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Neurobehavioral and Neurochemical of Effect of Azadirachta Indica on Stress Induced Anxiety in Mice

(Azadirachta Indica on Stress Induced Anxiety in Mice)

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Abstract: Anxiety disorder is common mental disorders that share extreme or pathological anxiety as the primary disturbance in mood or emotional tone. Biochemical alteration are observed in anatomical centers, which are responsible for processing of emotions and are connected to ―stress‖ axis , which is strongly activated in response to threat or fear. The imbalances in the neurotransmitters like GABA, Serotonin, nor adrenaline, Dopamine etc mainly causes anxiety disorders. Synthetic drugs available for treatment of anxiety have various adverse effects including drowsiness, ataxia with benzodiazepines and insomnia, libido with selective serotonin reuptake inhibitors. Azadirachta indica A. Juss, common name Neem, Neem tree, Indian-lilac (Canada), Margosa, Nimtree and mMrgosier, family Maliacea, responsible for some of the already mentioned neem benefits, they are known to possess immunomodulatory, antiinflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anti-carcinogenic properties being one of the most active parts of the plant. Behavioral parameters are the primary evidence to confirm anxiety as well as anti-anxiety effect of treatments. All the parameters are based on pathophysiology of anxiety because anxiety or fear is evaluated through stress or immobilization of animal like mice and rats. On the bases of behavioral parameters as well as biochemical estimation, study concludes that Azadirachta indica shows significant effect in plasma nitrates and other chemical messenger in anxiety at dose of 200mg/kg

Index Terms **- Azadirachta indica A. Juss, Synthetic drugs, Mice, Extract, Effect, Dose, Toxicity.**

INTRODUCTION

Anxiety disorder is common mental disorders that share extreme or pathological anxiety as the primary disturbance in mood or emotional tone. Biochemical alteration are observed in anatomical centers, which are responsible for processing of emotions and are connected to ―stress‖ axis , which is strongly activated in response to threat or fear. Anxiety refers to an overwhelming sense of apprehension or fearfulness **(American Psychiatric Association 2000)**.

The term anxiety covers four aspects of experiences an individual may have: mental apprehension, physical tension, physical symptoms and dissociative anxiety. Anxiety disorder is divided into generalized anxiety disorder, phobic disorder, and panic disorder; each has its own characteristics and symptoms and they require different treatment **(American Psychological Association 2004)**. The emotions present in anxiety disorders range from simple nervousness to bouts of terror. The imbalances in the neurotransmitters like GABA, Serotonin, nor adrenaline, Dopamine etc mainly causes anxiety disorders. Selective serotonin reuptake inhibitors, the drugs most commonly used to treat depression, are frequently considered as a first line treatment for anxiety disorders **(Augustin 2005).**

Synthetic drugs available for treatment of anxiety have various adverse effects including drowsiness, ataxia with benzodiazepines and insomnia, libido with selective serotonin reuptake inhibitors **(Baldessarini 2001)**.

Panic disorder appears to be a genetically inherited neurochemical dysfunction that may involve autonomic imbalance; decreased GABA-ergic tone ; allelic polymorphism of the catechol-o-methyltransferase (COMT) gene; increased adenosine receptor function; increased cortisol ; diminished benzodiazepine receptor function; and disturbances in serotonin, serotonin transporter (5-HTTLPR) and promoter (SLC6A4) genes, norepinephrine, dopamine, cholecystokinin, and interleukin-1-beta. Some theorize that panic disorder may represent a state of chronic hyperventilation and carbon dioxide receptor hypersensitivity. Some epileptic patients have panic as a manifestation of their seizures (Barbotte et al., 2001). Genetic studies suggest that the chromosomal

regions 13q, 14q, 22q, 4q31-q34, and probably 9q31 may be associated with the heritability of panic disorder phenotype. The cognitive theory regarding panic is that patients with panic disorder have a heightened sensitivity to internal autonomic cues (eg, tachycardia) (Barlow, 2001). The efferent pathways from the central nucleus of the amygdale travel to a multiplicity of critical brain structures, including the Para brachial nucleus (resulting in dyspnea and hyperventilation), the dorsomedial nucleus of the vagus nerve and nucleus ambiguous (activating the parasympathetic nervous system), and the lateral hypothalamus (resulting in SNS activation) (Bhatnagar 2013).

NEED OF THE STUDY.

Common mental disorders are increasing worldwide. Between 1990 and 2013, the number of people suffering from depression and/or anxiety increased by nearly 50%, from 416 million to 615 million. Close to 10% of the world's population is affected and mental disorders account for 30% of the global non-fatal disease burden. WHO estimates that, during emergencies, as many as 1 in 5 people are affected by depression and anxiety (Bhattacharyya 2008).

According to WHO, herbs are very useful to treat anxiety because of less/minimum side effect, greater availability and cost effective. Synthetic drugs available for treatment of anxiety have various adverse effects including drowsiness, ataxia with benzodiazepines and insomnia, libido with selective serotonin reuptake inhibitors. Anxiety is a major neurological disorder in which biochemical parameters like plasma nitrate, AchE, GABA, are mainly affected (Brannon and Feist 2004).

The effect of Azadirachta indication on plasma nitrates and other neurotransmitters are not studied and reported yet, only anxiolytic activity is reported in some research articles on the bases of behavioral models like elevated plus maze (EPM), light & dark apparatus, open field test (OFT), etc. (Calixto et al., 2010). The aim of study is to screen the effect of Azadirachta indica on these transmitters in stress induced anxiety in mice, by which we will confirm how the Azadirachta indica increase or decrease the level of these transmitters. Behavioral parameters were employed to evaluate the physiological effect of Azadirachta indica and the biochemical parameters were validating the biochemical bases of the activity.

MATERIAL AND METHOD

Plant leafs were collected from medicinal garden in Sagar Institute of Pharmaceutical Sciences, Sagar (M.P.) and authenticated from Dr. H. S. Gour University, Department of Botany, Sagar (M. P.)

Extraction Method

Petroleum ether:- The shad dry the coarse powder of leaves was packed in extraction thimble of soxhlet apparatus and were subjected to continuous hot extraction with petroleum ether for 18 hour or till the clear extraction obtained. After that, remove solvent and extract too. Store it for recycle. Shade dries the powdered herb for second step of extraction with ethanol (Carlini, 2003).

Ethanol extract :- Mark left after petroleum ether were dried below 50^0C in hot air oven and then packed well in extraction thimble of soxhlet apparatus and subjected to continuous hot extraction with ethenol for 18 hour or till the clear extraction obtained. The extract was filtered while hot and resultant extract was distilled in vacuumed under reduced pressure in order to remove the solvent completely. Dry it and kept in the desiccator till the experimentation. Obtained extract was weighed and calculate percentage yield in terms of air dried powdered crude material. (Cates et al., 1996) After extraction, we performed phytochemical test like test for alkaloids, glycosides, terpenoids, etc to confirm and validate extraction procedure.

PHARMACOLOGICAL SCREENING

Animal: Mature Sprague-Dawley Mice (20–25gm) was used for the study. All animal was kept in standard plastic polypropylene cages with stainless steel coverlids and wheat straw was used as bedding material. The animal was facilitated with standard environment of photoperiod (12:12 hr dark: light cycle) and room temperature (23±20 C). The animal assists free to feed and purified water ad libitum. All experiment was according to CPCSEA guidelines and approved by IAEC.

Animal model:- (Immobilization Stress or stress induced anxiety) Animals was immobilized (IMO) for 3 hr. by taping all the four limbs on board by putting them on their backs using zinc oxide hospital tape. The animals were released by unraveling the tape after moistening with acetone in order to avoid pain and discomfort. In unstressed group, the mice will be handled without any stress.

Description of groups

Control Group (Vehicle Treate)
Negative Control (Disease Induced)
Standard (Diazepam, 4mg/kg i.p.)
Test group-1 (Ethanolic Extract of Azadirachta Indica 100mg/kg)
Test group-II (Ethanolic Extract of Azadirachta Indica 200mg/kg)

Statistical Analysis

The statistical analysis was carried out as per standard method. Results was expressed as Mean± SEM compared with the analysis of variance (ANOVA) followed by Dunnet's test value for statistical significance.

RESULTS AND DISCUSSION

Behavioral Parameters

Behavioral parameters are the primary evidence to confirm anxiety as well as anti-anxiety effect of treatments. All the parameters are based on pathophysilogy of anxiety because anxiety or fear is evaluated through stress or immobilization of animal like mice and rats.

Elevated Plus Maze (EPM)

After immobilization of animals for 3hr, the drug treatment was started for all groups except standard and test groups. Time spent in open arm and closed arm were observed. In standard group, time spent in open arm were significantly increased (P>0.001) after administration of ethanolic extract of Azadirachta Indica at dose of 200 mg/kg (167±2.5201) as compared with standard group (194±5.1307). In fear, animal is more favorable to dark area which was shows in negative control group (41±3.4021).

Table 1: Result of Elevated Plus Maze (EPM) experiment

Group	Elevated Plus Maze (5Min.)		Actophotometer
	Time spent in Open arm (Sec.)	Time spent in Close arm (Sec.)	Locomotors activity (5 min.)
Positive control	237±5.0382	63±2.0126	76±2.2108
Negative control	41±3.4021	260±3.2017	35±2.5421
Standard	194± 5.1307***	106± 6.2016***	64± 3.5041***
***A. Indica* 100 mg/kg**	118± 3.0124*	184± 5.3026*	49± 4.2401*
***A. Indica* 200 mg/kg**	167± 2.5201**	133± 4.2103**	58± 4.240***

Values are expressed MEAN±SEM, n=6, ** = P<0.01, *** = P<0.001 when compared to normal control group,, Standard:- Diazepam (4mg/kg).

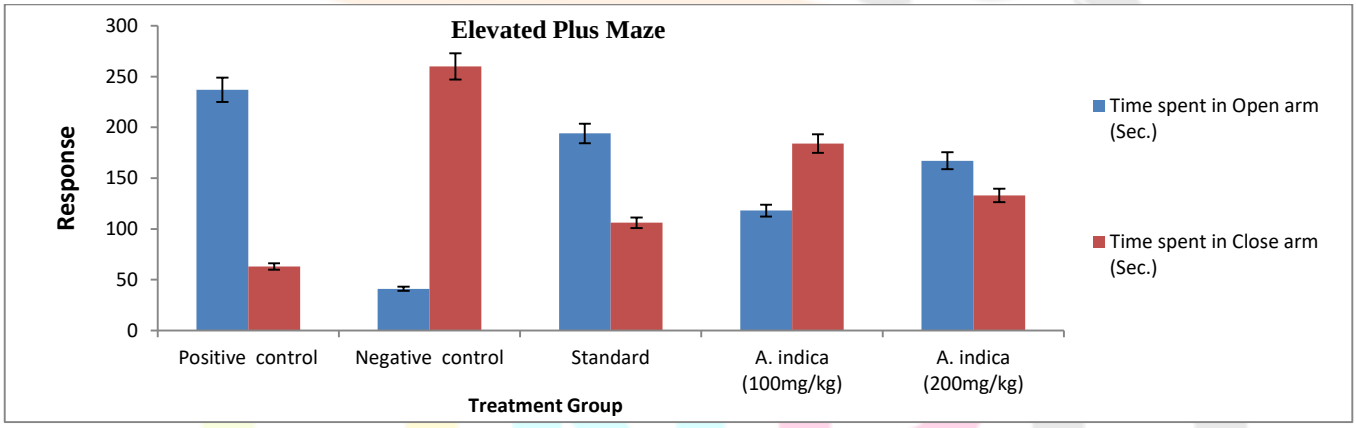


Figure 1: Effect of ethanolic extract of *Azadirachta Indica* on EPM parameters in stress induced anxiety in mice

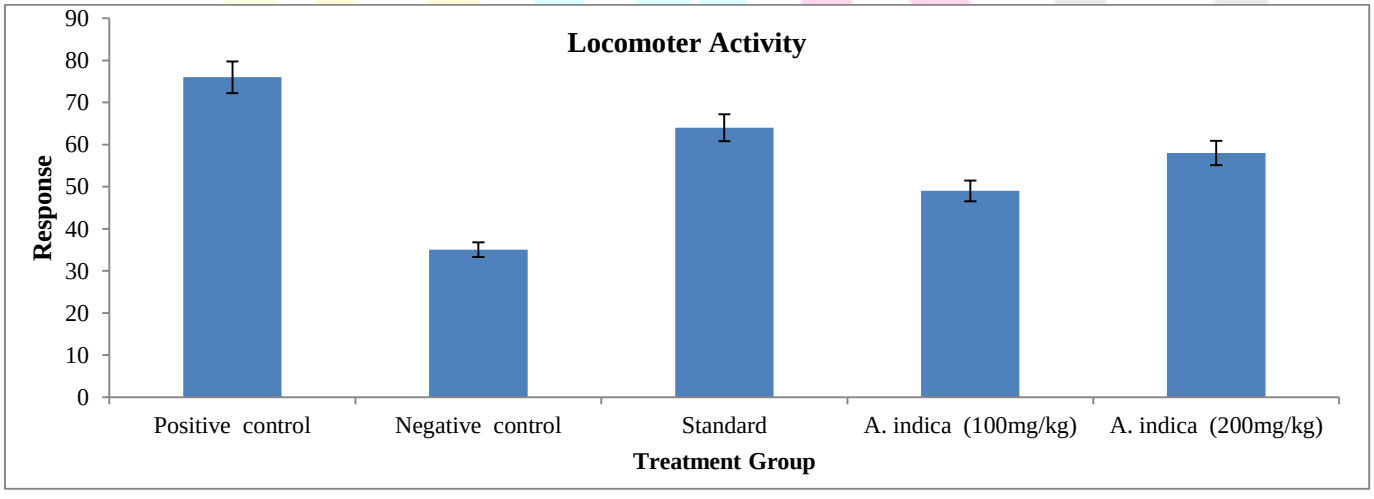


Figure 2: Effect of ethanolic extract of *Azadirachta Indica* on locomoter activity in stress induced anxiety in mice

Light and Dark Test

After immobilization of animals for 3hr, the drug treatment was started for all groups except standard and test groups. Time spent in light and dark area was observed. In standard group, time spent in light area were significantly increased (P>0.001) in light area after administration of ethanolic extract of Azadirachta Indica at dose of 200 mg/kg (177±2.3054) as compared with standard group (169±3.0554). In fear, animal is more favorable to dark area which was shows in negative control group (56±2.1245).

Open Field Test (OFT)

OFT is the test to evaluate anti-anxiety effect as well as to compare the statistics with actophotometer because each squire in OFT is 10 x 10cm and each electrode's difference in actophotometer is 6 cm so the reading should be double in OFT. Animal in control group were shows significant walk fullness in OFT (48±2.2450). After administration of ethanolic extract of Azadirachta Indica at dose of 200 mg/kg, the animal was shows significant effect (P>0.001). Rearing is the parameter in OFT which shows alertness of animal. After administration of ethanolic extract of Azadirachta Indica at dose of 200 mg/kg, the animal was shows significant effect (P>0.001) in OFT (34±2.0454) compared with standard group.

Table 2: Result of Light & Dark Test

Group	Light & dark Test (5 Min.)		Open Field Test (5 Min.)	
	Time spent in Light Area (Sec.)	Time spent in Dark Area (Sec.)	No. of Squire Cross	No. of Rearing
Positive control	217± 2.3024	83± 4.2015	48±2.2450	24±5.0545
Negative control	56±2.1245	244± 2.5045	14±2.5402	39±5.2540
Standard	169± 3.0554**	131± 4.2045**	38±2.2405***	27±5.2451***
***A. indica* 100mg/kg**	135± 6.0215*	165± 4.3024*	31± 2.0245*	35± 2.2405*
***A. indica* 200mg/kg**	177± 2.3054**	123± 4.2402**	34± 2.0454**	32± 5.4545**

Values are expressed MEAN±SEM, n=6, ** = P<0.01, *** = P<0.001 when compared to normal control group,, Standard:- Diazepam (4mg/kg).

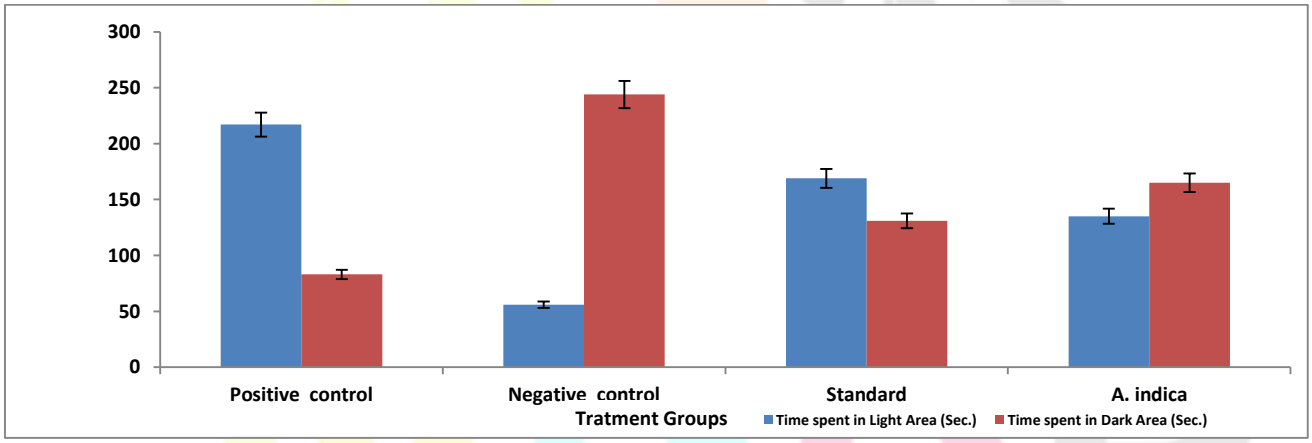


Figure 3: Effect of ethanolic extract of *Azadirachta indica* on light and dark test in stress induced anxiety in mice

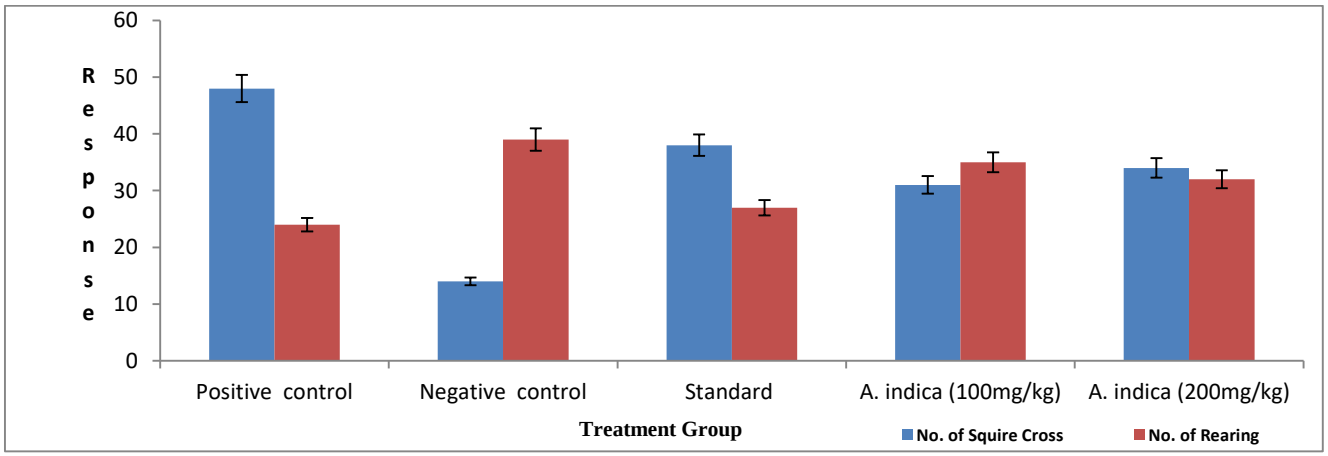


Figure 4: Effect of Ethenolic extract of *Azadirachta indica* on OFT in stress induced anxiety in mice

6.2. *Biochemical Estimation*

Actophotomete (Locomoter Activity). Locomoter activity after administration of ethanolic extract of Azadirachta indica at dose of 200mg/kg, the animal was shows significant effect (P>0.001). After administration of ethanolic extract of Azadirachta indica at dose of 200mg/kg, the animal was shows significant effect (P>0.001) in locomoter activity (58±4.240) compared with standard group (64±3.5041).

The gaseous messenger molecule nitric oxide (NO) is synthesized from its precursor L-arginine by a family of three NO synthases (NOS), designated as ‘‘neuronal’’ NOS-I, ‘‘inducible’’ NOS-II and ‘‘endothelial’’ NOS-III. In the adult brain, the inducible iso form NOS-II is present only at very low levels in microglia and immune cells, while ‘‘endothelial’’ NOS-III is expressed predominantly in the vasculature. Whether or not this isoform is also expressed in neural cells, is still a matter of debate but data arguing for this are only sparse. The quantitatively major source for NO in the CNS thus is the ‘‘neuronal’’ isoform NOS-I present in approximately 1% of all neurons. Nitrinergic transmission is especially important in limbic structures, in the basal ganglia where NO regulates striatal output and in the cerebellum.

NO exerts multiple actions in the CNS and from animal studies, it has been suggested that it is involved in behavioral processes such as learning and memory formation. Pathologies of the NO pathway have been implicated in almost every major neuropsychiatric disorder including schizophrenia, affective disorders, alcoholism, Alzheimer’s dementia, Parkinson’s and Huntington’s disease. For some of these disorders, NOS-I has also been identified as a risk gene in human case-control association stud. The role of NO in the regulation of normal human brain functioning however is still unclear, although first genetic studies argue for a function of NOS-I in the regulation of impulsive behaviors. In a second series of experiments, we investigated whether NOS1 knockdown animals have cognitive deficits.

Table 3:

Groups	Plasma Nitrate (µmol/l)	iNOS (µmol/l)	AchE (mg/dl)	GABA (ng/gm of Tissue)
Positive Control	24.38± 2.3540	103.71± 2.84550	54.31± 2.2505	3078.7± 2.2012
Negative Control	73.25± 2.2405	26.23± 2.5470	81.23± 3.0245	1634.2± 2.2102
Standard	48.73± 1.9058***	98.16± 2.2405***	48.72± 3.2301***	2826.4± 2.2015***
***A. Indica* 100 mg/kg**	61.58 ± 2.2405*	43.29± 3.1450*	63.17± 2.0540*	2424.4± 2.3201*
***A. Indica* 200 mg/kg**	53.41± 2.2450**	71.96± 2.5402**	51.92± 2.3054**	2683.5± 2.2230**

Values are expressed MEAN±SEM, n=6, ** = P<0.01, *** = P<0.001 when compared to normal control group„ Standard:- Diazepam (4mg/kg).

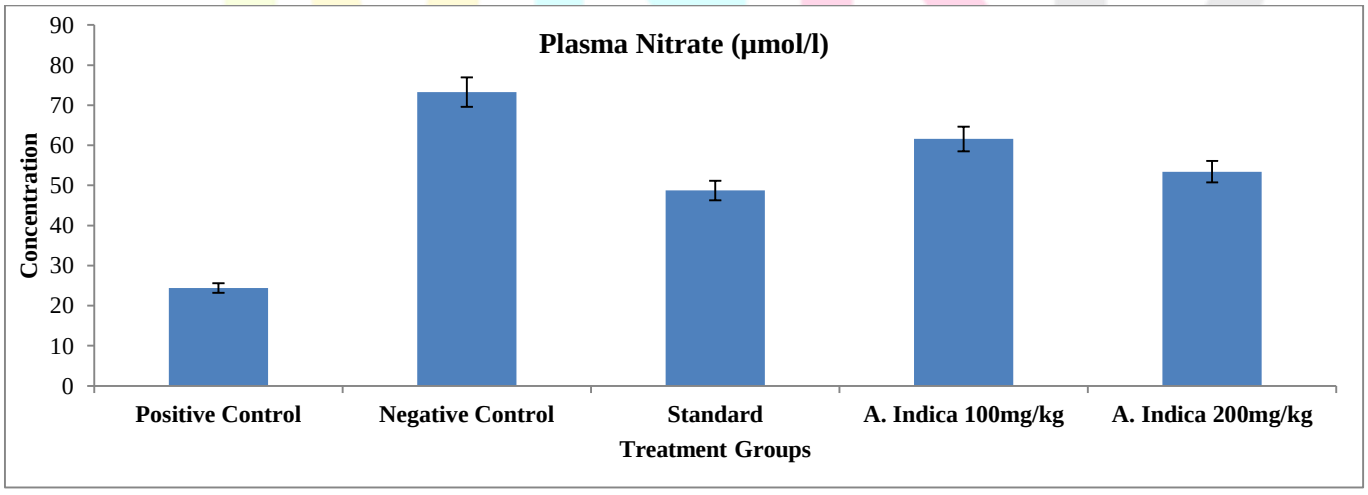


Figure 5: Effect of ethanolic extract of *Azadirachta Indica* on plasma nitrate in stress induced anxiety in mice.

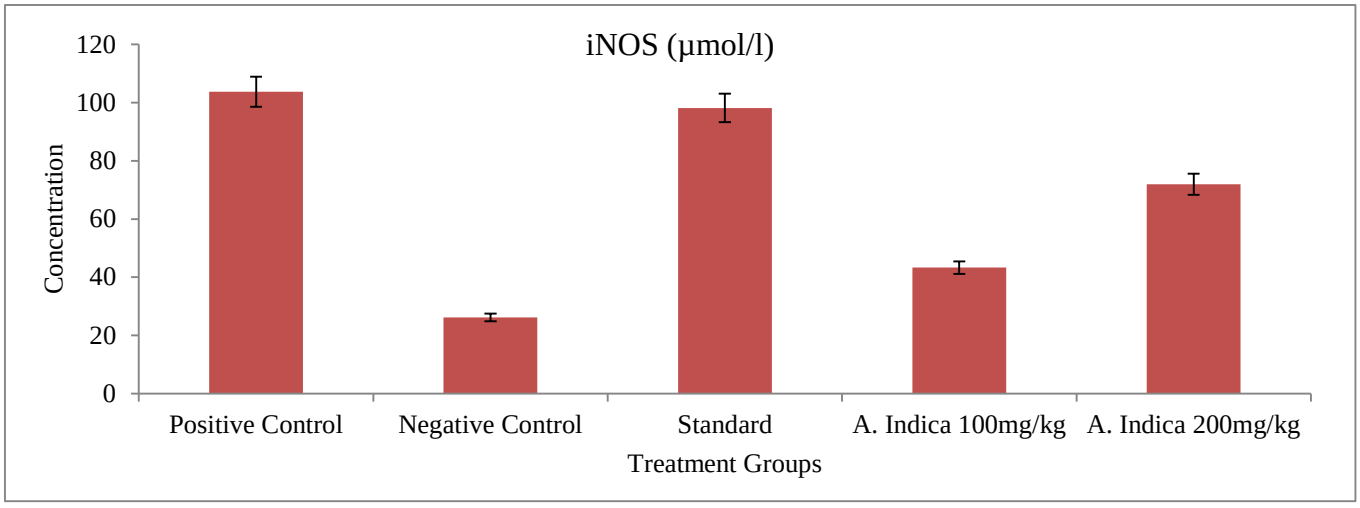


Figure 6: Effect of Ethanolic Extract of *Azadirachta indica* on iNOS in stress induced anxiety in mice

As shown in Table 03, AChE activity was increased significantly by administration of ethanolic extract of Azadirachta indica at dose of 200mg/kg (P>0.001) treated group compared to controls. In addition to its role in cholinergic transmission, substantial evidence has accumulated over the last two decades which suggests a non-cholinergic neuromodulatory function for AChE. Few studies have demonstrated that the expression of AChE during early development correlate closely with the major phase of neurite outgrowth. Layer et al. w26x have showed that AChE can stimulate neurite outgrowth in chick nerve cells. On the contrary, treatment with AChE inhibitors has been shown to retard neuritic outgrowth in a dose dependent manner in retinal ganglion cells, dorsal root ganglion and sympathetic ganglion neurons. There is a growing body of evidence supporting the morphogenic effects of AChE in both in vivo and in vitro systems. AChE is known to regulate the neuritic outgrowth and survival of cultured neurons and also has morphogenic and axogenic role in the developing nervous system. In addition, AChE has a role in cell growth and survival. These functions are considered to be the non-classical roles of this classical enzyme.

Furthermore, ACh is also known to enhance the neuritic outgrowth and in turning of the nerve growth cones. These studies, together with the present demonstration of increased dendritic arborization in the hippocampus, suggest that chronic drug administration induces AChE activity which in turn might modulate dendritic branching pattern in specific brain regions. GABA appears to play an important role in the pathogenesis of several neuropsychiatric disorders. Many of the traditional agents used to treat psychiatric disorders are known to act, at least in part, by enhancing GABA activity, while some of the newer agents may exert their therapeutic effects exclusively through GABAergic actions. In our present study, treatment with extract and further GABA estimation in brain showed significant enhancement of GABA levels in whole brain compared to control group.

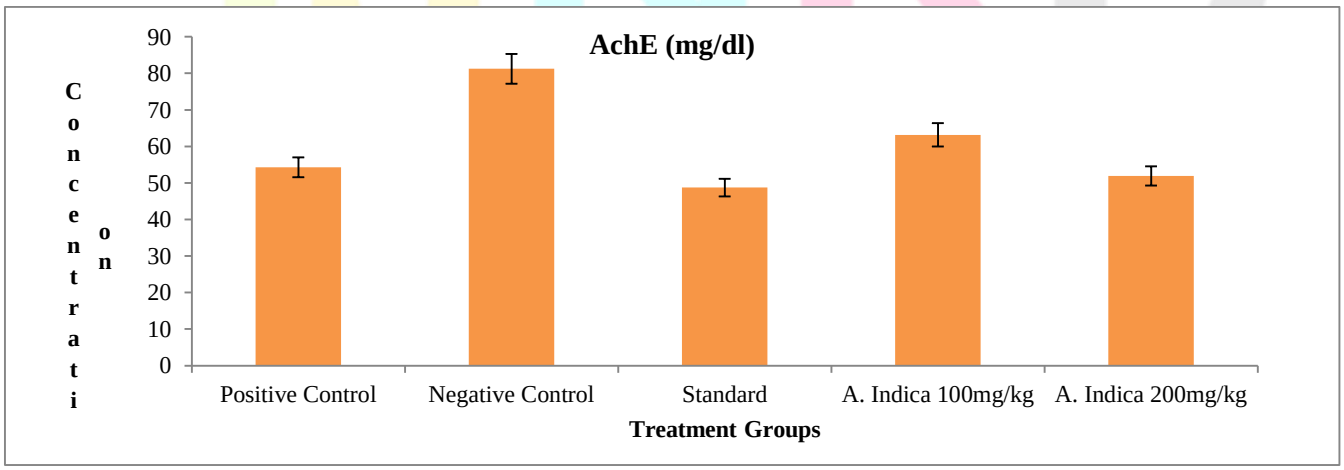
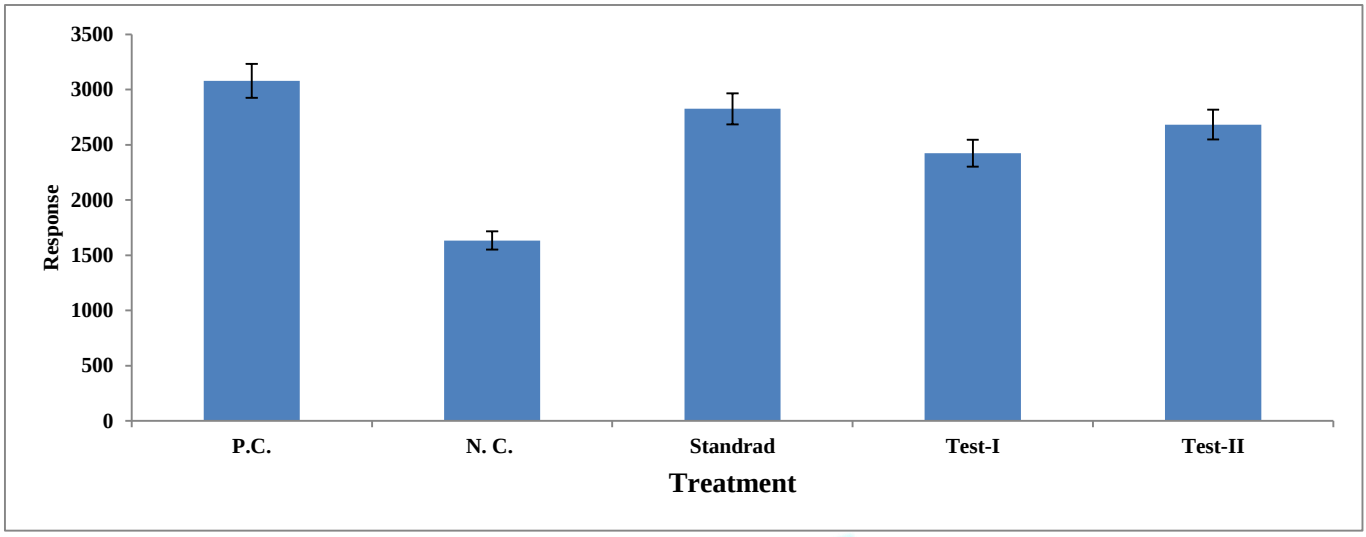


Figure 7: Effect of Ethenolic extract of *Azadirachta Indica* on AchE in stress induced anxiety in mice.



P.C.:- Positive Control; N.C.:- Negative Control, Standard:- Diazepam (4mg/kg), Test-I:- *A. Indica* (100mg/kg), Test-II:- *A. Indica* (200mg/kg).

Figure 8: Effect of ethenolic extract of *Azadirachta indica* on GABA in stress induced anxiety in mice.

On the bases of behavioral parameters as well as biochemical estimation, study concludes that Azadirachta indica shows significant effect in plasma nitrates and other chemical messenger in anxiety at dose of 200 mg/kg.

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