EFFECT OF ETHANOLIC EXTRACT OF FLOWERS OF NYCTANTHES ARBOR-TRISTIS LINN. ON VARIOUS OXIDATIVE STRESS PARAMETERS IN FORCED SWIMMING MODEL

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Article History: **Received:** 01/11/22 **Revised:** 16/11/22 **Accepted:** 28/11/22

Abstract

AIM- The aim of the present investigation is to evaluate the effect of oxidative stress parameters in forced swimming model.

MATERIAL & METHODS- Forced swim test is commonly used pharmacological model to study antidepressant activity (Porsolt *et al*., 1997). The apparatus consisted of transparent cylinder (50cmhigh x20cmwide) and water at room temperature was filled to 30 cm depth. 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.

RESULTS- The cut off value of 200 and 1/5th dose double of 400 mg/kg were selected for activity. 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. 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. Imipramine treatment also significantly ($p<0.01$), increased the catalase level compared to the corresponding diseased groups respectively.

CONCLUSION- 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.

Keywords: Ethanolic Extract, Flowers, *Nyctanthes arbor-tristis* Linn., Oxidative stress parameters, Forced Swimming Model

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DOI: 10.53555/ecb/2022.11.12.192

INTRODCUTION

Oxidative stress is caused by an imbalance in the pro-oxidant and antioxidant systems that can occur as a result of an increase in oxidative metabolism (Gotz *et al*., 1994). High content of unsaturated fatty acids and iron are present in the nervous system. The high lipid content of nervous tissue and presence of high aerobic metabolic activity will make it more prone to oxidative damage. The adequate increase in the level of iron is a important requirement during the development of brain, but it may also cause injury to the brain cells and release ions of iron, that can cause oxidative stress through the iron-catalyzed formation of reactive oxygen species (ROS) (Andorn *et al*., 1990). Brain regions that are rich in catecholamines are more prone to free radical generation. The catecholamines can auto-oxidize to free radicals or due to metabolism by the endogenous enzymes such as monoamineoxidases. In substantia nigra (SN), there is a attachment that has been established between antioxidant depletion (including GSH) and tissue degeneration. A number of *in vitro* investigations have shown that antioxidants both endogenous and dietary can defend nervous tissue from damage by oxidative stress (Contestabile A., 2001).

ROS are particularly active in the mind and neuronal tissue as the metabolism of excitatory amino acids and neurotransmitters can serve as sources of oxidative stress. ROS affects glial cells and neurons and leading to neuronal impairment. It has been reported that deleterious consequences of ROS on human cells may result in oxidative injury that can also lead to programmed cell death i.e. apoptosis (Salganik RI., 2001). Antioxidants are compounds that remove free radicals and scavenging ROS (GilgunSherki *et al*., 2001). Among them, oxidative metabolic reactions and their by products have been consistently implicated in AD pathogenesis and represent the biological basis for the oxidative stress hypotheses of Alzheimer's disease. Various studies have shown that different biomarkers of oxidative stress mediated events are elevated in the AD brain (Selkoe DJ., 2007). The role of abnormal metabolic oxidative reactions in the central nervous system may be considered as pathological cause of sporadic AD. AD brains exhibit constant evidence of reactive oxygen species and reactive nitrogen species (RNS) mediated injury (Pratico & Sung, 2004). In our previous study, an attempt was made to evaluate the Neuropharmacological investigation of ethanolic extract in various models. In the continuation of the previous work, here we have evaluated the effect of ethanolic extract on various oxidative stress parameters in forced swimming model.

MATERIAL & METHODS

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).

Preliminary Phytochemical Tests

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

Acute toxicity studies

The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guideline 423 and as per approved protocol by IAEC and CPCSEA.

The acute oral toxicity study was carried out by using Wistar rats (150-200 gm). The temperature in the experimental room was around 25^0C. Lighting was in natural sequence i.e. being 12 h darkness, 12 h light. The substance was tested using a stepwise procedure, each step using five animals of each dose.

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 impiramine (15 mg/kg, p.o). Forced swim test is commonly used pharmacological model to study antidepressant activity (Porsolt *et al*., 1997). The apparatus consisted of transparent cylinder (50 cm high x20 cm wide) and water at room temperature was filled to 30 cm depth. In pre test, rats are placed in cylinder for 15 min 24 hr prior to 5min swim test.

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. 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)

Glutathione peroxidase was assayed by the method. 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^oC 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).

Assay of glutathione reductase (GRD)

Glutathione reductase was assayed by the method. The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml of EDTA, 0.5 ml of oxidized glutathione and 0.2 ml of NADPH was made up to 3 ml with water. After the addition of 0.1 ml of suitably diluted tissue, the change in optical density at 340 nm was monitored for 2 minutes at 30 sec intervals. The activity of GRD is expressed as n moles of NADPH oxidized / minute / mg protein (Beutler *et al*., 1963).

Estimation of reduced glutathione (GSH) Reagents

Glutathione was measured according to the method. The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid), and 0.4 ml of distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of glutathione was expressed as µg/g. tissue (Ellman GL., 1959).

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

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.

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, $1/10^{th}$ and $1/5^{th}$ dose was selected as effective dose or therapeutic dose. The cut off value of 200 and $1/5^{th}$ dose double of 400 mg/kg were selected for activity.

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.

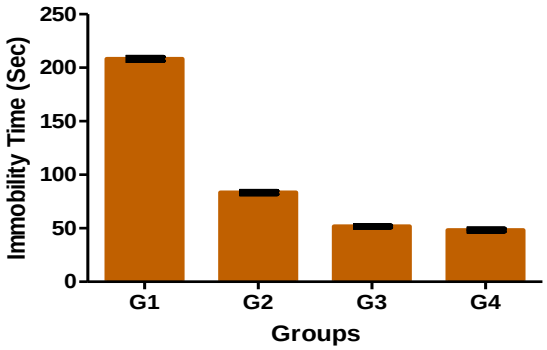

Figure No. 1: Effect of ethanolic extract on immobility time (Sec) in rats

Statistical significance test was done by ANOVA followed by Dunnet's 't' test (n=6) Values are mean ± SEM of 6 animals per groups *P< 0.05 vs control

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 and results indicated in figure 2.

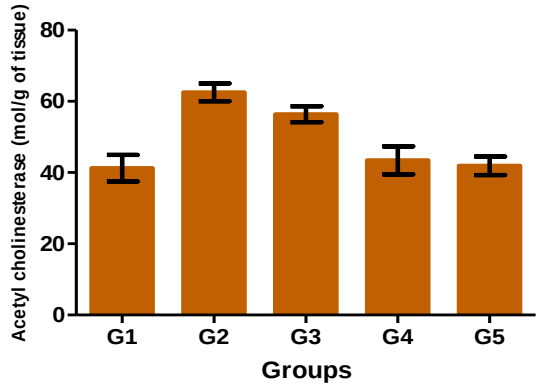

Figure No. 2: Effect of ethanolic extract on Acetylcholinesterase level in rats

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.

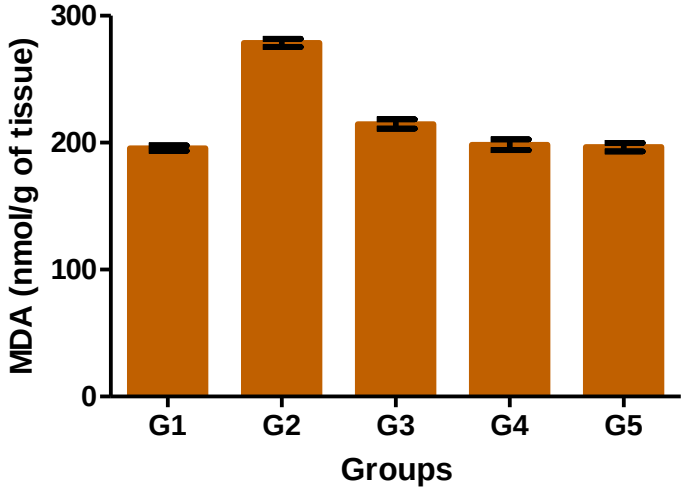

Figure No. 3: Effect of ethanolic extract on MDA level in rats

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.

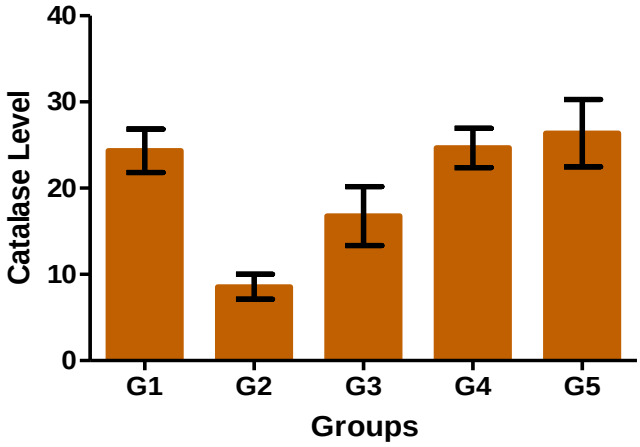

Figure No. 4: Effect of ethanolic extract on Catalase level in rats

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.

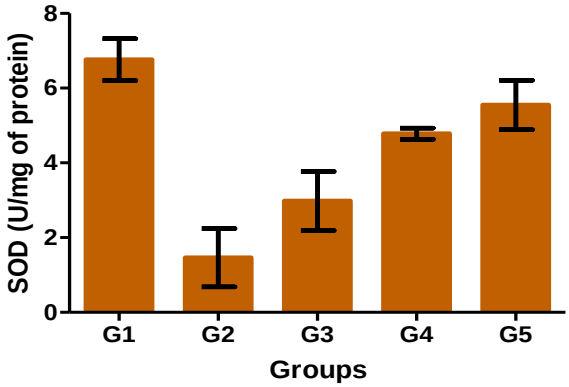

Figure No. 5: Effect of ethanolic extract on SOD level in rats

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.

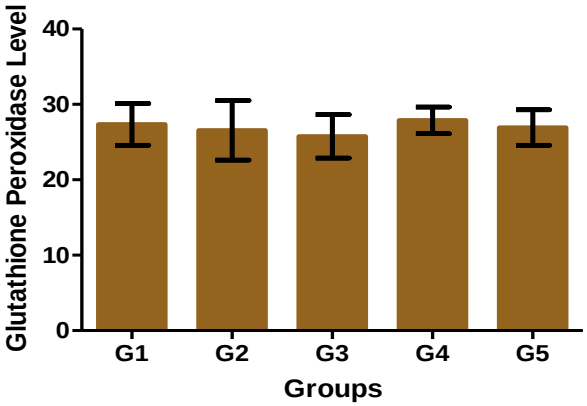

Figure No. 6: Effect of ethanolic extract on Glutathione peroxidase level in rats

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

Diseased rats significantly ($p<0.01$) decreased brain glutathione reductase 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 glutathione reductase level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding diseased rats groups respectively. Imipramine treatment also increased the glutathione reductase level significantly ($p < 0.01$) when compared to the corresponding diseased groups.

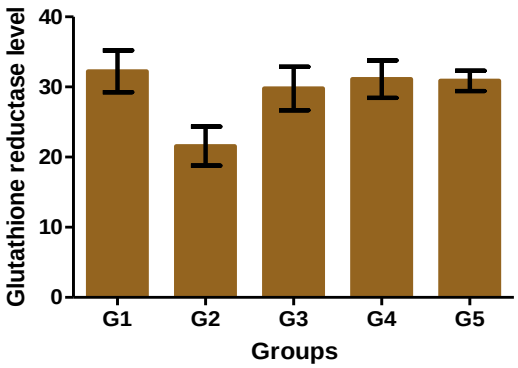

Figure No. 7: Effect of ethanolic extract on Glutathione reductase level in rats

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

Diseased animals significantly ($p<0.01$) decreased brain reduced glutathione level as compared to control groups. Imipramine and ethanolic extract of *Nyctanthes arbor-tristis* at dose of 200 mg/kg and 400 mg/kg p.o treatment increased the reduced glutathione level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding diseased groups respectively.

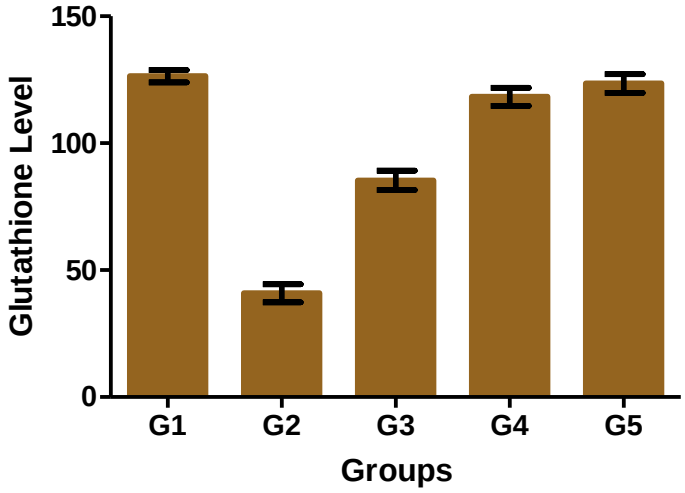

Figure No. 8: Effect of ethanolic extract on Glutathione level in rats

DISCUSSION

Phytotherapeutic products are often considered as safe because they are natural (Gesler WM, 1992). However, these products may contain bioactive principles with potential to cause adverse effects (Bent S & Ko R., 2004). In supplement, the poor Pharmacogivilane surveillance in this field makes it difficult to determine the adverse effects caused by the utilization of phytotherapeutic products (Eisenberg DM *et al*., 1998).

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, GRD and GSH 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 GRD and 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).

Intracellular GSH status served as a sensitive indicator of the health of a cell or tissue. Our results indicate that GSH levels decreased significantly after applicable of forced swimming test. There was a significant reduction in levels of glutathione, a tripeptide found in all cells, which reacts with free radicals to protect cells from superoxide radical, hydroxyl radical and singlet oxygen (Schulz *et al*., 2000). Depletion of brain GSH level due to the treatment with forced swimming was restored significantly by *Nyctanthes arbor-tristis*. Thus, it can be postulated that *Nyctanthes arbor-tristis* scavenges ROS and exhibit a protective effect against oxidative damage induced by selective model by restoring the activities of glutathione reductase and SOD. 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.

In addition, *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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