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NEUROPHARMACOLOGICAL EVALUATION OF ETHANOLIC EXTRACTS OF FLOWERS OF NYCTANTHES ARBOR-TIRSTIS WITH SPECIAL REFERENCE TO ANTI-ANXIETY & ANTI-DEPRESSANT ACTIVITY

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ABSTRACT

Aim- The present study was designed to evaluate the neuropharmacological activity of ethanolic extracts of flowers of Nyctanthes arbor-tristis with special reference to anti-anxiety and antidepressant effects in experimental animal models.
Material & Methods- The flowers were successively extracted using different solvents, and the ethanolic extract was selected for pharmacological evaluation based on phytochemical constituents. Preliminary phytochemical screening revealed the presence of flavonoids, phenolic compounds, tannins, glycosides, steroids, and terpenoids. Acute toxicity studies performed according to OECD guidelines demonstrated the safety of the extract up to 2000 mg/kg body weight. Neuropharmacological evaluation was carried out using Elevated Plus Maze (EPM) and Forced Swim Test (FST) in Wistar albino rats. The ethanolic extract was administered orally at doses of 200 mg/kg and 400 mg/kg, while diazepam and imipramine were used as standard drugs for anxiolytic and antidepressant studies, respectively.
Results- In the Elevated Plus Maze model, the extract did not produce significant anxiolytic activity as compared to the control group. However, in the Forced Swim Test, the extract significantly reduced immobility time in a dose-dependent manner, indicating marked antidepressant activity comparable to imipramine. Biochemical investigations demonstrated significant inhibition of acetylcholinesterase activity along with reduction in malondialdehyde (MDA) levels, suggesting attenuation of oxidative stress. Furthermore, treatment with the ethanolic extract significantly increased antioxidant enzyme levels such as catalase and superoxide dismutase (SOD), while glutathione peroxidase activity showed minimal changes. The findings suggest that the antidepressant and neuroprotective effects of Nyctanthes arbor-tristis may be mediated through cholinergic modulation and antioxidant mechanisms.
Conclusion- In conclusion, the ethanolic extract of Nyctanthes arbor-tristis flowers possesses significant antidepressant and neuroprotective activity with potential memory-enhancing properties, although no appreciable anxiolytic effect was observed

GRAPHICAL ABSTRACT

KEYWORDS: Neuropharmacological evaluation, Antidepressant activity, Anxiolytic activity, Forced Swim Test, Elevated Plus Maze, Oxidative stress, Acetylcholinesterase, Antioxidant enzymes

INTRODUCTION

The field of Neuropharmacology primarily focuses on the interaction of chemical agents with neuronal pathways, neurotransmitters, receptors, ion channels, and signalling mechanisms involved in central and peripheral nervous system functions. Neuropharmacological evaluation plays a crucial role in the discovery and development of novel therapeutic agents for neurological and psychiatric disorders such as depression, anxiety, epilepsy, schizophrenia, Parkinson's disease, Alzheimer's disease, and neuropathic pain (Dang *et al*., 2024). Conventional Neuropharmacological agents including antidepressants, anxiolytics, antipsychotics, and sedatives are effective but are often associated with several adverse effects such as sedation, tolerance, dependence, cognitive impairment, sexual dysfunction, and withdrawal symptoms (Picheta *et al*., 2024). These limitations have stimulated growing interest in medicinal plants and phytoconstituents as alternative therapeutic options. Recent scientific investigations have demonstrated that plant-derived compounds can modulate several neurochemical pathways including serotonergic, dopaminergic, noradrenergic, cholinergic, GABAergic, and glutamatergic systems (Alizadeh *et al*., 2024).

In recent years, advances in molecular neuropharmacology have further expanded the understanding of the mechanisms underlying plant-based neurotherapeutics. Several phytochemicals have been reported to exhibit neuroprotective effects through antioxidant activity, reduction of neuroinflammation, inhibition of monoamine oxidase enzymes, modulation of hypothalamic-pituitary-adrenal (HPA) axis, enhancement of brain-derived neurotropic factor (BDNF), and regulation of neuroplasticity (Estela-Zape *et al*., 2024). Herbal medicines are gaining considerable attention due to their multitarget mechanisms, lower toxicity, better patient compliance, and synergistic therapeutic effects. Recent systematic reviews and meta-analyses have highlighted the growing evidence supporting the efficacy of medicinal plants in depression and anxiety disorders (Nurzyńska-Wierdak R, 2024; Shringi *et al*., 2024).

Among medicinal plants, *Nyctanthes arbor-tristis* Linn., commonly known as Parijat or Night Jasmine, has attracted significant pharmacological interest due to its CNS depressant, antioxidant, anti-inflammatory, anxiolytic, and antidepressant activities (Kenda *et al*., 2022). The flowers contain flavonoids, iridoid glycosides, tannins, and phenolic compounds that may contribute to Neuropharmacological effects through modulation of neurotransmitter systems and oxidative stress pathways (Biton *et al*., 2024). Therefore, Neuropharmacological evaluation of *Nyctanthes arbor-tristis* flowers may provide scientific evidence for its traditional use in nervous disorders and may aid in the development of safer herbal neurotherapeutic agents.

MATERIAL & METHODS

Plant materials Collection & Authentication

The flowers of *Nyctanthes arbor-tristis* were collected from local area of Hyderabad in the month of August & September. The plant materials i.e. flowers were taxonomically identified by Senior Scientist, College of Horticulture, Mandsaur, MP.

Preparation of Total Crude Extract

The plant material i.e. flowers were dried under shade and subjected to coarse powder for extraction process. Accurately weighed quantity of flower powder of *Nyctanthes arbor-tristis* were extracted using petroleum ether for the removal of fats and then different solvents were used according to polarity charts i.e. DCM, Ethyl acetate, ethanol and finally water by soxhlet apparatus for 72 h. After drying, the respective extracts were weighed and percentage yield was determined (Mukherjee, 2002). The percentage yields of all dried extracts were determined by using the following formula.

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Preliminary Phytochemical Tests

Qualitative chemical tests of different extracts were subjected to various chemical tests to detect various phytoconstituents (Kokate, 2003; Khandelwal, 2006).

Selection of animals

Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 g were used which were procured from the central animal house of College of Pharmacy, India. All animals were housed in an animal room under normal condition of 25±1^{0}C, 12-h light and dark cycle. The animals were allowed free to access commercial rat pallet diet (Lipton India Ltd, Mumbai, India) and water *ad libitum.* The bedding materials of the cages were changed every day. All the experimental procedures were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study designs were approved by the Institutional Animal Ethical Committee.

Acute toxicity studies

The acute oral toxicity study was carried out by using Wistar rats (150-200 gm). The temperature in the experimental room was around 25^{0}C. Lighting was in natural sequence i.e. being 12 h darkness, 12 h light. All extracts were prepared as a suspension by triturating dried extracts with 1% Tween 80, prepared in distilled water. Wistar rats (150-200 g) were used

for acute toxicity study to determine the acute toxicity of all extracts. The extracts were administered in a single dose by gauge into the stomach tube. Prior to dosing, animals were kept for 12 h of fasting. Then animals were weighed and test substances were administered. Extracts of all plants were administered orally to albino rats at dose of 2000 mg/kg. In first step each dose was tested on single rat and then administered to other four rats.
One tenth and one fifth of the lethal dose was taken as effective dose (therapeutic dose) and cut off value was selected as 200 and 400 mg/kg to evaluate the dose dependent action for the evaluation of Neuropharmacological activity (OECD guidelines, 2001).

Assessment of Anxiolytic activity in rats using the Elevated plus maze
The elevated plus maze test has been widely validated to measure anxiety in rodents (Lister RG, 1990).
The elevated plus maze consists of two open arms and two closed arms with the open arm perpendicular to the closed one. Rats were divided into different groups as per the protocol of study consisting of 6 rats per groups. Treated groups received the Ethanolic extract at a dose of 200 and 400 mg/kg body weight. The other two groups received control vehicle 2%v/v Tween 80 and standard drug (Diazepam 2 mg/kg, i.p) and 30 minutes later, the animals were individually placed at the center of the plus maze and observed for 5 minutes. The number of entries and time in seconds spent by the animals in the open arm were noted and compared with the control groups.

Assessment of anti-depressant activity of extracts using Forced swimming test
Rats were divided into different groups as per the standard protocol of the study and each group consisting of 6 rats per groups. Treated groups received the Ethanolic extract at a dose of 200 and 400 mg/kg body weight. The other two groups received control vehicle 2%v/v Tween 80 and standard drug imipramine (15 mg/kg, p.o). Forced swim test is commonly used pharmacological model to study antidepressant activity (Porsolt *et al*., 1997). Extracts and standard dose was administered 30 minutes prior to swim test Duration of immobility was recorded during 5min swimming test, a rat was assigned to be immobile when it floated in an upright position and making little movements to hold its head above water. Increase in active response such as climbing or swimming and reduction in immobility are considered as behavioral profile consistent with antidepressant like action (Cryan *et al*., 2002).

Biochemical estimations
For preparation of homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 minutes and the resultant cloudy supernatant liquid was used for estimation of cholinesterase and *In vivo* antioxidant level.

Estimation of Acetylcholinesterase (AchE) enzyme in rat brain
Acetylcholinesterase (AChE) enzyme activity was estimated (Ellman *et al*., 1961). A 0.4 ml aliquot of brain homogenate was taken in a cuvette containing 2.6 ml of 0.1M phosphate buffer (pH 8).100µl of the DTNB reagent was added and the absorbance was measured at 412 nm. 20µl of the acetyl thiocholine iodide was added. Changes in absorbance were measured and the change in absorbance per minute was estimated. The enzyme activity is expressed as millimoles/minute/mg. tissue.

Estimation of antioxidant status in rat brain
Estimation of MDA
Malondialdehyde (MDA) is a measure of lipid peroxidation and was measured (Ohkawa *et al*., 1979). Reagents were added to 0.1 ml of processed tissue samples, then heated at 100 °C for 60 min. Mixture was cooled with tap water and 5 ml of n-butanol pyridine (15:1), 1 ml of distilled water was added and vortexes vigorously. The mixture was centrifuged at 4000 rpm for 10 minutes and the organic layer was separated. The absorbance was measured at 532 nm using a spectrophotometer and concentration of MDA was expressed as nmol/g tissue.

Estimation of catalase
Catalase of brain homogenate was estimated according to the method (Aebi H., 1974). 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started after the addition of 1.0ml of freshly prepared 30mM H_2O_2. The decomposition rate of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm. Activity of catalase was expressed as units/mg protein.

Assay of super oxide dismutase (SOD) Reagents
To 50 µl of the suspension, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 minutes by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance per minutes at 420 nm. The activity of SOD is expressed as units/mg protein (McCord & Fridovich, 1969).

Assay of glutathione peroxidase (GPx)
The reaction mixture consisting of 0.2 ml each of EDTA, sodium azide and H_2O_2, 0.4 ml of phosphate buffer, 0.1

ml of suitably diluted tissue was incubated at 37^{O}C at different time intervals and 0.5 ml of TCA was added and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml DTNB were added and the color developed was read at 420 nm immediately. The activity of GPx is expressed as µmoles of glutathione oxidized / minutes /mg protein (Lawrence & Burk, 1976).

STATISTICAL ANALYSIS

Data were expressed as the mean standard error of mean (S.E.M.) of the means and statistical analysis was carried out employing one-way ANOVA. Differences between the data were considered significant at $P < 0.05$.

RESULTS & DISCUSSION

Extractive Value Determination

Dried flowers of *Nyctanthes arbor-tristis* were extracted using petroleum ether, DCM, Ethyl acetate, ethanol and finally water. The percentage yields of all dried extracts were determined by using the following formula.

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Table No 1: Different extracts with their appearance and % yield (in gm)

S. No.	Extracts	Color of dried extracts	Consistency of dried extracts	% Yield (W/W)
1	Pet. ether extracts of *Nyctanthes arbor-tristis*	Dark Brown	Sticky	10 %
2	DCM extracts of *Nyctanthes arbor-tristis*	Dark Orange	Sticky	8.8 %
3	Ethyl acetate extracts of *Nyctanthes arbor-tristis*	Dark Orange	Sticky	2.6 %
4	Ethanolic extracts of *Nyctanthes arbor-tristis*	Dark Brown	Sticky	10 %
5	Aqueous extracts of *Nyctanthes arbor-tristis*	Light Color	Sticky	8.9 %

Preliminary Phytochemical Screening

The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolics compounds.

Table No 2: Qualitative chemical analysis of extracts by chemical tests

S. No	Phytoconstituents	Chemical Tests	DCM Extract	Ethyl acetate Extract	Ethanol Extract	Aqueous Extract
1	Alkaloids	Wagner's test	+	+	-	+
		Dragendorff's test	+	+	-	+
		Mayer's test	+	+	-	+
		Hager's test	-	-	-	+
2	Amino Acid	Millon's test	+	+	-	-
		Ninhydrine test	-	-	-	-
3	Flavonoids	Shinoda test	+	+	+	+
		Alkaline reagent test	+	+	+	+
		Zinc hydrochloride test	_	+	+	-
4	Phenolics (Tannins)	Gelatin test	+	+	+	+
		Phenazone test	-	-	-	-
		Ferric chloride test	+	+	+	+
5	Protein	Biuret test	+	+	-	-
		Hydrolysis test	+	+	+	-
		Test with trichloroacetic acid	-	-	-	-
6	Triterpenoids & Steroids	Libermann-Burchard test	+	+	+	+

		Salkowski test	+	+	+	+
7	Carbohydrates	Benedict's test	+	+	+	+
		Fehling's test	+	+	+	+
		Molish's test	-	-	-	-
8	Anthraquinone glycosides	Borntrager's test	+	+	+	+
		Modified Borntrager's test	+	+	+	+
9	Coumarin glycosides	_____	-	-	-	-
10	Saponin glycosides	_____	+	+	+	+
11	Cardiac glycosides	Baljet's test	+	+	+	+
		Legal's test	+	+	+	+
		Keller-killiani test	+	+	+	+

Where, (-) Negative, (+) Positive

Acute Toxicity Studies of Plant Extracts

No toxic effects were observed at a higher dose of 2000 mg/kg body weight of Wistar rats. Hence, 1/10th and 1/5th dose was selected as effective dose or therapeutic dose. The cut off value of 200 and 1/5th dose double of 400 mg/kg were selected for anti-arthritic and anti-inflammatory activity.

Table No 3: Acute toxicity studies of plant extracts

S. No.	Treatment	Dose (mg/kg)	Number of animals	Mortality			Toxicity Profile
				After 24 hrs	After 7 days	After 14 days	
1	Petroleum Ether Extract	2000	5	0	0	0	Safe
2	DCM Extracts	2000	5	0	0	0	Safe
3	Ethyl acetate	2000	5	0	0	0	Safe
4	Ethanolic Extracts	2000	5	0	0	0	Safe
5	Aqueous Extracts	2000	5	0	0	0	Safe

NEUROPHARMACOLOGICAL EVALUATION OF EXTRACTS

Assessment of Anxiolytic activity in rats using the Elevated plus maze

The results obtained indicate that the groups treated with ethanolic extract of *Nyctanthes arbor-tristis* at dose level of 200 mg/kg and 400 mg/kg, p.o did not produce significant increase in number of entries in open arm compared to the control groups. However diazepam treated groups showed statistically significant increase ($p < 0.01$) in entries in open arm.

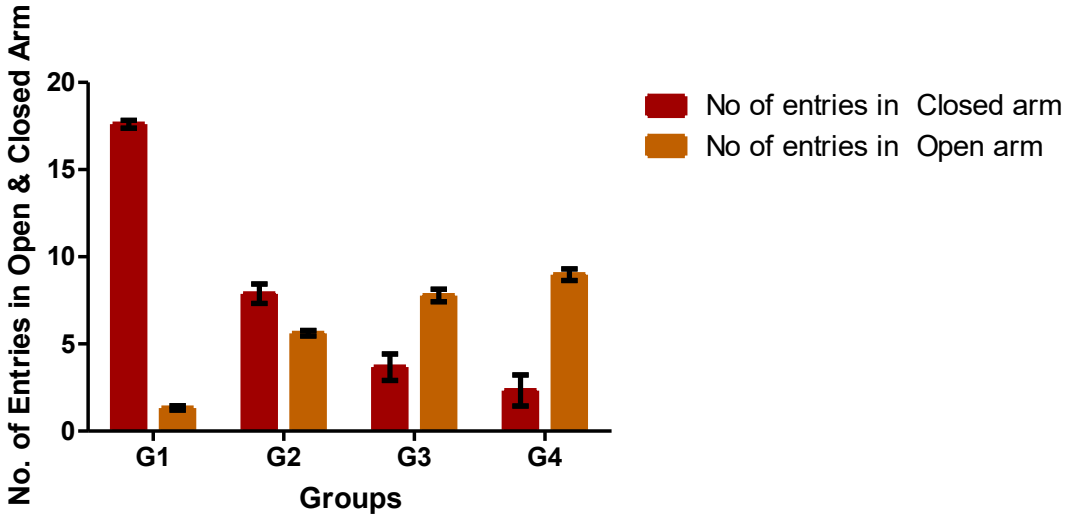

Figure No. 1: Effect of ethanolic extract on No. of entries in open & closed arm

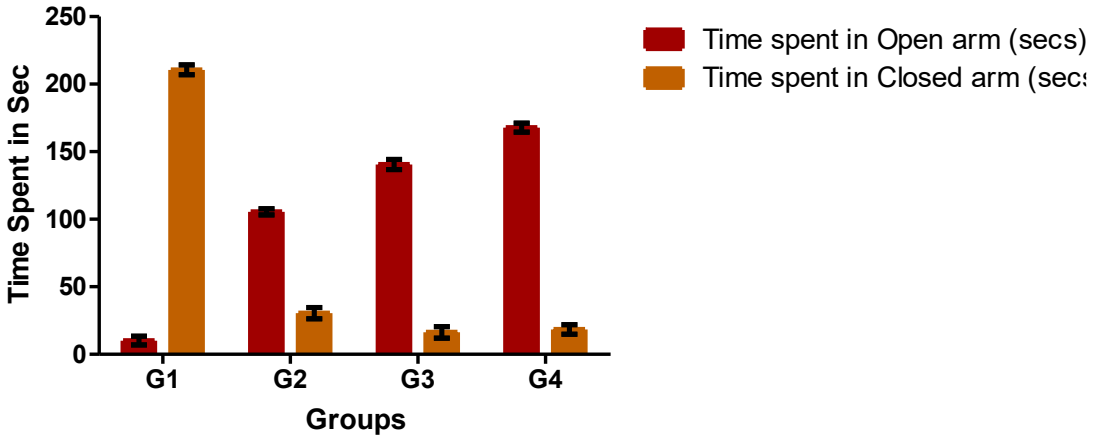

Figure No. 2: Effect of ethanolic extract on time spent in open & closed arm

Assessment of antidepressant activity in rats using Forced swim test

The effect of ethanolic extract of *Nyctanthes arbor-tristis* at dose level of 200 mg/kg, 400 mg/kg, p.o and impiramine on active behaviors in forced swim test of rats is shown in figure. Ethanolic extract significantly ($p < 0.01$) shortened the immobility time in dose dependent manner in comparison to the control values. However impiramine treated groups showed significant reduction ($p < 0.01$) in immobility time as compared to the control.

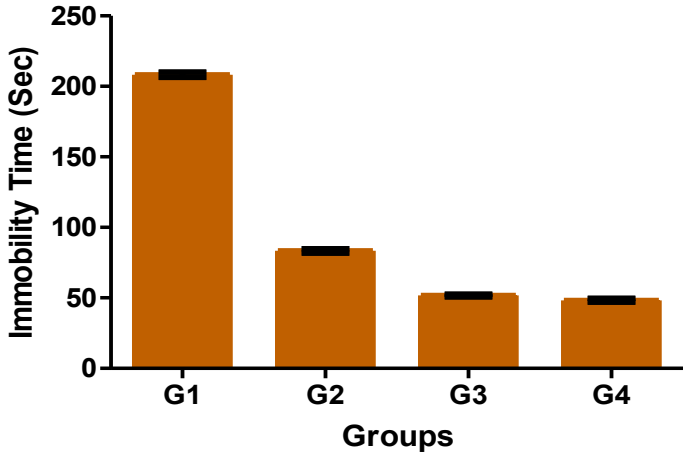

Figure No. 3: Effect of ethanolic extract on immobility time (Sec) in rats

Estimation of the cholinesterase level in the brain homogenate in rats

Effect on brain cholinesterase activity

Ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o significantly ($p<0.01$) reduced the levels of cholinesterase as compared to scopolamine treated groups by Ellman's kinetic calorimetric method, which is considered as indicator of inhibition of cholinesterase activity in rat brain after 14 days of treatment. Impiramine significantly ($p<0.01$) reduced the levels of cholinesterase.

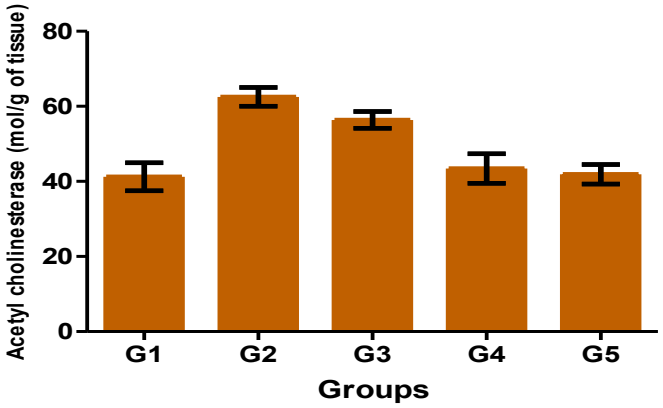

Figure No. 4: Effect of ethanolic extract on Acetylcholinesterase level in rats

Estimation of the antioxidant enzyme level in the brain homogenate

Effect of ethanolic extract of *Nyctanthes arbor-tristis on* MDA level

Disease control rats significantly ($p < 0.01$) increased the brain MDA level compared to control groups. Standard drug imipramine and ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o treatment, ($p < 0.01$) significantly decreased brain lipid peroxide level in dose dependent manner when compared to their corresponding diseased groups, respectively.

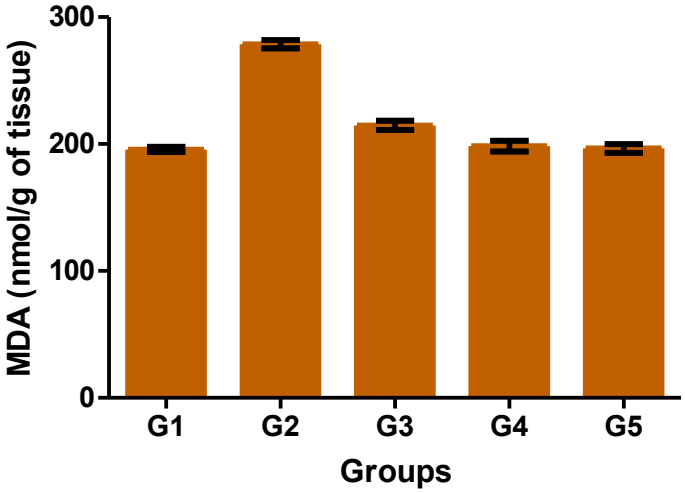

Figure No. 5: Effect of ethanolic extract on MDA level in rats

Effect of ethanolic extract of *Nyctanthes arbor-tristis on* catalase level

Disease control rats significantly ($p < 0.01$) decreased brain catalase level as compared to control groups. Ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o treatment increased the catalase level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding scopolamine treated groups respectively. Imipramine treatment also significantly ($p<0.01$), increased the catalase level compared to the corresponding diseased groups respectively.

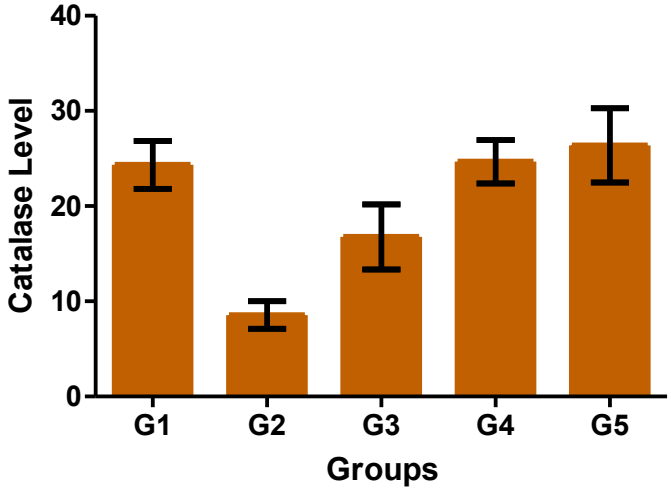

Figure No. 6: Effect of ethanolic extract on Catalase level in rats

Effect of ethanolic extract of *Nyctanthes arbor-tristis on* super oxide dismutase

Disease control rats significantly ($p<0.01$) decreased brain super oxide dismutase level as compared to control groups. Treatment with standard drug imipramine and ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o respectively increased the super oxide dismutase level significantly ($p<0.01$) in dose dependent manner when compared to the corresponding diseased groups.

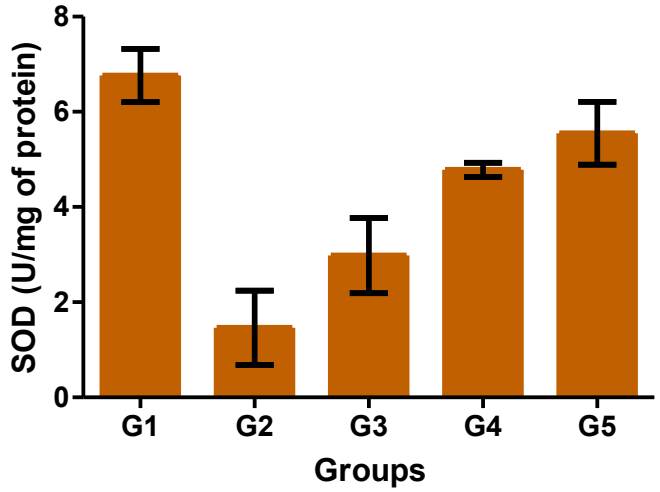

Figure No. 7: Effect of ethanolic extract on SOD level in rats

Effect of ethanolic extract of *Nyctanthes arbor-tristis on* glutathione peroxidase

Imipramine and ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o treatment did not show significant change in the glutathione peroxidase level when compared to the corresponding diseased groups.

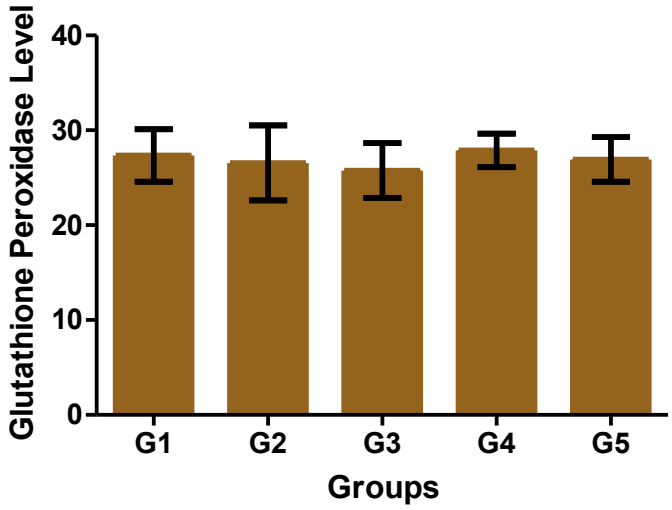

Figure No. 8: Effect of ethanolic extract on Glutathione peroxidase level in rats

All the extracts were also evaluated for the acute toxicity studies as per OECD guidelines and all the extracts were found to be safe at the dose of 2000 mg/kg and no chances of any behavioural and other effects were seen.

In our investigation, the extracts did not produce any significant change or increase in the exploratory activity of the rats in the elevated plus maze method; hence we can conclude that the extract does not possess Anxiolytic activity. Generally most of the Anxiolytic agents have an adverse effect on memory as seen with the benzodiazepines, commonly used as Anxiolytic (Murgnandam *et al*., 2000).

Neurological diseases are associated with decline in cognitive abilities, patient also have non cognitive symptoms such as depression, apathy and psychosis that impair learning (Fodale *et al*., 2006). The forced swimming test demonstrated that ethanolic extract of *Nyctanthes arbor-tristis* clearly acted as antidepressant in rats. The reduction of immobility was comparable to observed effects after administration of reference antidepressant drug imipramine, a putative catecholaminergic involvement in the antidepressant like effects of *Nyctanthes arbor-tristis* extracts could be suggested. Considering the lack of need of drugs with proven effect in improving learning, specific memory improving and antidepressant effect of *Nyctanthes arbor-tristis* can be of enormous interest for neurochemical investigation which can unravel the mechanism of action of plant drug with respect to activity.

In the present study ethanolic extract of flowers of *Nyctanthes arbor-tristis* inhibited Acetylcholinesterase enzyme, there by elevating acetylcholine concentration in the brain homogenate and ultimately improved memory in rats. It had been suggested that the varying degrees of behavioral impairments are associated with aging and age associated neurodegenerative diseases. Oxidative stress due to free radicals generation is responsible for producing the neuronal changes mediating these behavioral deficits (Cantuti *et al*., 2000).

Oxidative stress in brain generates oxygen radicals like superoxide anion, hydroxyl radical and hydrogen peroxide, which

acts on polyunsaturated fatty acids in brain, there by propagating the lipid peroxidation (Coyle & Puttfarcven, 1993). The oxidative free radical scavenging enzymes like glutathione, SOD and catalase plays an important role to reduce oxidation stress in brain.

The forced swimming test resulted in a significant increase in lipid damage which was determined by estimation of MDA level, which is a measure of lipid peroxidation and free radical generation. Elevation of brain oxidative status of amnesic rats resembled the clinical situation where considerable studies reported the incidence of oxidative stress and membrane lipid peroxidation in demented patients (Palmer AM. 1999). More specifically, the entire brain of patients with neurological disease was shown to be subjected to an oxidative challenge (Balazs & Leon, 1994). In addition, the overall peroxidation activity in brains of neurological patients was significantly elevated compared to normal subjects (Marcus *et al*., 1998). Inhibition of MDA by *Nyctanthes arbor-tristis* suggests its neuroprotective properties.

The status of endogenous antioxidant enzymes were investigated as free radicals mediate oxidative damage. The antioxidant enzymes like SOD, GPx serves as the first line of protection against free radical damage during the oxidative stress conditions. Forced swimming method induced a significant decrease in the enzymatic activity of antioxidant enzymes like SOD, but there was only slight change in GPx activity. *Nyctanthes arbor-tristis* at a dose of 200 mg/kg, 400 mg/kg and also Imipramine inhibited the decrease in the activity of GRD, whereas reduced SOD activity induced by forced swimming was not only restored, but also increased higher than that of normal control rats. Studies have also reported significantly lower levels of SOD activity than that of non-demented control in the cerebellum, frontal cortex and hippocampus (Chen *et al*., 1994).

SOD is the only enzyme that uses the superoxide anions as the substrate and produces hydrogen peroxide as a metabolite. Super oxide anion is more toxic than H_2O_2 and has to be removed. Pretreatment with *Nyctanthes arbor-tristis* significantly prevented the reduction of SOD activity in brain during swimming test. The cognitive enhancing activities of *Nyctanthes arbor-tristis* might be due to the inhibition of AChE activity and the decrease in ROS by restoring the antioxidative defense system.

CONCLUSION

Nyctanthes arbor-tristis ethanolic extract, as an antioxidant medication may be of value for demented elderly patients with elevated brain oxidative status. Since depression usually coexists with dementia, *Nyctanthes arbor-tristis* extract as an antidepressant medication with added advantage of preventing oxidative stress could be a better alternative for depressed demented patients. This study may also provide more evidence that *Nyctanthes arbor-tristis* extract, as an antioxidant medication, can be a novel type of antidepressant with memory enhancing properties.

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