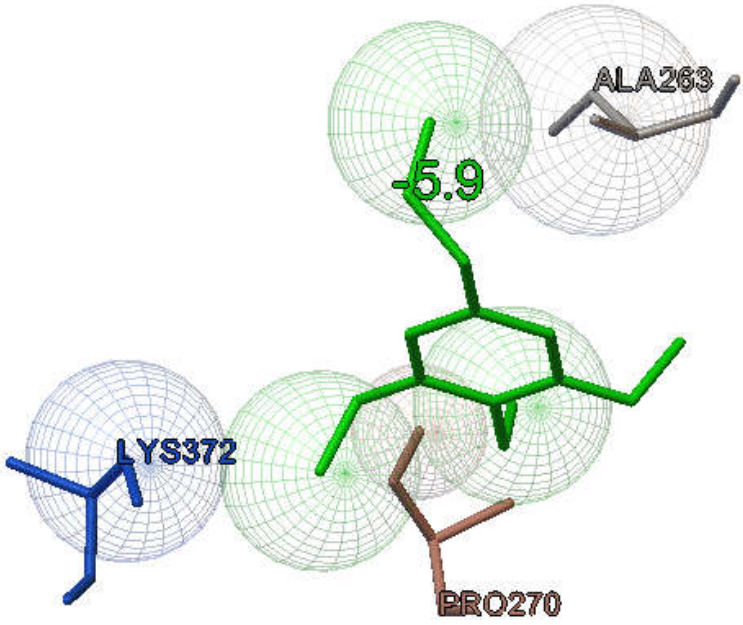
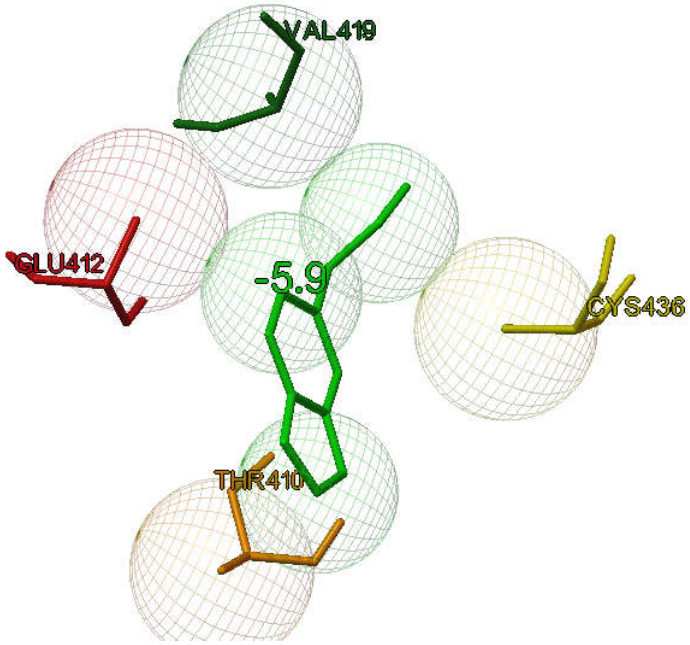


Figure 4.47: Best binding pose between GluN1B/GluN2B and Safrole.



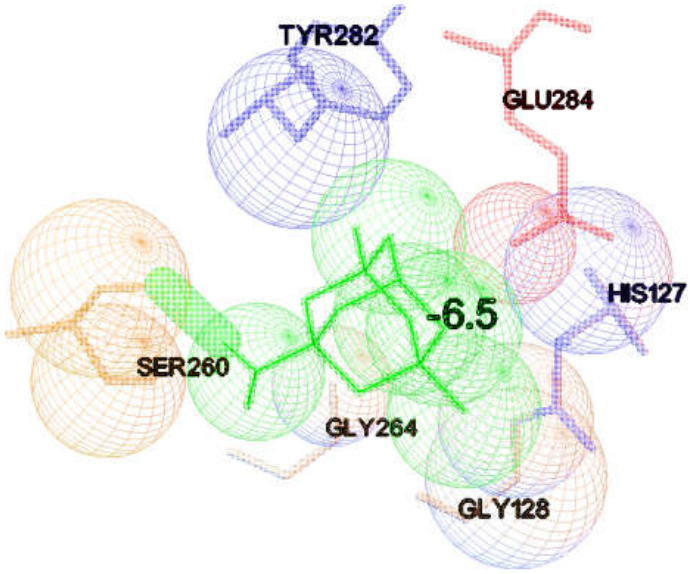


Figure 4.48 Best binding pose between GluN1B/GluN2B and Memantine.

B. Neuroprotective effects of decoction of mace of *Myristicafragrans*Houtt.

Body weight

Figure 4.49: Evaluation of body weight of MSG and mace fed rats **(n=6).**

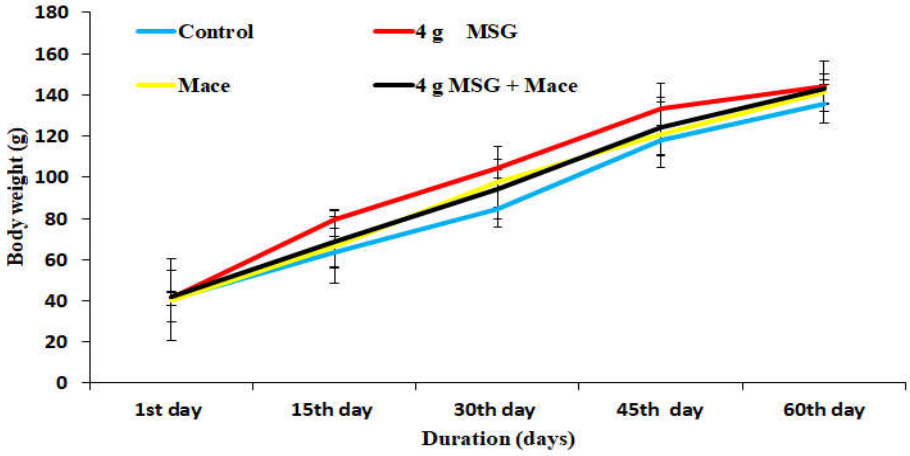
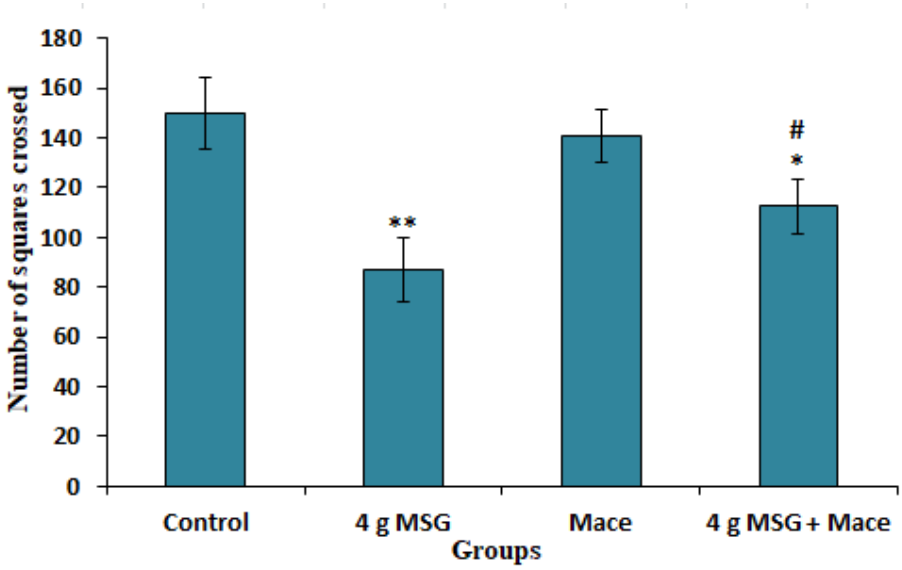


Figure 4.49 shows the comparison of the body weight of the MSG fed rats and MSG along with mace fed rats. In both the groups there did not appear any significant difference in the body weight when compared to the control group or MSG fed group.

Behavioural experiments

Figure 4.50: Number of squares crossed during the six minutes of open field exploration (n = 6).

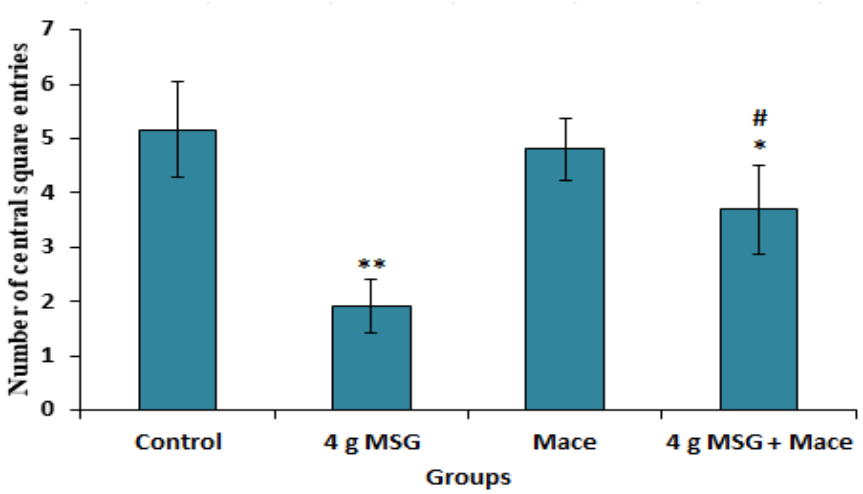


*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.
#p ≤ 0.05 compared with the 4 g MSG group.

Figure 4.50shows the effect of mace on the total number of squares crossed in the open fieldof MSG treated rats. The rats fed with 4 g MSG showed a highly significant ($p<0.01$) decline in the total number of squares crossed in the open field and the rats treated with 4 g MSG along with mace showed a significant ($p<0.05$) decline in the total number of squares crossed in the open field when compared to the

control group. The rats fed with 4 g MSG along with mace also showed a significant ($p<0.05$) increase in the total number of squares crossed in the open field when compared with the 4 g MSG fed rats.

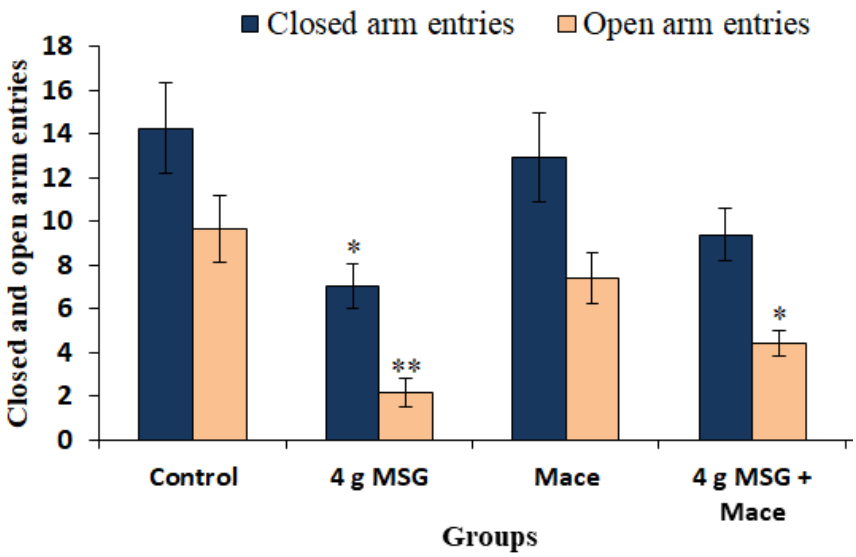
Figure 4.51: Number of center square entries during the six minutes of open field exploration (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.
#p ≤ 0.05 compared with the 4 g MSG group.

Figure 4.51shows the effect of mace on the total number of central square entries in the open fieldof MSG treated rats. The rats fed with 4 g MSG showed a highly significant ($p<0.01$) decline in the number of central square entries in the open field, whereas the rats fed with 4 g MSG along with mace showed a significant ($p<0.05$) decline in the total number of central square entries in the open field when compared to the control group. The rats fed with 4 g MSG along with mace also showed a significant ($p<0.05$) increase in the total number of central square entries in the open field when compared with the 4 g MSG fed rats.

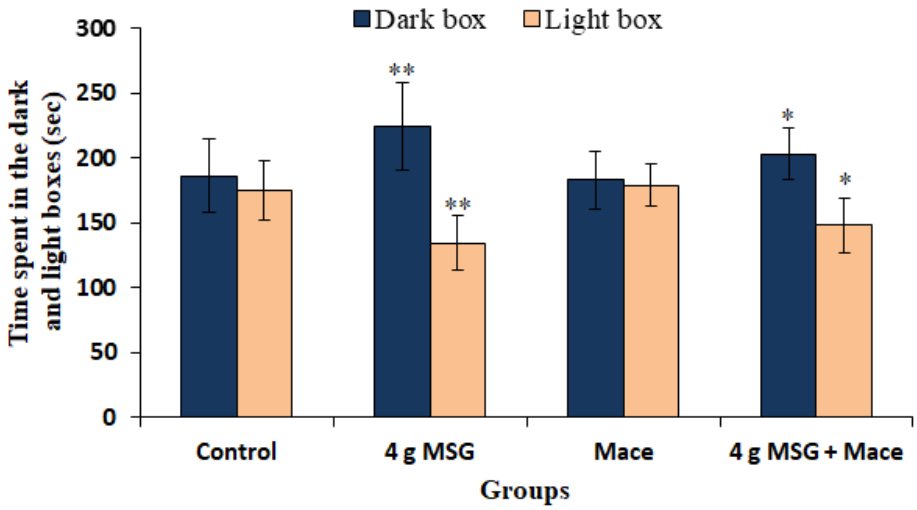
Figure 4.52: Number of closed and open arm entries during the six minutes of exploration in elevated plus maze (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.

Figure 4.52shows the effect of mace on the number of entries into the open and closed arms in the elevated plus mazeof MSG treated rats. The rats fed with 4 g MSG showed a significant ($p<0.05$) decline in the number of closed arm entries and a highly significant ($p<0.01$) decline in the number of open arm entries, whereas the rats fed with 4 g MSG along with mace showed a significant ($p<0.05$) decline in the number of open arm entries and no significant difference was observed in closed arms when compared to the control group.

Figure 4.53: Duration of stay in the dark and light box during the six minutes of exploration in the dark/ light transition apparatus (n =6).

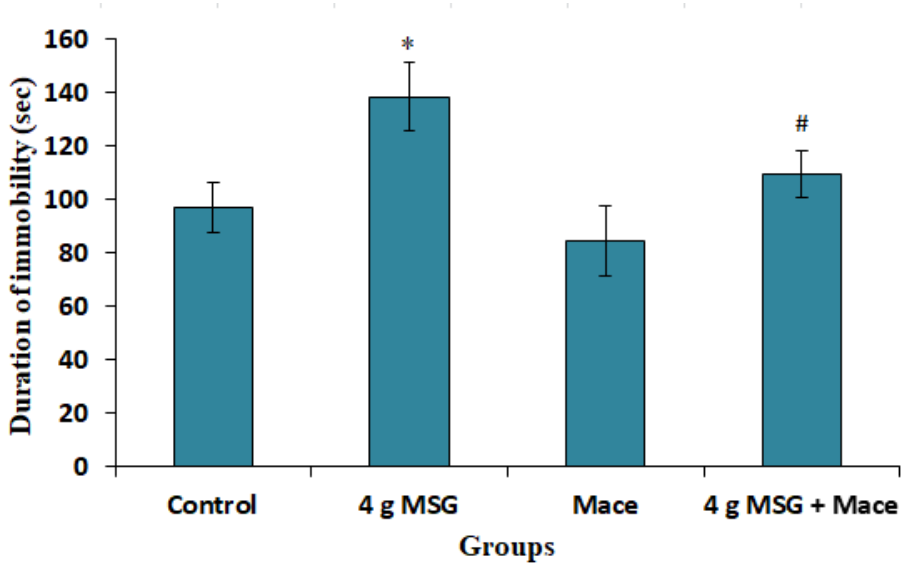


*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.

Figure 4.53shows the effect of mace on the duration of stay in the dark and light box of dark/ light transition apparatus of MSG treated rats. The rats fed with 4 g MSG showed a very high significant ($p<0.01$) increase in the duration of the time spent in the dark box and a very highly significant ($p<0.01$) decline in the duration of the time spent in the light box when compared to the control group. The rats fed with 4 g MSG along with mace showed a significant ($p<0.05$) increase in the duration of the time spent in the dark box and the significant decline in the duration of the time spent in the light box when compared with the control group. However, the rats fed with 4 g MSG along with mace showed a non-significant decrease in the duration of the time spent in the dark box and a non-significant

increase in the duration of the time spent in the light box when compared with the 4 g MSG fed rats.

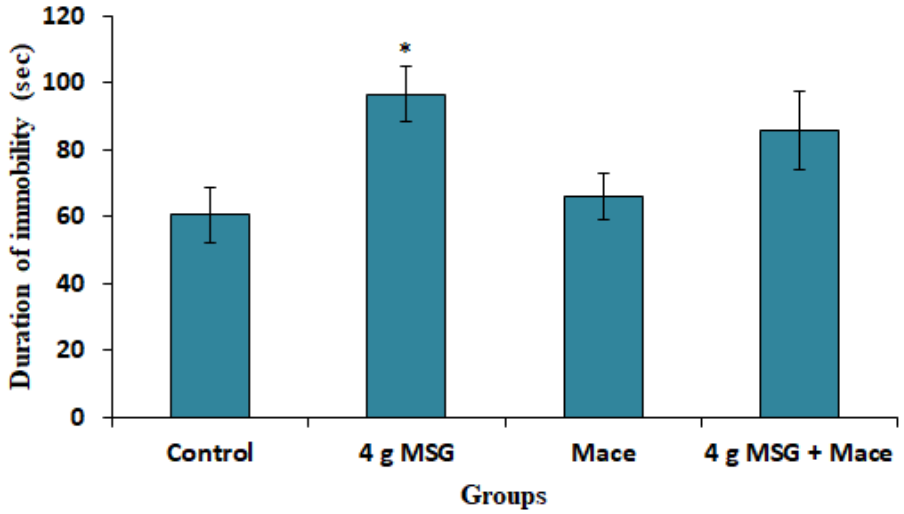
Figure 4.54: Immobility time during the six minutes of forcedswim test (n = 6).



*p ≤ 0.05 compared with the control rats.
#p ≤ 0.05 compared with the 4 g MSG group.

Figure 4.54shows the effect of mace on the immobility time during the forced swim of MSG treated rats. The rats fed with 4 g MSG showed a significant ($p<0.05$) increase in the immobility time and the rats fed with 4 g MSG along with mace did not show any significance when compared with the control group. However, the rats fed with 4 g MSG along with mace showed a significant ($p<0.05$) decrease in the immobility time when compared with the 4 g MSG fed rats.

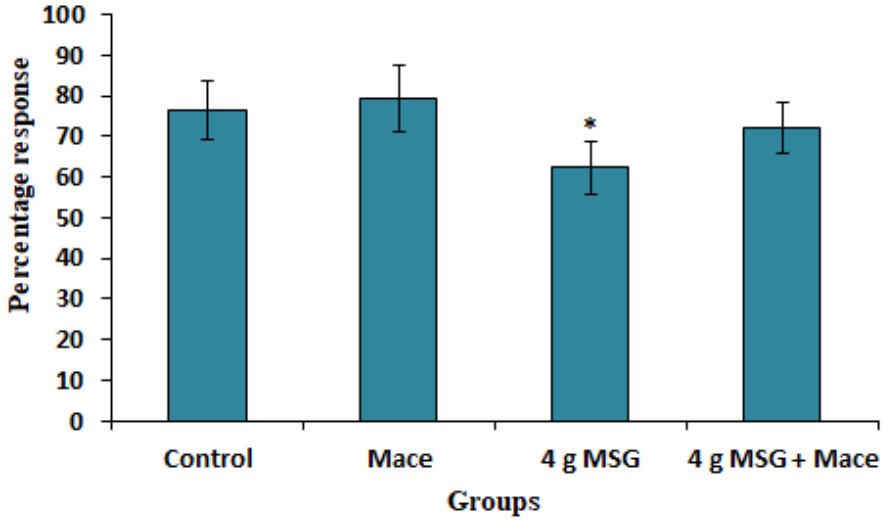
Figure 4.55: Immobility time during the six minutes of tail suspension test(n = 6).



*p ≤ 0.05 compared with the control rats.

Figure 4.55shows the effect of mace on the immobility time of MSG treated rats during the tail suspension**.** The rats fed with 4 g MSG showed a significant ($p<0.05$) increase in the immobility time, while the rats fed with 4 g MSG along with mace did not show any significance when compared with the control group. However the rats fed with 4 g MSG along with mace showed a non-significant decrease in the duration of immobility when compared with the 4 g MSG fed rats.

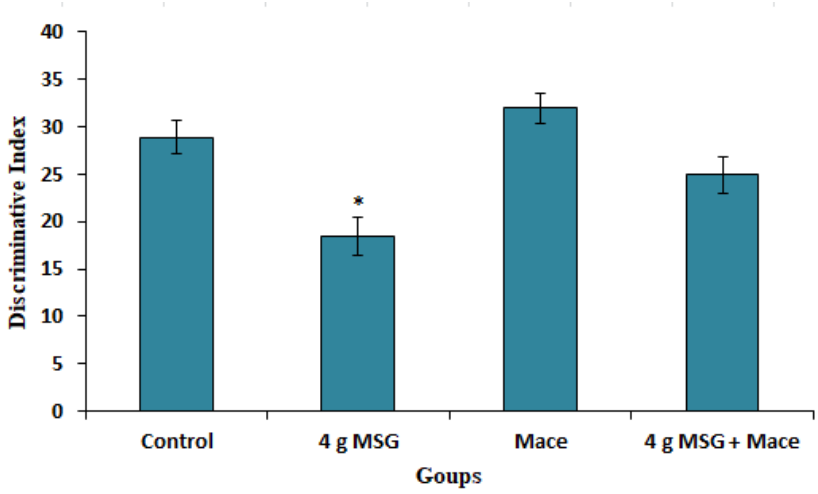
Figure 4.56: Percentage response in the T maze exploration (n = 6).



*p ≤ 0.05 compared with the control rats.

Figure 4.56 shows the effect of mace on the T maze exploration of MSG treated rats. The rats fed with 4 g MSG showed a significant ($p<0.05$) decline in the percentage response, whereas the rats fed with MSG along with mace did not show any statistical significance when compared to the control group. It was also showed that, the MSG fed rats along with mace had an improved performance than MSG alone, when compared to the control group.

Figure 4.57: Discrimination index during the six minutes of novel object recognition test(n = 6).

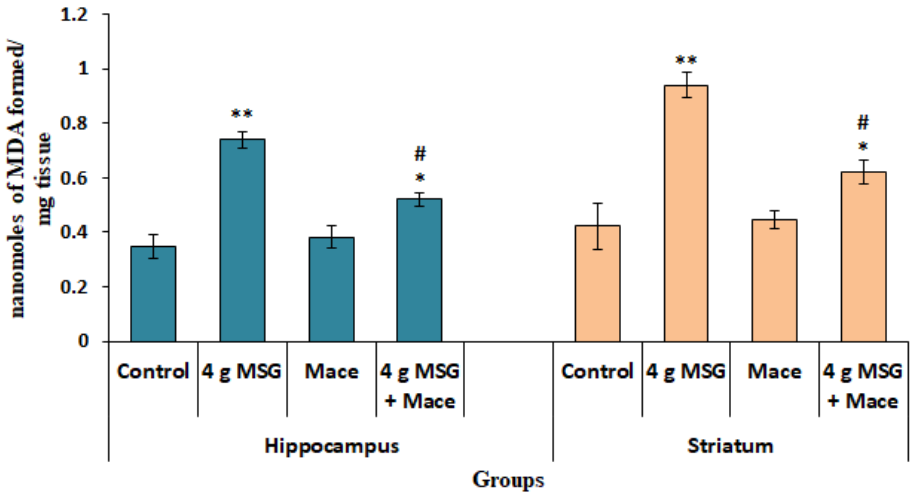


*p ≤ 0.05 compared with the control rats of group.

Figure 4.57 shows the effect of mace on the discrimination index of novel object recognition test of MSG treated rats. The rats fed with 4 g MSG showed a significant ($p<0.05$) decline in the discrimination index, whereas the rats fed with MSG along with mace did not show any statistical significance when compared to the control group. It showed a non-significant increase in discrimination index of MSG along with mace fed rats when compared with 4 g MSG fed rats.

Biochemical analysis

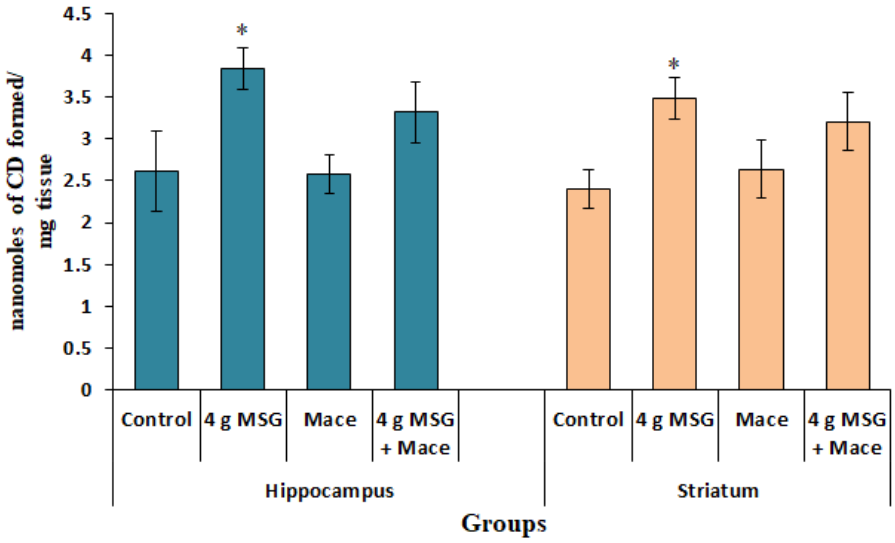
Figure 4.58: Lipid peroxidation marker malondialdehyde level in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.
#p ≤ 0.05 compared with the 4 g MSG group.

Figure 4.58 shows the effect of mace on MDA level in the striato-hippocampal region of MSG fed rats. The striatum and hippocampal region of the brain showed a highly significant ($p<0.01$) increase in the MDA activity in 4 g MSG fed rats and a significant ($p<0.05$) MDA activity was observed in 4 g MSG fed rats along with mace when compared to the corresponding control groups. The rats fed with 4 g MSG along with mace also showed a significant ($p<0.05$) decline in the MDA level when compared with the 4 g MSG fed rats.

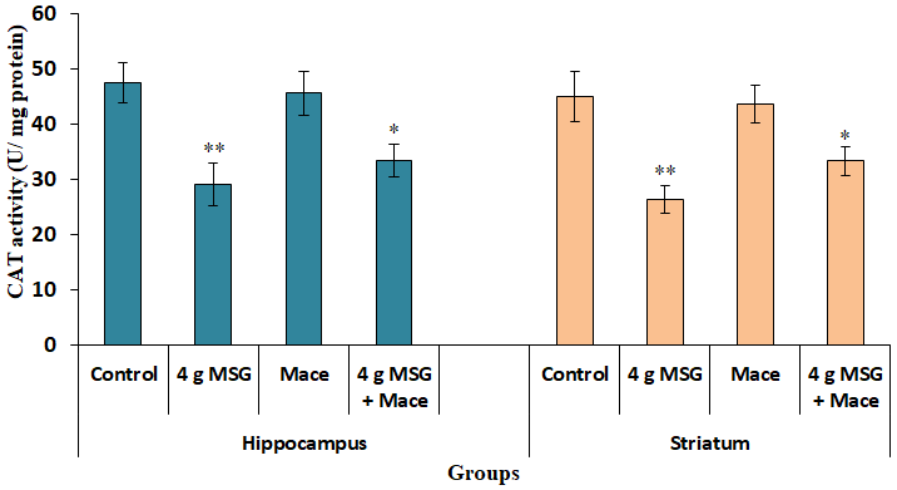
Figure 4.59: Lipid peroxidation marker conjugated dienes level in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 compared with the control rats

Figure 4.59 shows the effect of mace on CD level in the striato-hippocampal region of MSG fed rats. The striatum and hippocampal region of the brain showed a significant ($p<0.05$) increase in the CD activity in 4 g MSG fed rats, whereas the rats fed with MSG along with mace did not show any statistical significance when compared to the control group.

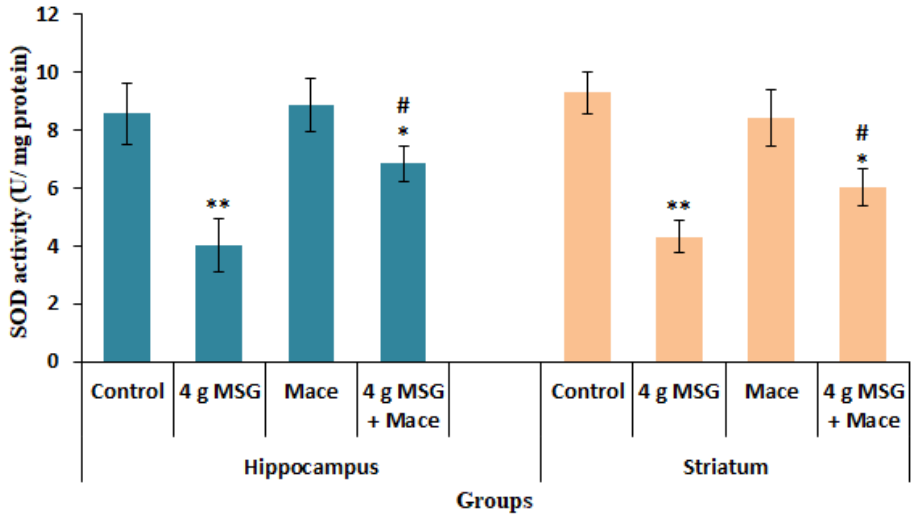
Figure 4.60: Antioxidant enzyme catalase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.

Figure 4.60 shows the effect of mace on CAT level in the striato-hippocampal region of MSG fed rats. The striatum and hippocampal region of the brain showed a highly significant ($p<0.01$) increase in the CAT activity in 4 g MSG fed rats and a significant ($p<0.05$) CAT activity was observed in 4 g MSG along with mace fed rats when compared to the corresponding control groups.

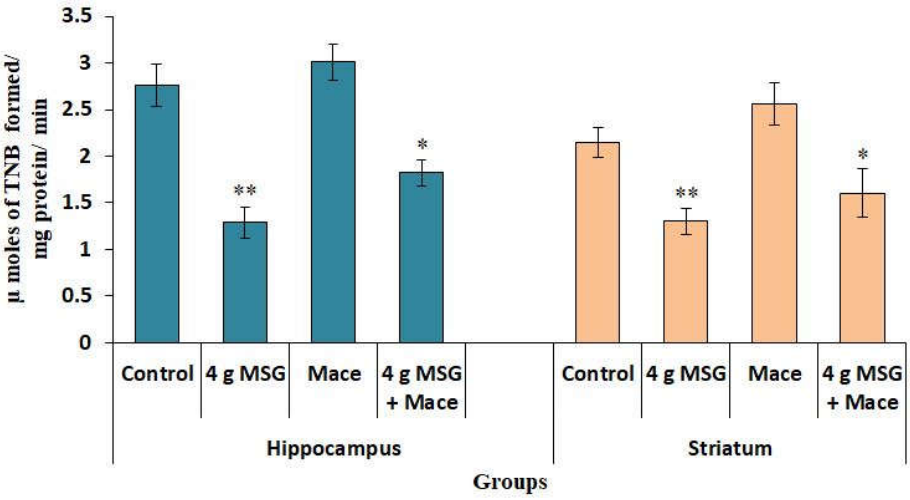
Figure 4.61: Antioxidant enzyme superoxide dismutase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.
#p ≤ 0.05 compared with the 4 g MSG group

Figure 4.61 shows the effect of mace on SOD level in the striato-hippocampal region of MSG fed rats. The striatum and hippocampal region of the brain showed a highly significant ($p<0.01$) increase in the SOD activity in 4 g MSG fed rats and a significant ($p<0.05$) SOD activity was observed in 4 g MSG along with mace fed rats when compared to the corresponding control groups. The rats fed with 4 g MSG along with mace also showed a significant ($p<0.05$) increase in the SOD activity when compared with the 4 g MSG fed rats.

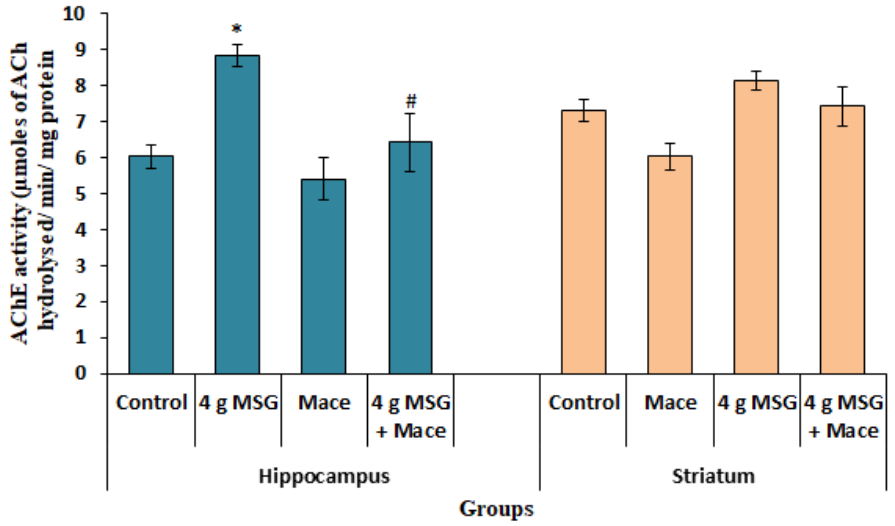
Figure 4.62: Antioxidant glutathione level in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 and **p ≤ 0.01 compared with the control rats.

Figure 4.62 shows the effect of mace on GSH level in the striato-hippocampal region of MSG fed rats. The striatum and hippocampal region of the brain showed a highly significant ($p<0.01$) increase in the GSH level in 4 g MSG fed rats and a significant ($p<0.05$) GSH level was observed in 4 g MSG along with mace fed rats when compared to the corresponding control groups.

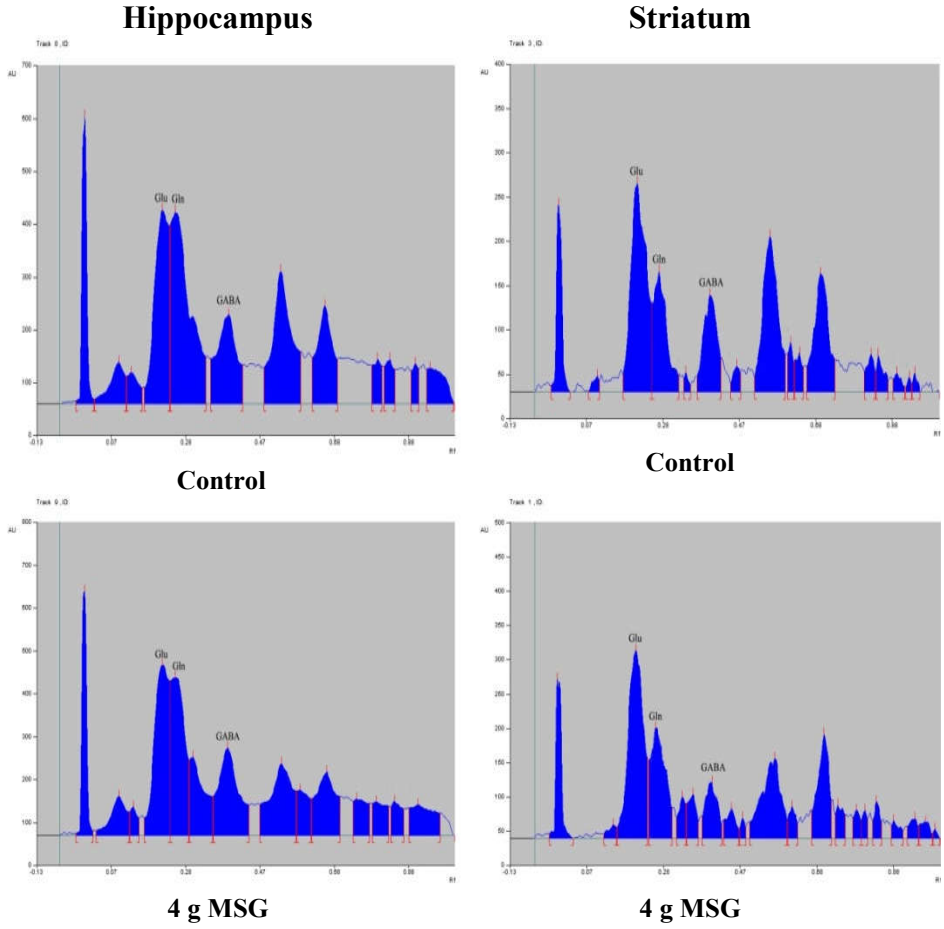
Figure 4.63: Enzyme Acetylcholinesterase activity in the striato-hippocampal region of the brain (n = 6).



*p ≤ 0.05 compared with the control rats of group I.
#p ≤ 0.05 compared with the 4 g MSG group.

Figure 4.63 shows the effect of mace on AChE level in the striato-hippocampal region of MSG fed rats. In the hippocampal region, the rats fed with 4 g MSG showed a significant ($p<0.05$) increase in the AChE activity and no significant activity was observed in 4 g MSG along with mace fed rats when compared to the control group. However the rats fed with 4 g MSG along with mace showed a significant ($p<0.05$) decline in AChE activity when compared with the 4 g MSG fed rats. In striatum, both 4 g MSG fed rats and 4 g MSG along with mace fed rats did not show any statistical significance when compared to the corresponding control groups.

Figure 4.64: Estimation of glutamine, glutamate, and GABA level in striato-hippocampal region of the brain using HPTLC.



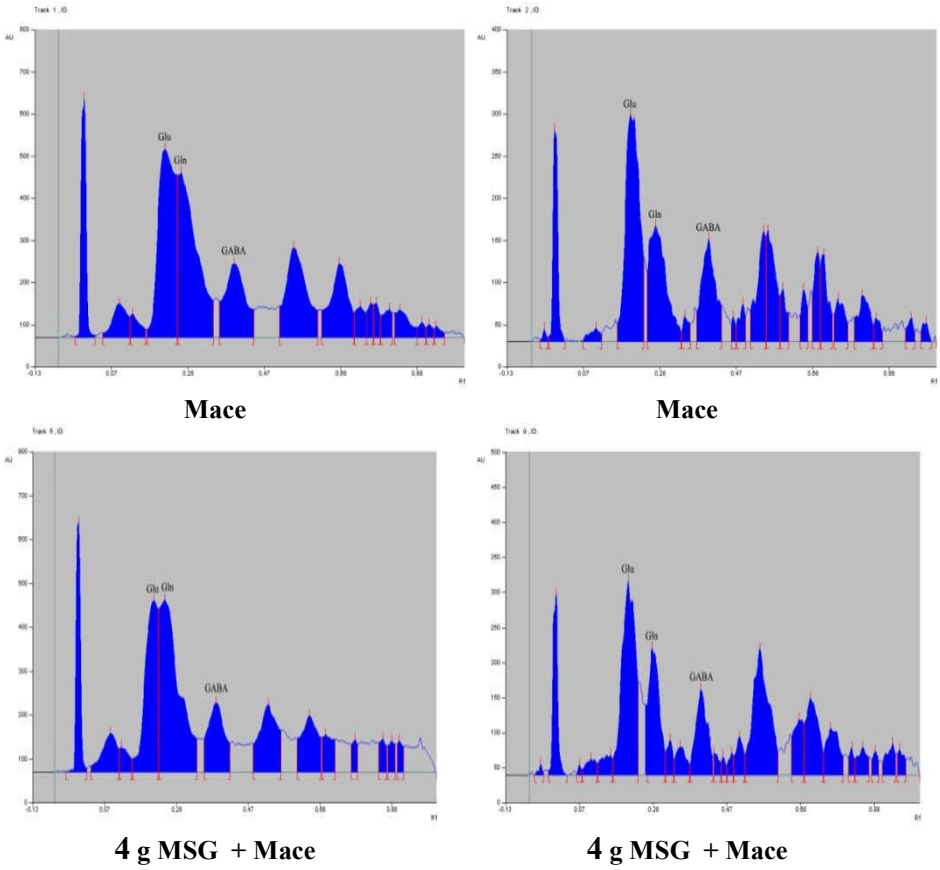


Figure 4.64 shows the effects of MSG along with mace on glutamine, glutamate and GABA levelin the striato-hippocampal region of the brain. The glutamine, glutamate and GABA levelin hippocampus and striatum region of group I and II did not show any statistical significance when compared with the control group.

Table 4.5: Retention time and peak area of glutamine, glutamate and GABA level in striato-hippocampal region of the brain using HPTLC.

MSG treated groups	**Hippocampus**					
	Glu		Gln		GABA	
	Rf	A.%	Rf	A.%	Rf	A.%
Control	16	15.7+0.4	23	16.6+1.2	34	8.2+0.6
4 g MSG	17	14.04+2.2	26	17.26+2.2	37	6.9+1.2
Mace	16	16.5+1.8	25	16.2+3.02	37	6.5+0.9
4 g MSG + Mace	16	15.2+1.2	25	16.9+2.3	36	6.2+2.4
MSG treated groups	**Striatum**					
	Glu		Gln		GABA	
	Rf	A.%	Rf	A.%	Rf	A.%
Control	16	15.82+0.8	26	7.8+2.2	37	7.26+0.8
4 g MSG	16	14.28+2.4	26	6.64+2.8	34	4.62+3.2
Mace	17	14.8+2.6	27	7.2+3.1	34	6.9+3.4
4 g MSG + Mace	15	14.6+1.8	27	6.8+1.2	34	6.1+1.8

DISCUSSION

Monosodium glutamate is a sodium salt of L-glutamate, a well-known flavor enhancer and a source of the fifth taste "*umami*". Because of its palatability and taste enhancing capacity, it is an essential part of the human diet and found in most Asian diets. Children are more addictive to the foods such as chips, soups, noodles, meats, canned foods etc., which are marinated by MSG. Glutamate is a very essential substance for our body, it is never possible to discriminate the glutamate coming from the monosodium glutamate and other natural foods. It is an important oxidative fuel for enterocytes and additionally is a precursor for many essential substances in the body including arginine, proline, glutathione etc. (Jinap&Hajeb, 2010). It is an abundant excitatory neurotransmitter in brain acting through NMDA and AMPA receptors and plays a pivotal role in learning and memory (Riedel *et al*., 2003).

In addition to the food industry, humans are exposed to glutamate through cosmetics, pharmaceutical, fertilizers, pesticides etc. The use of MSG in food has no much restrictions or regulations in many countries. The restaurants and other food industries for increasing taste and deliciousness, for their huge profits, adding MSG in food without any proper measurement, thus the amount of glutamate entering into the body is much higher than the normal limit. Young children are more vulnerable to the risk of MSG, because their blood brain barrier is not much developed and has poor capacity to prevent

toxins. In 1958 the U.S. Food and Drug Administration has determined MSG as a safe ingredient for the general population and has not set a specified acceptable daily intake. However, an unknown percentage of the population might react to MSG and develop symptoms that constitutes a syndrome including burning sensation at the back of the neck, forearms, chest, headache, chest pain, facial pressure /tightness, nausea, palpitation, numbness at the back of the neck, radiating to the arms and back, tingling, warmth, weakness in face, temples, upper back, neck and arms, drowsiness and weakness collectively called MSG symptom complex (Walker and Lupien, 2000). Animal studies also showed that high dose of MSG administration increases blood glutamate level and cause damages to vital organs of the body (Bogdanov*et al*., 1996). The present study investigated the dose dependent effect of oral ingestion of monosodium glutamate on the striato-hippocampal region of the brain of male *Wistar albino* rats and its long term implications after MSG treatment by investigating the behavioural alterations, changes in the brain antioxidants, lipid peroxidation status, neurochemicals and changes in the histo-architecture of striato-hippocampal region of brain. It also assessed the neuroprotective effect of *Myristicafragrans*Houtt.

The present study in group I and II did not show any significant increase in the body weight when compared to the control group which is supported by Tordoff *et al.,* (2012), where it was shown that body weight of a total of 32 groups of 10-12 adult rats or mice given various doses of MSG does not alter the body weight or body composition. On

the contrary, studies also reported that long term administration of MSG increases body weight associated with obesity, fat accumulation, increased cholesterol, dyslipidemia and insulin resistance (Alarcon-Aguilar *et al*., 2007; Lobato *et al*., 2011; Sari *et al*., 2018). A clinical trial conducted by Anderson and co-workers showed that MSG increase fullness and reduced desire to eat (Anderson *et al*., 2018). In rodents, experimental studies showed that arcuate nucleus and median eminence of the hypothalamus was the most prone area for MSG toxicity and obesity but fail to show hyperphagia (Olney, 1969; Seress, 1982; Dawson and Annau, 1983). Hyperphagia is a direct function of a hormone Ghrelin, acting through the arcuate nucleus of the hypothalamus (Tamura *et al*., 2002).

Animal studies indicated that high dose of MSG has the capacity to induce hypothalamic damage in the brain and leptin resistance, essential for food intake and energy balance, can cause over weight and obesity. Dawson *et al*., (1997) showed that neonatal administration of MSG for fourteen days damaged the arcuate nucleus of hypothalamus and subsequent increase in body weight and neuroendocrine dysfunction. Holzwarth-Mcbride *et al*., (1976) showed that postnatal administration of MSG from 5^{th} to 10^{th} day decreased the perikarya in the arcuate nucleus of adult mice resulted in a reduced gonadotropic hormone release along with obesity. Subcutaneous injection of MSG to 4 days old mice at a dose of 2 mg/ g body weight showed an immediate peak upsurge in glutamate level at arcuate nucleus than any other parts of the thalamus and hypothalamus. This transient accumulation of subcutaneously administered glutamate

might be selectively destroying the hypothamic anterior neuronal perikarya(Perez and Olney, 1972). Neonatal administration of MSG produces hypothalamus induced obesity (Braga *et al*., 2001). Destruction of hypothalamic nucleus by the neonatal MSG administration also cause dysfunctioning of hypothalamic–pituitary-thyroid axis associated with reduction in growth and obesity (Miskowiak and Partyka, 1993). Another study evidenced that an intravenous administration of MSG produces obesity, increased blood glucose, insulin, total cholesterol and triglyceride levels (Nagata *et al*., 2006), but it did not show any polyphagia which may be due to the excessive stimulation of the vagus nerve (Balbo *et al*., 2000).

Behavioural studies

Anxiety and depression disorders are the most common multifactorial psychiatric concern in the society. Both disorders show a diverse set of symptoms, but they are amalgamated and often occur together to experience a complex set of emotional and functional challenges. The prevalence of anxiety and depression appeared to have increased over the past years and this may be considered as an artifact of the modernity and change in food habits. Early studies had reported that food additives especially monosodium glutamate, most widely used, is one among the cause for this anxiety and depression. The anxiety-like behavioral in rodents are elicited by using psychopharmacological tests such as open field test (OF), elevated plus maze (EPM), dark/light transition test (DLT), free-exploratory paradigm (FEP) and marble burying test exploiting the natural aversion of rodents to unprotected open areas (Carola *et al.,* 2002). The basic

instinct of rodents to explore novel environments and an innate aversion from large areas, brightly lighted areas, open and elevated environments, were considered as dangerous environments. A significant avoidance from these areas and increased thigmotaxis (tendency to stay close to the walls) considered as anxiety like behaviour (Campos *et al.,* 2013; Seibenhener and Wooten, 2015).

The results of this study showed that in group I, oral administration of MSG at a dose of 2 g and 4 g/ Kg body weight daily for sixty days, altered behaviour and showed an anxiety like behaviour in rats. However, in group II, sixty days of MSG feeding period followed by sixty days of non MSG feeding period, showed a positive alteration in behaviour which indicates a reduction in anxiety. However, it does not show a complete recovery from anxiety like behaviour although it observed that the slow decline in anxiety like behaviour taken as a positive sign of recovery.

MSG induced changes in the locomotor activities of group I was demonstrated by using OF test and showed a significant reduction in the number of squares crossed and increased immobility period in the OF. In addition to this a reduced locomotor activity was also demonstrated by using EPM and DLT, which showed a reduced number of entries into the closed and open arms in the EPM and decline in the number of transitions in DLT. The reduced locomotor activities and exploratory behaviour was considered as a direct reflection of a suppressed and stressed brain which is taken as a sign of anxiety (Bhosale *et al*., 2011; Galani & Patel, 2010; Öztürk *et al*., 1996). The decline in the locomotor activities in the adult rats due

toMSG treatment is reported in many other studies (Frieder& Grimm, 1984; Klingberg *et al*., 1987; Kiss *et al*., 2007). Moreover, the other parameter includes reduced frequency and duration of stay in the central square of OF, increased rearing and reduced grooming in OF suggesting an anxiogenic-like behavior. In support of this later assumption that rats treated with high dose of MSG showed a reduced duration of stay in the open arm, increased duration of stay in the closed arms of EPM, increased duration of stay in dark chamber and decreased duration of stay in light chamber of DLT, reduced duration of stay in the novel compartment of FEP and increased number of marbles buried in marble burying apparatus (Deacon, 2006) are related to anxiety-like behaviors. The present study results in accordance with the previous studies suggests that the intraperitoneal or subcutaneous injection of high dose of MSG in neonatal *Wistar* rats produced an elevation of anxiogenic parameters in EPM, OF and DLT at its 60^{th} and later days of life and which was more prone in male rats (Dubovicky*et al*., 1997; Dubovický *et al*., 1999; Hliňák *et al*., 2005; Cui *et al*., 2013; Onaolapo*et al*., 2015; Suzan *et al.,* 2016). The rats fed with 400 mg MSG also showed a change in the number of squares crossed and immobility period in OF and increased duration of stay in dark chamber and decreased duration of stay in light chamber of DLT, which may be an indication of a mild type of anxiety or the tendency to get anxious, nevertheless other parameters in OF, EPM and DLT was shown to be negative.

MSG induced changes in the locomotor activates of group II was also taken as an anxiety like behaviour, which includes reduction

in the number of squares crossed and increased immobility period in the OF test, increased duration of stay in dark chamber, reduced duration of stay in light chamber of DLT and decline in the number of transitions in DLT, though other parameters in OF, EPM, DLT, MB and FEP was shown to be negative when compared to the control group. The observed anxiety like parameters was more prone in 4 g MSG fed rats; however, when compared with group I, it is lesser. It is indicated that after sixty days of MSG treatment followed by a non MSG period, there was an improvement in the behavioural pattern and showed an anxiolytic effect but does not show a complete cure.

It is known that neonatal rats are more prone to MSG toxicity than adult rats due to the poor development of blood brain barrier (Boonnate*et al.,* 2015). Blood brain barrier never allows the transport of glutamate into the brain although the administration of glutamate in neonatal stage or in gestational period increases the free glutamate level in the brain, which cause excitotoxic effects on vital parts of the brain and later develop neurodegenerative disorders (Bogdanov *et al.,* 1996; Narayanan *et al.,* 2010). Hypothalamus, hippocampus and striatum are more vulnerable excitotoxic areas in the brain due to glutamate flood. Early reports suggests that hypo locomotor activities in the OF and EPM was due to MSG attributed activation of rostro-caudal axis of the arcuate nucleus in the hypothalamus (Park *et al.,* 2000; Cortese and Phan, 2005). Excess glutamate also reduced locomotor activities by exerting an inhibitory effects on dopamine, serotonin, epinephrine and nor-epinephrine by interfering with dopamine and 5-HT receptors (Abu-Taweel *et al.,* 2014). A

subpopulation of serotonergic neurons in the brain are more sensitive to the glutamate and cause an increased uptake of 5-HT leading to a dysfunctioning of serotonergic system in the brain, which could be associated withanxiogenic and depressive like behaviour in rodents (Phelix and Hartle, 1990; Quines *et al.,* 2014). Early literatures also report the importance of GABA-glutamate balance in anxiety disorders (Millan, 2003). Suzan *et al*., (2016) reported that neonatal MSG administration increased hippocampal GABA uptake and decrease of hippocampal GABAergic transmission. It also reduces the number of GABA-positive cells and increases the GAD activity (Beas-Zárate *et al.,* 1998). It is also validated by Dalia *et al.,* (1996) and reported that injection of glutamate antagonists showed to induce locomotion although this induced locomotion was eliminated by dopamine antagonists, thereby suggesting that glutamate retards locomotion by interaction with dopamine. Cools *et al.,* (1988) reported that the visual increase in the grooming behaviour in rats are associated with decreased dopamine level and release of peptides such as adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), or endorphin (Dunn *et al.,* 1985). Stimulation of glutamatergic neurons in the posterior dorsal part of the amygdala and hypothalamus induced repetitive self-grooming in mice and suppressed social interaction (Kalueff *et al*., 2016). The present study also showed an increase in the rearing behaviour. However, there is no clear indication that rearing behavior is either anxiolytic or anxiogenic. Some studies indicate increased rearing is in concordance with increased anxiety levels in mice while others postulate decreased

rearing behavior is indicative of increased anxiety (Seibenhener and Wooten, 2015).

Anxiety is mostly connected with fear, shyness and a common struggle to be social and difficult to face people around. Stress induced anxiety can increase cortisol level in the body. Cortisol has the capacity to inhibit aggression (Øverli *et al*., 2002; Montoya *et al*., 2012; Dantzer *et al*., 2017). In agreement with the Øverli *et al*., (2002) and Dantzer *et al*., (2017) our data shows that high dose MSG treatment decreases agressive behaviour, social intraction and an increase in non-social exploration analysed by resident intruder paradigm and the three-chamber sociability and social novelty test. However, in group II, it showed a recovery and reaches to that of control level. Carter and Levesque, (1979) reported that 2 and 4 g MSG/ kg body weight reduces agression associated with reduction in locomtor activity. Nishigaki *et al*., (2018) testified that MSG addministration to a spontaneously hypertensive rats (SHR) exhibited decrease in aggressive behaviour, social intraction and an anxiety like behavior.

Forced swim test (FST) and tail suspension test (TST) are behavioural tests used to study depressive behaviour in rodents. The test involves the scoring of passive or immobile behaviour when rodents are subjected to short term inescapable stress, first it struggles to escape and eventually stops trying and become immobile. Longer periods of immobility are characteristic of a depressive behaviour. The results of forced swim test (FST) and tail suspension tests (TST) revealed that high dose of MSG in group I induced depression like

behaviour demonstrated by increased duration of immobility period during the test. However, group II rats showed a non-significant immobility period and suggests a reduction in depressive behaviour. It is also supported by earlier studies(Umukoro*et al*., 2015; Quines *etal*., 2016). The dysfunctioning of limbic structures especially hypothalamus and the up regulation of HPA axis and related up surge in cortisol may also contribute to the depression like behaviour (Quines *et al.,* 2014).

The effect of MSG on memory performance in rats was further assessed in this study by using T-maze (TM) and Novel object recognition test (NOR).The TM and NOR tests are widely used for the assessment of the spatial working memory and the recognition memory in rodents. The TM test is based on the ability of rodents to remember the sequence of arm entries called spontaneous alternation. This is a type of spontaneous alternation using an external stimuli such as hunger, electric shock etc (Deacon and Rawlins, 2006). Whereas, NOR test utilizes the innate preference for novelty, if the familiar object is recognized (Lueptow, 2017). It is a non-force drived and doesn't use any external stimuli. The ratio of exploration time between familiar and novel objects in NORis called discrimination index (DI). Increase in the DI indicates increased exploration of the novel object and successful retention of memory for the familiar object. Discrimination index of "zero" indicate equal exploration of both objects (Ennaceur and Delacour, 1988; Win-Shwe and Fujimaki, 2012).

The results of the present study revealed that high dose MSG fed rats in group I significantly impaired the response in TM and discrimination index in NOR test, whereas in group II it did not show any significant difference when compared to the control group. However, the low dose fed rats in both groups did not show any significant difference when compared to the control group. The present results are also supported by the earlier animal works (Wong *etal*., 1997; Collison *et al*., 2010). It has been reported that the MSG administration from postnatal days attributed a poor performance in long-term recognition memory for novel objects and short term working memory or spatial memory (Araujo *et al*., 2017). Hermawati *et al*., (2015) reported that MSG administration at a dose of 2 g/ kg body weight impaired the spatial working memory and reduced the size and number of pyramidal cells in the CA1 region of the hippocampus. It also disturbed the glutamate receptors in the hippocampus and caused impaired acquisition and retention of discrimination learning (Kubo *et al*., 1993). Though the cerebral cortex, striatum and hippocampus primarily involved in the development of learning and memory, the hippocampus is the vital area involved in the modulation and reinforcement of memory (Monfort *et al*., 2015). The CA1 area in the hippocampus carry a crucial role in brain plasticity and memory formation through long-term potentiation (LTP) and is a more sensitive area to MSG toxicity (Beas-Zárate *et al.,* 2002; Kandel *et al*., 2014). Sanabria *et al*., (2002) reported that neonatal administration of MSG exhibited a drastically decreased LTP field excitatory postsynaptic potentials (fEPSPs) in adult rats.

Studies demonstrated that learning deficit and memory impairments are more severe in male rats exposed to MSG during its neonatal stage by intraperitoneally or intravenously (Chow *et al*., 2013). Park *et al*., (2000) reported that high dose MSG fed rodents exhibited impaired memory performance with histopathological changes in arcuate nucleus of hypothalamus. However, cerebral cortex and hippocampus did not show any pathological changes. Gestational MSG administration also produced memory impairments in its offsprings at 60^{th} days of their life, while it does not carry to the second generation by the mating of memory impaired first generation offsprings (Yu *et al*., 1997).

Sensory and motor coordination was also assessed by hot plate test and rotarod test and the results showed that neither low nor high dose MSG administration did not show any significant alteration in the motor and sensory coordination. Kiss *et al*., (2005) also reported that MSG does not cause any motor sensory deficit. In certain cases it is reported so, but it does not cause permanent alterations in the reflex performance and motor coordination. However, contradictory results are also reported to have a decreased latency period in rotarod and reduced thermal nociceptive threshold in hot plate tests (Kiss *et al*., 2005; Rosa *et al*., 2015; Araujo *et al*., 2017; Zanfirescu *et al.,* 2017).

Biochemical studies

Glutamate toxicity is a major contributor to pathological cell death within the nervous system and appears to be mediated by free radicals and reactive oxygen species (ROS)(Coyle *et al*., 1981). There

are two pathways contributing to the glutamate toxicity: excitotoxicity pathway depend on the hyper-activation of glutamate receptors mediated through calcium ions and nonreceptor mediated oxidative glutamate toxicity (Murphy *et al.,* 1989). Oxidative glutamate toxicity is initiated by high concentrations of extracellular glutamate that prevent cystine uptake into the cells *via* the cystine/glutamate antiporter system, resulting in the depletion of intracellular cysteine and glutathione. Glutathione (GSH) depletion induces cellular accumulation of free radicals and ROS (Rajagopal *et al*., 2013).

Oxidative damage occurs, when the imbalance between the production of free radicals and effectiveness of antioxidant defense system in the body. The free radicals and ROS cause the lipid peroxidation (LPO) of bio membrane through a chain of reactions (Singh and Ahluwalia, 2012). The oxygen free radicals take out the hydrogen atoms from fatty acid molecule of lipids to form fatty acid radical, which tends to be stabilized by a molecular rearrangement to form conjugated dienes (CD) (Ayala *et al.,* 2014). These formed conjugated dienes subsequently react with oxygen molecules to form a lipoperoxyl radical. Lipoperoxides are unstable and decompose to form a wide range of compounds including malondialdehyde (MDA) that damage cells by the binding the free amino groups of amino acids of proteins. The formed lipoperoxyl radical further reactswith another molecule of fatty acid, from which a hydrogen atom is detached under formation of lipid hydroperoxides(Sochor *et al*., 2012).

In the present study, the extent of LPO had been determined by assessing the LPO byproducts such as MDA and CD. The rats in

group I fed with 2 g and 4 g MSG/ Kg body weight showed a very high significant ($p<0.01$) increase in the MDA level in the hippocampus and striatum region, whereas in group II it showed a significant ($p<0.05$) increase in MDA level, which is lower than group I. The rats fed with 400 mg MSG in group I also showed a significant increase in the MDA level ($p<0.05$) but it is not shown in group II. The rats in group I fed with 4 g MSG showed a significant increase in the CD level in the hippocampus ($p<0.01$) and striatum ($p<0.05$) while the rats fed with 2 g MSG also expressed a significant increase in the CD level in the hippocampus ($p<0.05$) and striatum ($p<0.05$) region. In group II, both 2 g and 4 g MSG showed a significant increase in CD level in hippocampus whereas in striatum it showed a significant increase in CD only at 4 g MSG fed rats. The increased amount of MDA and CD level indicate a high rate of lipid peroxidation which leads to cell damage (Calis *et al*., 2016; Umukoro *et al*., 2015; Farombi and Onyema, 2006). This cell suppression especially in the hippocampus and striatal region may cause the behavioural alterations especially anxiogenic and depressive behaviours (Kazmi *et al.,* 2017). But group II showed the presence of MDA and CD level, however it is less when compared to the group I.

GSH, a tripeptide, is the major free thiol and electron donor in most living cells and is involved in maintaining cellular oxidation state by detoxifying ROS and removing hydroperoxideswith the help of antioxidant enzymes such as SOD, CAT, GST etc. Excessive glutamate present in the extracellular space reduces GSH level and elevates the free oxygen radicals, for attenuating this generated free

radicals, as GSH donates electrons to become GSSH (oxidized glutathione). This reaction again reduces the level of GSH. In agreement with the earlier literature, our study also showed a very high significant ($p<0.01$) decline in GSH level in hippocampus and striatal region fed with 2 g and 4 g MSG. The hippocampal region also showed a significant decline in the GSH level treated with 400 mg MSG. However in group II, hippocampal and striatal region showed a decline ($p<0.05$) in the GSH level fed with 2 g and 4 g MSG i. e., the concentration of glutamate in the extracellular space slowly started to reduce and lead to a corresponding decrease in the inhibition of cystine/glutamate antiporter system and free radicals (Conrad and Sato, 2012; Lewerenz *et al*., 2006) So GSH level gradually started to increase and which may reduce the MDA level and CD level observed in group II.

Along with GSH in group I, CAT and SOD also shown to be decreased and an increase in GST. SOD are metalloenzymes that catalyze the dismutation of the superoxide anion (O_2^-) to molecular oxygen and hydrogen peroxide. Catalase is another metalloenzymecatalyzing the decomposition of hydrogen peroxide (H_2O_2), a harmful byproduct of many normal metabolic processes, to water and oxygen and thereby protecting the cell from oxidative damages. Group I rats fed with 4 g MSG showed a highly significant increase in the CAT and SOD level in hippocampus and striatum regions, whereas in 2 g MSG fed rats showed a significant increase in CAT at striatum region and a very high significant increase in SOD at both striatum and hippocampus regions. However, in group II the rats

fed with 4 g MSG showed a significant increase in CAT at hippocampal region and a significant increase in SOD at both hippocampus and striatum. The rats fed with 2 g MSG also showed a significant increase in the SOD level at hippocampus and striatum region (Owoeye and Salami, 2017; Soliman, 2011). The antioxidant enzyme GST also were found to be elevated in 2 g and 4 g MSG fed rats in group I at hippocampus and striatum region. Apart from this, 400 mg MSG fed rats also showed an elevated GST level at hippocampal region. But group II, did not show any significant difference in the GST level when compared to the control group. Earlier studies reported that MSG reduced the antioxidant level especially GSH, CAT and SOD level, but a mixed response is observed in the case of GST (Onaolapo *et al*., 2016). Apart from brain, kidney, liver and other organs of the body also showed thedepletion of the antioxidants by MSG(Sharma and Amod, 2015; Ahluwalia *etal*., 1996).

Glutamate transporters in the brain are sodium-dependent proteins that putatively rely indirectly on Na^+/K^+-ATPase to generate ion gradients that drive glutamate uptake. Hence the increase in one depletes the other one. The depleted activity of Na^+/K^+-ATPase thereby reduced the energy that could be derived from hydrolysis of ATP to pump Na^+ out of and K^+ into the cell resulting in the accumulation of glutamate in the extracellular compartment of the astrocytes, and alterations in Na+-coupled transport systems, osmoregulation and ion concentrations in excitable tissues (Rose *et al*., 2009; Rajagopal *et al*., 2013). The result of the present study showed

that rats in group I fed with 4 g MSG showed a significant decline in Na^+/K^+-ATPase at hippocampus and a visual decline was observed in striatal region. However, rats fed with different doses of MSG in group II did not show any difference in Na^+/K^+-ATPase level at hippocampus and striatal region. Due to high concentration of glutamate in the blood,it may block or slow down the passive transport of glutamate from the astrocytes and neuron through glutamate transporters,which may lead to either damage of glutamate transporter system or suppressed activity of Na^+/K^+-ATPase system (Stelmashook *et al*., 1999).

AChE is a carboxylesterase family of enzyme, located at the neuromuscular junction and function as to cease synaptic transmission by catalyzing the breakdown of acetylcholine (ACh) and allowing activated cholinergic neurons to come to a resting state. ACh act as a neurotransmitter to control the behaviour as well as learning and memory. In the present study, group I rats fed with 4 g MSG/ KG body weight showed a significant increase in the AChE level in hippocampal region, however, it does not significantly altered at striatal region or in group II striato-hippocampal regions. The present study reports that MSG administration increase the AChE level in the hippocampal region leading to a decline in the cholinergic neurotransmission due to a decrease in the acetyl choline level in the synaptic cleft, thus contributing to the progressive cognitive impairment. Other reports also state that MSG has the capacity to cause cognitive impairments, which is revealed through T-mace test, Y maze test, Novel object recognition tests etc., (Abu-Taweel *et al*.,

2014; Hae-Nim *et al*., 2015; Moneim *et al*., 2018). The present study also reveals that intake of high dose of MSG also increases the blood glutamate level significantly while it does not alter the brain glutamate, glutamine and GABA. It indicates that glutamate present in the MSG is elevating blood glutamate level but does not enter into the brain directly. However, it elevates the lipid peroxidation markers and depletes the antioxidant status (Onaolapo*et al*., 2016). Histo-pathological studies also revealed that MSG does not interfere with the morphology of the neurons.

MSG induced ROS production and oxidative stress in the striato-hippocampal region of the brain in rats were demonstrated by significant increase in the MDA and CD level associated decrease in the GSH, CAT, SOD and significant increase in the GST level, which leads to severe behavioral deficits including anxiety, depression and cognitive impairments. However, the high dose of MSG elevates the concentration of glutamate in the plasma, nevertheless it does not affect the brain glutamate, glutamine and GABA levels. That means that glutamate does not directly enter the brain, but by using certain indirect mechanism to increase the ROS production and oxidative stress. Whenever the blood glutamate level reaches normal level, the brain ROS generation and oxidative stress was reduced and lead to a natural cure which also revealed by the group II rats in the present study.

Neuroprotective effects of mace from *Myristica fragrans*Houtt.

Phytochemicals are naturally occurring biologically active plant chemicals that contribute vastly to protection against predators but they have the ability to interact with one or more compounds of a living tissue and provide a wide range of protective effects. *M. fragrans* is a popular herbal plant and a folk medicine which possess a spectrum of pharmacological activities. It is not a native of India but both nutmeg and mace are frequently mentioned in Ayurvedic literature. Mace is the aril, bright red, fleshy and branched lacy covering of the seed nutmeg from *Myristica fragrans*Houtt. The bright scarlet colour of the mace is because of the bioflavonoid lycopene, a dominant carotenoid in human blood (Prajapathi*et al*., 2003; Pimenta, 2003; Nybe *et al*., 2007; Šojić *et al*., 2015;). It contains a large amount of alkaloids, flavonoids, phenols, steroids, terpenoids, tannins, saponnin and essential oils which are biologically potent compoundsand has the capacity to attenuate free radicals and act as antioxidant. Phenolic and terpenoid rich food decreases the risk of depression and act as a neuroprotective agent(Szwajgier *et al.,* 2017).

The antioxidant activity of the decoction of mace was analyzed by DPPH radical scavenging assay showing a very good antioxidant activity with an IC_{50} value of 0.3 mg/ ml, while ascorbic acid was used as a positive control with a very low IC_{50} value of less than 0.025 mg/ ml. Other extracts such as methanol, acetone, chloroform and ethanol possess high antioxidant activity than water extracts due to the presence of phenolic contents and essential oils (Tan *et al.,* 2013). The phytochemical screening of the decoction of mace from *Myristica fragrans*revealed the presence of phenols, saponins, alkaloids,

terpenoids, coumarins, steroids, carbohydrates (Olaleye *et al.,* 2006) and proteins possess high amount of antioxidant activity along with anti-inflammatory, antidiabetic, hypolipidemic, analgesic, insomnia, hypocholesterolemic, anticonvulsant and carminative (Jaiswal *et al*., 2009). GC-MS analysis revealed that there is presence of trace amounts of essential oils including Terpinen-4-ol, Elemicin, Myristicin and Safrole. Essential oils especially Terpinen-4-ol possess anticonvulsive, neuroprotective effects (Nóbrega *et al.,* 2014), myristicin and safrole exhibit antidepressant and anxiolytic, psychoactive drugs (Weil, 1966). It is also involved in the regulation of cognitive processes such as emotion, arousal through modulating the activity of AChE and other monoamines (Dhingra*et al*., 2006; Mukherjee *et al*., 2007; Plaingam *et al*., 2017). Docking studies with NMDA receptor subunits also showed that myristicin, elemicin and safrole have almost similar binding energy compared with memantine, a well-known NMDA antagonist. Hence the derivatives of these substances may be used to block NMDA receptor to avoid glutamate excitotoxicity. More studies are needed to prove such abilities of these substances (Gagliardi, 2000; Lipton, 2004).

The present study also reported that mace did not cause any significant change in the body weight; however it presented a non-significant reduction in the body weight induced by high dose of MSG. In agreement with earlier studies, our result did not show any significant decrease in body weight. Vangoori *et al.,* (2018) showed that *Myristica fragrans*extract has a very high clinical value in the treatment of obesity by reducing food intake and body weight by

inhibiting hypothalamic centers and pancreatic lipase. Arulmozhi *et al.,* (2007) reported that hydro-alcoholic extract of mace efficiently reduced the elevation of triglycerides and cholesterols thereby reducing hepatic lipoprotein secretion in high cholesterol fed rats. It is also reported that antioxidant, hypolipidaemic and hypocholesterolemiceffects of *M. fragrans*extractsare mainly due to the presence of myristicin (Yadav and Bhatnagar, 2007; Rahman *et al*., 2018). Patil *et al*., (2011) reported that regular use of nutmeg and mace enhances insulin secretion and thereby maintain the blood glucose level. It also preventselevation in glucose levels by inhibiting intestinal alpha-glucosidase. Nguyen *et al*., (2010) reported that nutmeg and its active constituents that stimulate AMP-activated protein kinase (AMPK), a metabolic enzyme which enhances glucose intake by cells and insulin secretion from pancreatic beta cells. Therefore, it is considered as a therapeutic target for metabolic disorders such as diabetes and obesity. Anti-diabetes factors isolated from methanolic extract of *M.fragrans*,namedmeso-dihydroguaiaretic acid and otobaphenol, are very effective to prevent protein tyrosine phosphatase 1B and up regulation of insulin receptor, a potent target for treating type-2 diabetes and obesity (Yang *et al*., 2006).

The results of behavioural studies showed that the decoction of mace has the ability to reduce anxiogenic behaviour demonstrated by OF, EPM and DLT tests. In open field tests, the rats fed with high dose of MSG along with mace showed a significant ($p \leq 0.05$) reduction in the total number of squares crossed and total number of central square entries when compared to the control group. However,

these parameters were significantly ($p \leq 0.05$) increased when compared to the 4 g MSG group considered as an anxiolytic effects. The above results showed that mace has the capacity to attenuate the toxic effects of MSG and thereby increasing locomotor activities, which is also taken as an anxiolytic effect. In EPM and DLT there showed a significant ($p \leq 0.05$) decline in the number of entry into the closed arms and reduced duration of stay in the dark chamber respectively. However, it showed a significant ($p \leq 0.05$) increase in the number of entries into the open arms of EPM and increased duration of stay in the light chamber of DLT when compared to the 4 g MSG group which is also considered as an anxiolytic effect. In agreement with earlier studies, it is clear that excess glutamate in the brain reduces the locomotor activities by interfering in the action of monoamines such as serotonin, dopamine and 5-HT receptors leading to a dysfunctioning of serotonergic system in the brain(Phelix and Hartle, 1990; Quines *et al.,* 2014;Abu-Taweel*et al.,* 2014). Plaingam *et al*., (2017) reported that *M. fragrans*volatile oil has the ability to increase brain monoamine neurotransmitters such as serotonin, norepinephrine and dopamine especially in the hippocampus, which are very crucial for the regulation of mood, emotions, arousal and certain types of memory. Wahab *et al*., (2009) observed that the volatile oil of nutmeg may be effective against grandmal and partial seizures, as it acts as an anticonvulsant and prevents seizure spread in a set of established animal seizure models. The myristicin and elemicin present in the extract also act like a psychoactive drug and is being used for the treatment of ADHD, anxiety, stress depression etc.

The results showed that mace has the capacity to reduce depression like behaviour in rodents evidenced by FST and TST. Both FST and TST showed that high dose MSG along with mace reduced the immobility period when compared with the 4 g MSG group. Dhingra & Sharma, (2006) reported that *n*-hexane extract of *M.fragrans*has the ability to decrease immobility time in TST and FST by modulating the monoamines such asadrenalin, dopamine and serotonin. It is also reported that 10 mg/ kg acetone extract of *M.fragrans*was found to be most potent and comparable with imipramine (15 mg/kg) and fluoxetine (20 mg/kg) (Jaiswal *et al*., 2009).

Dhingra *et al*., (2006) revealed that oral administration of 5 mg/ kg of hexane extract of *M. fragrans*inaSwiss male young albino mouse significantly reduced the Acetylcholinesterase(AChE)activityand thereby enhancing the learning and memory process by increasing the level of acetylcholine. Mukherjee *et al*., (2007) also showed that hydroalcoholic extract of *M. fragrans*suppressed the AChE activity in a hippocampal culture at an IC_{50} value of 133.28 ± 11.26. Another study reported that nutmeg extract inhibit AChE activity in penis and thereby increasing the activity of acetylcholine and acetylcholine positive fibers in penis (Odubanjo *et al*., 2018). Plaingam *et al*., (2017) showed that mace extract has the capacity to increase monoamines and thereby increasing cognitive performance. Parle *et al*., (2004) explained that 5 mg/ kg body weight of *M. fragrans*extract has the capacity to improve learning and memory in young and aged

mice and they also showed that this extract has the capacity to reverse the effect of scopolamine and diazepam induced learning and memory impairments in young mice. This memory enhancing activity may be due to the wide range of activity attributed to the plant including antioxidant, anti-inflammatory and procholinergic activity. The results of the present study showed that high dose MSG along with mace improved the percentage response in T-maze and increase in the DI of NOR test along with a decline inAChE level. In agreement with earlier studies, our results showed that mace has the capacity to improve ACh level and thereby improving learning and recognition memory. Jin *et al*., (2005) reported that lignan isolated from mace treated with murine hippocampal cell line and primary culture of rat microglial cells displayed a down regulation of cyclooxygenase-2 and suppress the production of nitric oxide synthase, inhibit the activity and synthesis of pro-inflammatory cytokines including tumor necrosis factor-α and interleukin-6. The suppression of such enzymes by mace lignan open a new therapeutic window to the neurodegenerative diseases associated with oxidative damage and neuro-inflammation.

The biochemical studies also showed that mace has a potent ability to reduce MSG induced lipid peroxidation and increase SOD and GSH level, however no much significant difference was observed in the Na^+/K^+ ATPase when compared to the high dose MSG fed group. Early literature showed that *M. fragrans*extracts excellent in scavenging radicals, reducing metal ions and inhibiting lipid oxidation (Capasso*et al*., 2000; Kapoor *et al*., 2013; Gupta *et al*., 2013). It

prevents lipid peroxidation in chicken tissue homogenates and egg yolk (Damien *et al*., 1995). Myristicin present in the extract has anti-inflammatory properties by inhibiting the production of calcium, nitric oxide, cytokines, interleukins (Lee & Park, 2011). Zheng *et al*., (1992) reported that Myristicin present in the essential oil of mace is responsible for the potent chemoprotective effect of *M. fragrans*. Oseni and Idowu, (2014) showed that water extract of nutmeg enhances GSH, GPx, catalase and SOD level, while lipid peroxidation and blood glucose level is declined in alloxan induced diabetic rats. The essential oil of mace exhibits a strong anti-inflammatory, anti-pyretic, antidiarrheal, sedative and analgesic effect in rats and mice. The pharmacological mechanisms behind the anti-inflammatory effects are similar to those of non-steroidal anti-inflammatory drugs (NSAIDs) (Olajide*et al*., 2000; Grover *et al*., 2002). Myristicin present in the volatile oil of mace exhibited significant hepatoprotective effects (Schenk and Lamparsky, 1981; Morita *et al*., 2003; Yimam *et al*., 2016). Chhabra and Rao, 1994 reported that the principal compound present in the water extract of mace, administrated through the trans-mammary route, enhanced the hepatic glutathione peroxidase and other hepatic xenobiotic metabolizing enzymes in the liver of mouse pups and potentiate the hepatic activity. Mace is also reported to modulate glutathione-s-transferase activity, control the development of *in vitro* aflatoxins induced DNA adducts and influenced the hepatic detoxification system in mouse liver (Shin *et al*., 1988; Kumari and Rao, 1989; Hashim *et al*., 1994).

Thus, it is observed that decoction of mace from *M. fragrans*contain potent phytochemical substance, probable synergistic effects of these substance ameliorating the anxiogenic, depressive and cognitive defects induced by high dose of MSG.

SUMMARY AND CONCLUSION

Monosodium glutamate is one of the most commonly used flavor enhancers and a controversial food additive found in almost all types of fast foods, packed Chinese foods, soups, canned vegetables and processed meats. MSG is a sodium salt of naturally occurring non-essential amino acid L glutamic acid, the most abundant amino acid found in nature, containing 78.2 % glutamic acid, 12.2 % sodium and 19.6 % water, which is water soluble and stable even under food-processing conditions. Glutamate is one of the most abundant excitatory neurotransmitter in the brain acting through NMDA and AMPA receptors and plays a pivotal role in learning and memory. It is the source for the unique flavor called "*umami*", a pleasant savoury taste, one of the five primary tastes. MSG adds the *umami* taste to the food through the stimulation of taste receptors by free glutamate and also enhances and intensifies the palatability of other tastes particularly salt, sweet and sour. Although health professionals and researchers have confusions and concerns whether these additives added to food stuffs is safe or not. The present study was designed to investigate the effect of MSG on neurobehavioral, neurochemical and histological alterations on striato-hippocampal region of the brain in *Wistar albino* rats and explore the possibility of recovery from MSG toxicity after 60 days of MSG administration followed by a 60 days of non-MSG treatment. The ameliorative effect of the decoction of mace from *Myristica fragrans*Houtt. against the MSG induced neurotoxicity was also investigated in this study.

The results showed that deleterious effect of MSG were dose dependant and more severe in group I, while the effects were mild and exhibited a recovery in group II animals. The MSG fed rats in group I showed an anxiety like behaviour indicated by decreased number of squares crossed, reduced frequency and duration of stay in the central square, increased immobility period, increased rearing and reduced grooming in the open field experiment, reduced number of entries into the closed and open arms, reduced duration of stay in the open arms and increased duration of stay in the closed arms of elevated plus maze, increased duration of stay in dark chamber, decreased duration of stay in light chamber of dark/light transition test (DLT) and decline in the number of transitions in DLT, increased number of marbles buried in marble burying apparatus and reduced duration of stay in the novel compartment of free exploratory paradigm. Increased immobility period exhibited by force swim test and tail suspension test revealed the depression level. Decreased percentage of performance in T- maze and decreased discrimination index in the novel object recognition test showed poor performance in spatial and working memory. Aggression and social interaction behaviour were shown to be decreased in MSG fed rats and no change was observed in the motor and sensory activities.

The biochemical parameters in the striato-hippocampal region of the brain revealed the influence of MSG to increased lipid peroxidation and oxidative stress established by malondialdehyde and conjugated diene level associated with decrease in the antioxidant status such as glutathione, superoxide dismutase and catalase, but a

slight elevation of glutathione-S-transferase level was observed. Acetylcholinesterase level showed to be increased and Na^+/K^+ ATPase level was decreased, while no significant difference was observed in glutamine, glutamate and GABA levels. Moreover, histological changes were also not observed to be different.

Group II showed a decreased number of squares crossed and increased immobility period in the open field and decreased number of transitions between dark and light chambers in the dark light transition test, while other tests such as elevated plus maze, marble burying test and free exploratory paradigm were not significant and failed to show anxiety like behaviour. Reduced immobility period in forced swim test and tail suspension test, improved percentage performance in T-maze and discrimination index of novel object recognition test showed reduced depressive behaviour and improved memory performance respectively. Aggression, social interaction, motor and sensory activities were also not-significant when compared to the control group. Striato-hippocampal lipid peroxidation level was reduced, glutathione, superoxide dismutase and catalase level was improved, acetylcholinesterase level was reduced and Na^+/K^+ ATPase level was improved when compared to the control group. Changes in glutamine, glutamate and GABA level and histological alterations were also not observed.

Rats treated with decoction of mace showed an ameliorative effect against the MSG induced neurotoxicity. The anxiety and depression like behaviour was also reduced, which was indicated by an increase in the number of squares crossed and increased number of

central square entries in the open field, improved number of entries into open and closed arms of elevated plus maze, increased duration of stay in light chamber, reduced immobility period in the forced swim test and tail suspension test. Improved memory performance was also demonstrated by T maze test. A reduction in the lipid peroxidation and oxidative stress by scavenging free radicals associated with an increase in the antioxidants.A decreased acetylcholinesterase level was also observed here.

Administration of MSG at high doses increased blood glutamate level, which may cause damage to the vital organs of the body that does not change the level of glutamate, glutamine and GABA level in the brain. It means that the strong blood brain barrier prevented the entry of blood glutamate into the brain. However, changes occurred in the behaviour and increased oxidative stress associated with a decrease in the antioxidant level, which may be due to some indirect mechanism involved or the reflection of effects occurring in the other parts of the body. Thus, the study conclude that dose dependant long term administration of MSG has a capacity to cause behavioural changes such as anxiety, depression, cognitive impairment, reduced aggression and social interaction associated with increased oxidative stress and antioxidant depletion by indirect mechanisms. This is not a perpetual and a reversible process during the life time. Due to normal diet and natural healing mechanisms in the body, there is amelioration of the toxic effects of MSG and showed gradual positive response and recovery in the behavioural and biochemical parameters. However, it is a time consuming

process.Decoction of mace from *Myristica fragrans* also has the capacity to improve antioxidant status and scavenging radicals associated with increase in acetylcholine level, thereby improving cognition and other behavioural parameters. Thus, the use of mace in food improves our antioxidant status and improves neurochemical levels thereby preventing the MSG induced excitotoxicity to a certain extent.

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