Investigations on light induced stress model and on the role of *Phyllanthus amarus* in attenuation of stress related depression with focus on 5HT$_{2A}$m- RNA expression

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ABSTRACT

Background: There is an increasing evidence that psychological stress and depression trigger changes in various biochemical parameters in animals and human subjects. Chronic stress in rats, and psychosocial stress in humans, is implicated in the pathophysiology of mood and anxiety disorders. **Purpose:** The current study was been designed to investigate the behavioral, anatomical status of rat brain and expression of serotonin receptor (5HT$_{2A}$) mRNA related to pathophysiology of stress after high light (HL) illumination and to evaluate the effect of *Phyllanthus amarus* (PA) over such stress induced changes in mid brain and prefrontal cortex region of rat brain. **Methods:** Bright light illumination was used to induce stress in wistar rats. Established methods were used to analyse biochemical and histopathological tests. **Results:** *PA* administration to HL stressed animals for 7 days prevented stress-induced oxidative damage, as evidenced by significant enhancement of key endogenous antioxidant defense components. HL stress also caused reduced activities of membrane bound enzymes in synaptic membrane along with reduced levels of lipid profile and glycoprotein in the midbrain region. Stress induced changes in the locomotor activity, time spent for exploring the center of the arena, frequency of rearing and grooming, and frequency of facing the corner of the arena, were altered on *PA* administration. **Conclusion:** The findings suggest that the therapeutic efficacy of *PA* may be mediated, atleast partially, via reversal of oxidative damage. Further the study demonstrates the promising intervention by *PA* on the mRNA expression of 5HT 2A in the brain. These preliminary results pave the way for further validation of *PA* against stress.

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Introduction

Stress is inevitable in every walk of life and reverberates in any individual's life, proving detrimental. Stress is a major contributor of depression and by the year 2020, it may rank second in morbidity among all illnesses worldwide. Depression may arise when neuronal systems do not exhibit appropriate, adaptive plasticity in response to external stimuli such as stress. Stress/depression has been shown to increase neuronal apathy/death. Stress exerts detrimental effects on several cell functions, through impairment of antioxidant defenses, leading to oxidative damage, which is central to many diseases.[1] Free-radical damage by reactive oxygen species has been suggested to play a critical role in the pathophysiology of diseases, neuropsychiatric disorders and stress induced depression. Although clinical depression, depressive symptoms and psychological stress should be distinguished, they are closely related with one another[2] and play an important role in the development of affective disorders. Repeated chronic stress has been associated with the development and manifestation of depression.[3]

Serotonergic neurotransmission in the central nervous system (CNS) is believed to be involved in this pathogenesis and recovery from depression.[4] Clinical findings suggest that enhancement of serotonergic neurotransmission underlies the antidepressant response associated with most agents presently available to treat depression.[5] Studies have shown that rats subjected to chronic stress exhibit behavioral, biochemical and physiological impairments.

Phyllanthus amarus (PA) is a rich source of plant chemical and all of its parts constitute biologically active compounds. It has a long back history documented in reducing pain and cholesterol maintanence. Some of the earlier studies showed that it possesses antioxidant, antidiabetic, antitumor properties and

that it suppresses the expression of hepatitis B viral mRNA in cell lines.[6] Traditionally, *PA* also has been used to treat jaundice, gonorrhea, uncontrolled menstruation, dysentery.[7] The aqueous extract of *PA* had been employed for treatment of nervous debility, epilepsy, as *medhya* (intellect promoting) and in *vata* disorders antiamnesic effects,[8] and thus the plant with multi-potentiated effects was selected for an evaluation as an anti-stress drug.

Light is an important environmental factor for regulation of mood. As rats are nocturnal and are sensitive to extreme bright light environment, light illumination was selected in the current study as a stressor for the stress-induced depression models. Serotonergic neurons may play a particularly important role in the facilitation of anxiety-related physiological or behavioral responses. A wealth of evidence supports an association between the neuronal activity of brainstem serotonergic neurons and the level of somatic motor activity or behavioral arousal on exposure to uncontrollable stress or other anxiety related stimuli including anxiogenic drugs and social defeat. Bright light can be used as an effective aversive stimulus leading to an increase in avoidance behaviors. Exposure of rats to the HL(400–500 lux) condition performed in the current study was comparable to previous behavioral studies using a similar behavioral test arena for an alternative anxiety paradigm, i.e. the social interaction test which will be a suitable model for analyzing the light induced variations in the brain.[9] The aim of the investigation is to analyze light induced variations in the brain of rats and the potential of *Phyllanthus amarus* in targeting the variations.

Methods

Animals. Adult Male albino rats of Wistar strain weighing about 150–200g were obtained from the Tamilnadu Veterinary and Animal Science University, Chennai, India. The animals were

acclimatized to animal house conditions, fed commercial pellet rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water. This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (360/01/a CPCSEA).

Stress Induction

To induce stress, rats were exposed to bright light illumination. In the present study, we attempted to manipulate the aversiveness of the test conditions by comparing undisturbed control and gently handled Wistar rats, that were exposed to bright light (400–500 lx) illumination in the box.

Each experimental animal was randomly assigned to one of four treatment groups: i.e. Group I -control (CO), Group II- high-light test (HL: 400–500 lx throughout the box), Group III- light and PA(200 mg/kg bwt) treated (LPA) and Group IV- drug alone (PA 200 mg/kg bwt)[10] treated groups. CO rats were left undisturbed on the test day. An hour before the stress induction treatment drug (PA) is given to LPA group. After acclimatization altogether 4 groups, HL and LPA groups were stressed per day (for a total of 7 days) between 9:00 a.m. and 1:00 p.m. During the stress procedure, the experimental group of rats were deprived of food and water, each group consisting of six animals.

Histopathological analysis

The brain tissue was excised and fixed in 10% phosphate buffered formalin. The tissue sections were embedded in paraffin wax and sectioned at 5–6 μm thickness. Sections were then stained with hematoxylin and eosin to evaluate the cellular pattern in the brain tissue of control and experimental groups.

Biochemical profile

100 mg of the whole brain tissue was weighed, uniformly homogenized with 1 ml of 0.1 M phosphate buffer, pH 7.4 and the homogenate was used for the following biochemical assays. Part of the assays was also performed using the synaptosomal plasma membrane isolated by gradient centrifugation techniques. 0.1 ml of the whole homogenate and 20 ml of synaptic plasma membrane diluted to 0.1 ml was used for the assay.

Isolation of synaptosomes

Fresh brain tissue was suspended in ice cold 5.0 mM Tris Hcl buffer containing 0.32 M sucrose (pH 7.4). The suspended tissue was gently disrupted in a Teflon glass homogenizer; w/v ratio 1:10. The homogenate was centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was taken and centrifuged at 17,000 g for 15 minutes at 4°C.

The pellet thus obtained was the synaptosomal fraction. It was suspended in 10.0 mM Tris Hcl buffer containing 136.0 mM sodium chloride, 5.0 mM potassium chloride, 0.16 mM calcium chloride, 0.1 mM ethylene diamine tetra acetic acid, 1.3 mM magnesium chloride and 10.0 mM glucose at a protein concentration of 1.0 mg/ml.

Isolation of synaptosomal plasma membrane

Synaptic plasma membrane was prepared by hypo-osmotic lysis of the synaptosomes, homogenization, and separation of the synaptic plasma membrane fron myelin and mitochondria by sucrose gradient centrifugation following the method of C.W. Cotman.[11]

Total protein was estimated by the method of.[12] Induction of oxidative damage was ascertained by measuring the extent of LPO in brain tissue extract. LPO was estimated by the method of Ohkawa et al.[13] Superoxide dismutase (SOD) was estimated by method of Markland.[14] Catalase (CAT) was estimated by the method of Sinha.[15] Glutathione-S-transferase (GST) was estimated by the method of Habig.[16] Glutathione peroxidase (GPx) was estimated by method of Rotruck.[17] Reduced glutathione (GSH) was estimated by the method of Moron[18] and vitamin C was estimated by the method of Omaye.[19]

Assay of ATP ases

The enzyme analyses were performed in the synaptic membrane of brain cells from mid brain of stress induced, drug treated and control rats. Na-k^{2+} ATP ase was estimated by the method of Bonting.[20] Mg^{2+} ATP ase was estimated by the method of Ohinishi.[21] Ca^{2+} ATP ase was estimated by the method of Desai.[22]

Assays of enzymes

The enzyme analyses were performed in the synaptic membrane of brain cells from mid brain of stress induced, drug treated and control rats. Activities of 5'ND by the method of Fini,[23] AChE was estimated by method of Ellman.[24]

Lipid profile

Estimation of lipid profile Total lipid was extracted from the brain sample according to the method of Folch.[25] Tissue cholesterol content was estimated by the method of Parekh and Jung.[26] Free cholesterol was estimated by the method of Leffler and Mc Dougald.[27] [Phospholipids were estimated by the method of Rouser, after digesting the lipid extract with perchloric acid. Triglycerides were estimated by the method of Rice.[28] Free fatty acid content was estimated by the method of Horn and Menahan.[29]

Estimation of PBCs

Hexose was estimated by the method of Neibes.[30] Hexosamine was estimated by the method of Wagner.[31] Fucose content was estimated by the method of Winzler.[32] The estimation of sialic acid was done by the method of Warren.[33]

RT-PCR analysis

Total RNA was extracted with the method of guanidine isothiocyanate and phenol. The content of c receptor mRNA was determined by reverse transcription-PCR presenting a modification of the method described elsewhere.[34] For reverse transcription 1 μg total RNA, 180 ng random primer, and 16 μl sterile 2.25 μmol KCl were mixed in a 0.5 ml tube, denatured at 94°C for 5 min and annealed at 41°C. A buffer (15 l) μ containing 200 U M-MulV reverse transcriptase (Biosan), 0.225 μ mol Tris-HCl (pH 8.1-8.3), 0.015 μmol each dNTP, 0.225 μmol dithiothreitol, and 0.03 μmol MnCl was added to the tube. The mixture (final volume 31 μl) was incubated at 41°C for 60 min. The cDNA obtained was stored at −20°C. An aliquot (1 μl) was mixed with 2.5 μl mixture of 5HT$_{2A}$ -receptor gene-specific primers(5´-TGCAGAATGCCACCAACTT and 5´-TGCCACAAAAGAGCCTATGAG, 5 pmol each) and 14 μl buffer containing 0.8 U Taq-polymerase (Medigen), 1 μmol KCl, 0.2 μmol Tris-HCl (pH 9.0), 0.004 μmol dNTP, 0.15% Triton X-100, and 0.03 μmol MgCl (final volume 17.53 μl). The mixture was denatured at 94°C, for 4 min and amplified (10 cycles: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C). β-Actin primers (5 GGGAACCGCTCATTGCC and 5 –

ACCCACACTGTGCCCATCTA, 2.5 μl, 5 pmol each) were added to tubes, denatured at 94ºC for 4 min and amplified in additional 25 cycles. Preliminary experiments under the same conditions revealed linear amplification of β-actin and 5HT$_{2A}$ receptor gene fragments within 27 and 38 cycles, respectively. Amplification products (15 μl) were mixed with 5 μl 0.35% orange G in 30% sucrose and analyzed by electrophoresis in 1 agarose with 0.5x TBE buffer. After ethidium bromide staining, the gels were scanned in UV and band intensity was determined with the help of Scion Image Program (Scion Corporation).

Statistical analysis

All statistical computations were performed using SPSS tool. Values of experimental groups are given as mean, (±) SD. Two-way ANOVA with Bonferroni post test analysis was used to determine statistical significance. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Exposure of rats to the high light condition increased multiple measures of anxiety-related behavior of group II rats. Increasing the intensity of illumination of the open-field arena reduced locomotor activity and increased avoidance of the center of the arena. In addition, rearing and grooming, sleep cycle were reduced under the condition while the duration of time spent in the corners of the apparatus and the frequency of a stereotypical behavior of facing the corners of the apparatus were increased. Facing the corner is interpreted as a coping style to avoid the exposure to the bright light and the open surface of the arena. Further, stress exposed Group II rats exhibited decreased ambulation and rearing which indicated reduced exploration and apathy respectively in these animals. In the Group III experimental rats showed better improvement in their behavioural alteration.

Figure 1 shows the brain tissue histopathology of (a) control group, (b) stress induced, (c) stress+drug treated, (d) PA treatment alone. Histopathology results from the midbrain region of rats under the group control (a) and (d) *PA* treated shows the normal architecture of the cells.In the group (b) stress induced group shows damaged neuron in the edematous cortical region. This damage to the neuron cells was apparently rescued to near normal in stress induced PA treated group.

In the present study, exposure to stress by light, appears to enhance lipid peroxidation in the animal system which could be due to the activation of immune cells by proinflammatory cytokines that leads to over production of ROS interfering with structure and ratio of PUFA,[35] that causes loss of fluidity in the biological membrane. Similar to the observation which showed that *PA* cause reduction in LPO, group III rats in the study registered a reduced LPO ($p < 0.05$) in comparison to stress group thus preventing the impairment neural activity.

LPO levels in cortex of brain are negatively correlated with GSH in the depression. Depression causes a significant decrease in cysteine and cystine in the brain because cysteine is the rate-limiting precursor for glutathione synthesis. An alteration in GST activity in various tissues in response to chronic stress is accompanied by significant decrease in GSH content, which could be attributed to the increase in conjugation reactions.[36]
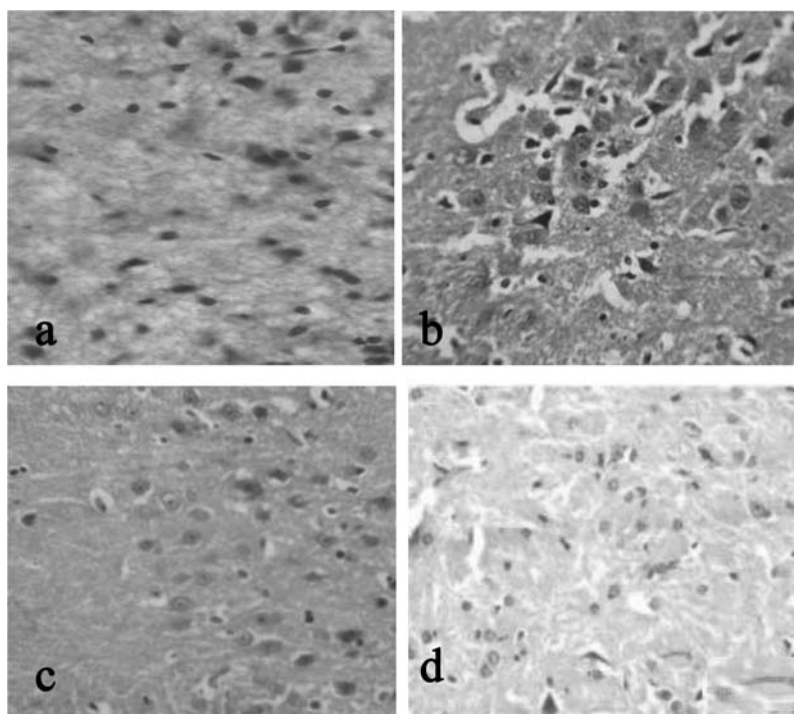

Fig. 1: Histopathological tissue section from mid brain of rat stained with H & E in 40X. **a** (CONTROL): Normal architecture of brain observed. **b** (STRESS INDUCED): rare normal cells present in edematous cortical region with scattered damaged neurons. **c** (STRESS+DRUG TREATED): more normal cells are present in edematous appearing cortical region when compared to that of group II. **d** (drug treated): drug control animal showing normal architecture of cell as that of control.

Depression is characterized with increased production of procytokines. Further, proinflammatory cytokine-induced increase production of ROS. Probably, this mechanism triggered during depression causes reduction in the activities of super oxide dismutase and catalase in stress induced rats in our study. Figure 2 a, b, c reflects the antioxidants activity in the brain tissue of control and experimental rats. In line with these experimental findings, clinical studies on patients with affective disorders have also revealed lower levels of superoxide dismutase[37] and catalase.[38] The protective activities can be mediated, at least partially, by their inductive effect on antioxidant enzymes such as CAT, SOD

and GST (endogenous scavengers of ROS) and that could be accomplished in group III rats by *PA* treatment.

Similar alteration was reflected in the study showing reduced activities of GST, GPX, reduced levels GSH and Vit C in group II rats. The mechanism under stress condition might be depletion in GR activity which catalyses NADPH-dependent conversion of GSSG to GSH which could be due to a decrease in NADPH levels, a secondary manifestation of cellular free radical stress. But the stress induced and *PA* treated group III rats showed different picture of increase in the enzyme activity and GSH levels strengthening the antioxidant defense which depicts the

(a)

(b)

(c)
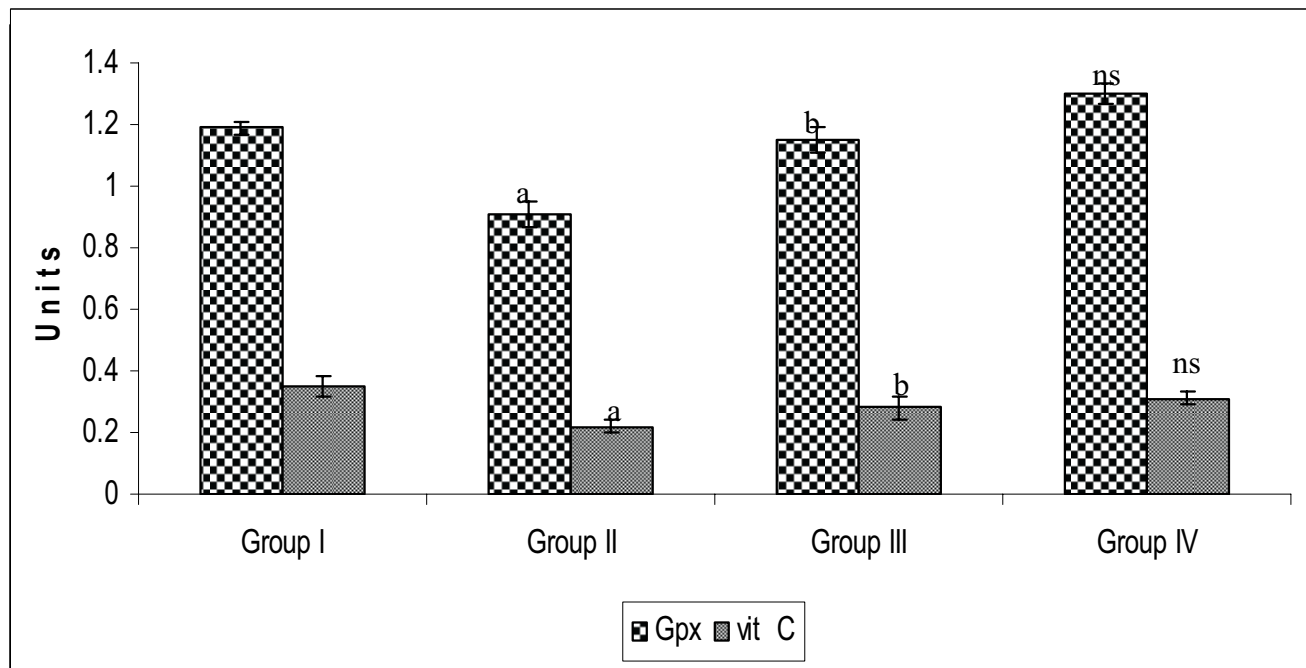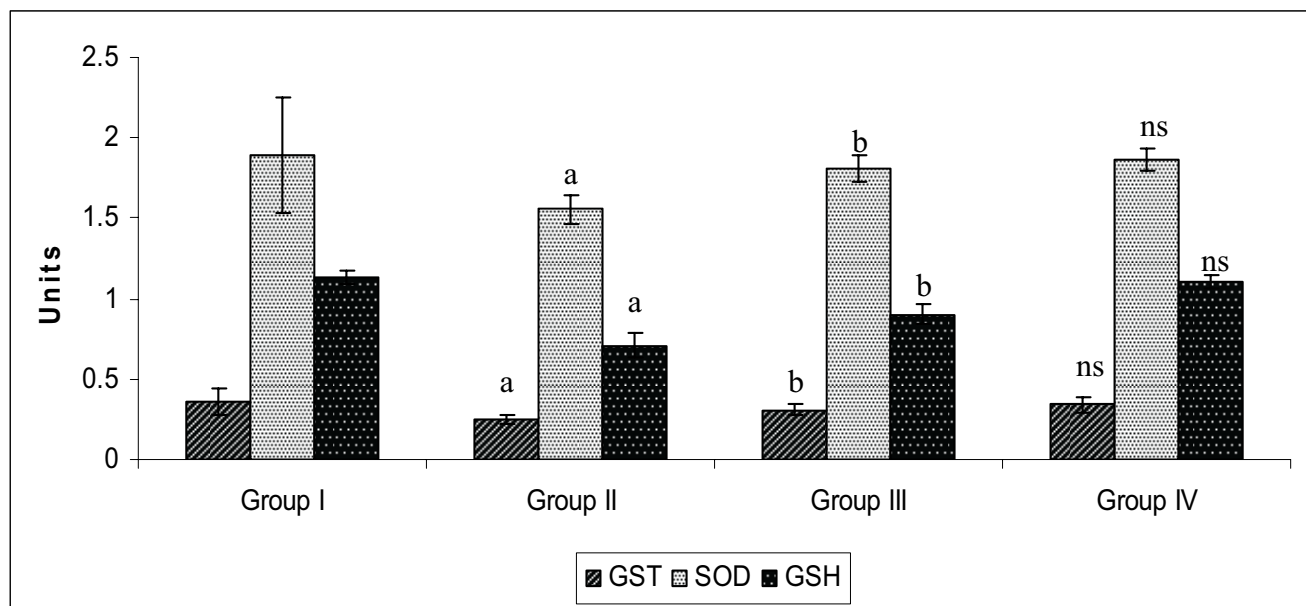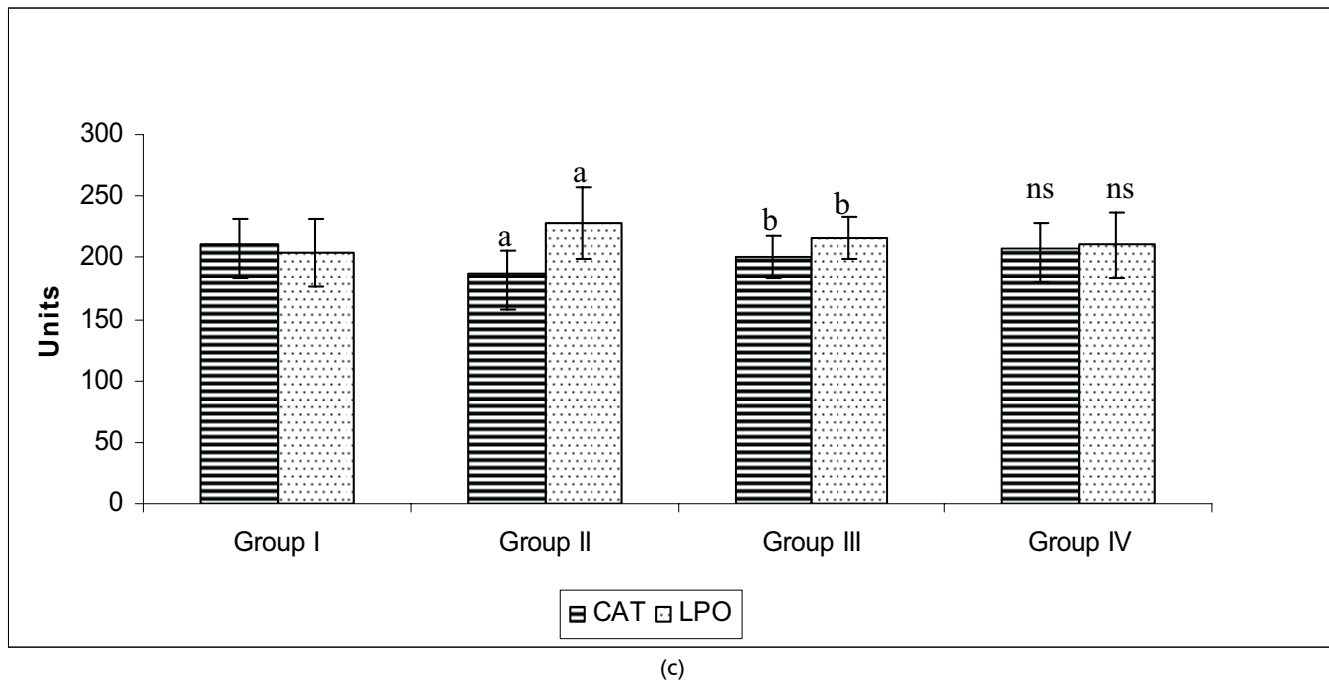
Fig. 2: Effect of *PA* on the activity of antioxidants in the brain tissue of control and experimental groups of rats.
SOD, units/min/mg protein; CAT, m mol of H_2O_2 consumed/min/mg protein; GPx, m mol of GSH oxidized/min/mg protein; GST, m mol of 1-chloro-2,4 dinitrobenzene conjugated/min/mg protein; GSH, m g/mg protein; LPO, nmol of MDA released/mg protein. Vitamin C, m g/mg protein. Statistical significance: * $p < 0.05$. a: Group II compared with Group I. b: Group II compared with Group III. NS-not significant. Group IV compared with Group I.

drug's ability to detoxify lipid per oxidation products accumulated during chronic stress. These findings are similar to results of other investigators studying GSH in relation to risk factors in depression illness in rats.[39]

Thus, in the Group III the protective effect of *PA*s may be observed which may be due to its antioxidant property by virtue of susceptible brain cells getting exposed to less oxidative stress resulting in reduced brain damage and improved neuronal function.

Figure 3 reflects the ATPases in the brain tissue of control and experimental groups of rats. The activities of synaptosomal membrane ATPases were assayed which showed a reduction in the stress induced group of rats. It was observed that these activities were reversed towards normal values ($p < 0.05$) to certain extent because of potent activity of *PA* thus challenging stress induced variation. The function of brain is based on ion homeostasis and ATPases are crucial for magnifying ionic gradients in neurons. The enzymes are involved in buffering after a period of hyperstimulation when the physiology is excited to hyperstate. Stress-induced disturbances were reported to alter ATP levels by the altered number of pumps, which in turn may be due to an increase or decrease in protein synthesis or degradation.[40] The above mentioned observations could be explained on the basis of depleted ATP levels on light-induced stress followed by reduction in the activities of ATPases which were found to be altered on *PA* treatment.

Figure 4 reflects the glycoprotein levels in the brain tissue of control and experimental groups of rats. Protein bound carbohydrates (PBC) such as hexose, hexose amine, sialic acid and fucose levels were found to be increased in the stress induced

rats ($p < 0.05$) when compared to control while *PA* treatment changed such clinical scenario.[43] It is evidenced that there is increase in the levels of PBC during stress thus arguing that the glycomoieties which involve in chief functions like brain from memory, sleep to anxiety/depression counteracted by *PA* may be due to its regulatory action on mechanism of glycoprotein release.

Figure 5 reflects the levels of lipid in the brain tissue of control and experimental groups of rats. Lipid profile showed variation among the experimental groups which is more related to the 5HT receptor function. A decreased content of cholesterol may induce a relatively increased membrane fluidity with increase in presynaptic 5-HT reuptake and decreased postsynaptic 5-HT function, resulting in antiaggressive depression. The connection between affective disorders and hypocholesterolemia may be associated most closely with alteration of cytokine mediators of inflammation produced by the brain which in turn, create changes in mood and behavior.[44] The decrease in cholesterol, triglycerides, free fatty acids and phospholipids were observed in stress induced rats that might be involved in modulating neurotransmitter function and these altered level of the lipids were found to be restored towards a normal in group treated with *PA*, highlighting that the drug might have an influence on serotonin utilization.

Figure 6 reflects the 5'ND and ACHe levels in the brain tissue of control and experimental groups of rats. The activities of enzymes namely acetylcholine esterase and 5'nucleotidase were found to be decreased in the synaptosomal plasma membrane. The activity of ACH was reported to be widely varying in different regions of brain after undergoing stress, suggesting that reduced cholinergic transmission might be partly responsible
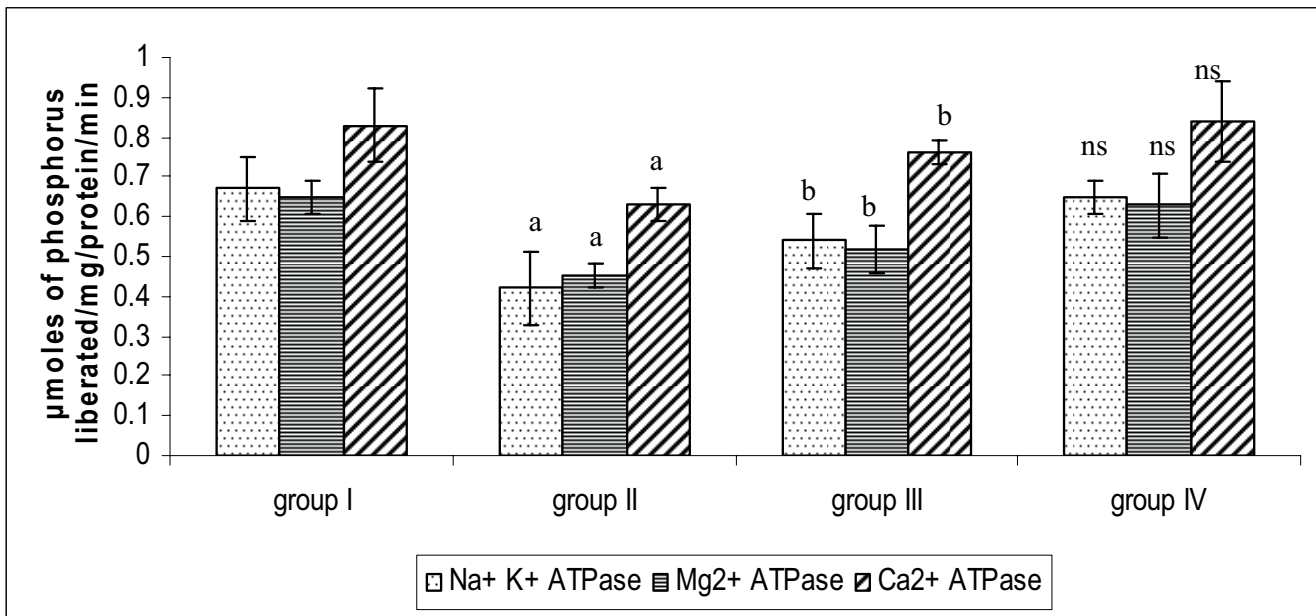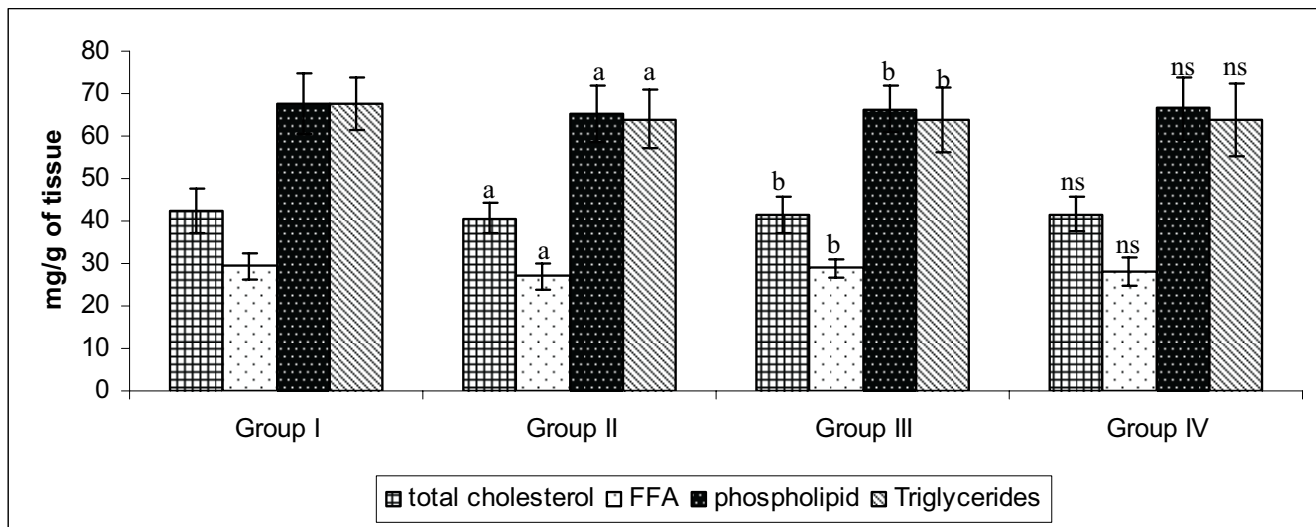
Fig. 3: Effect of *PA* on the activity of ATPases in the brain tissue of control and experimental groups of rats.
Each value is expressed as mean_S.D. for six rats in each group. ATPases are expressed as micromoles of phosphorus liberated/mg/ protein/min. Statistical significance: * $p < 0.05$. a: Group II compared with Group I. b: Group II compared with Group III. NS-not significant. Group IV compared with Group I.
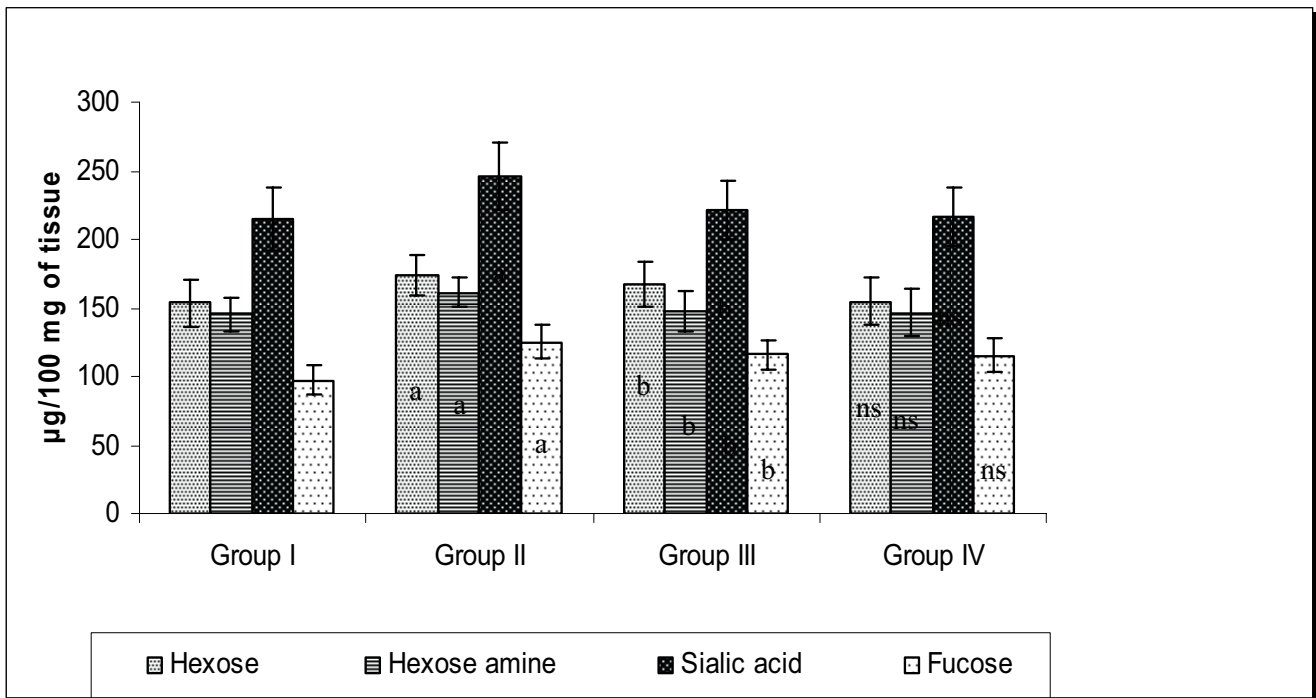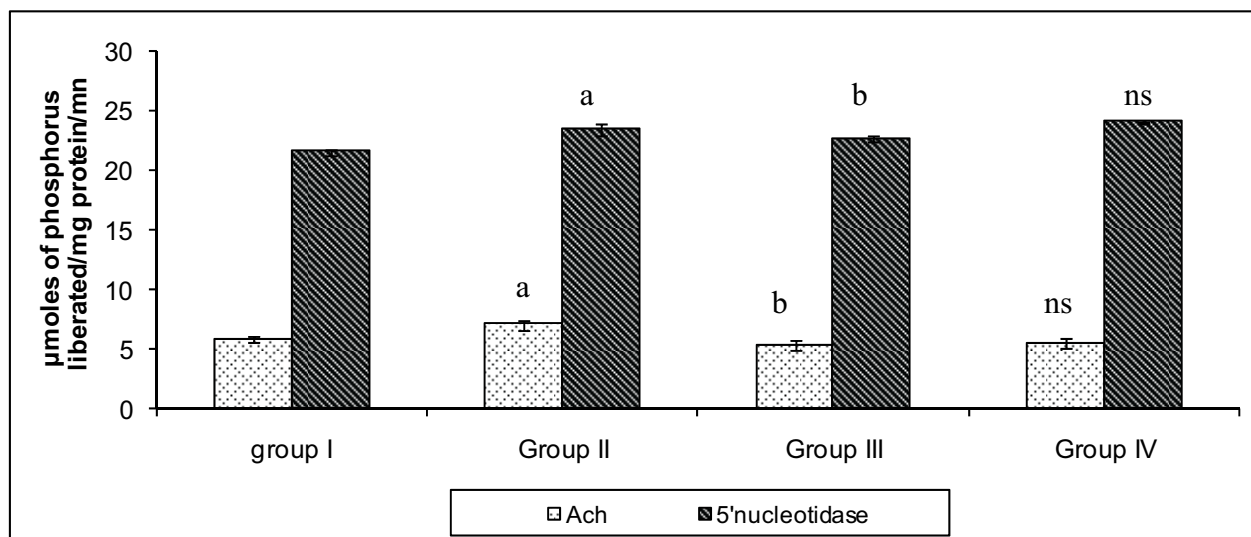

Fig. 4: Effect of *PA* on the levels of PBCs in the brain tissue of control and experimental groups of rats.
Each value is expressed as mean_S.D. for six rats in each group. Glycoprotein levels are expressed as µg/100 mg of tissue. Statistical Significance expressed as *P < 0.05. a: Group II compared with Group I. b: Group II compared with Group III. NS- not Significant. Group IV compared with Group I.

for the cognitive deficits. The result obtained in LPA can be viewed on the basis of decreased cholinergic transmission after stress-exposure.[41] However, such result was not encountered in LPA groups suggesting the potential of *PA*.

The activity of 5'nucleotidase alters with variations in membrane fluidity and on stress. The lowered activity of 5'ND was found in stress induced group when compared to control. Further, creatine kinase, on the other hand, showed an enhancement in the

Fig. 5: Effect of *PA* on the levels of Lipid profile in the brain tissue of control and experimental groups of rats.
Each value is expressed as mean±S.D for six rats in each group. Lipid levels are expressed as mg/g of tissue. Statistical significance expressed as *P < 0.05.
a: Group II compared with Group I. b: Group II compared with Group III. NS- not significant. Group IV compared with Group I.


Fig. 6: Effect of *PA* on the activity of marker enzymes in the brain tissue of control and   experimental groups of rats.
Each value is expressed as mean±S.D. for six rats in each group. Marker enzymes are expressed as MicroMoles of phosphorus liberated/mg/ protein/ min. Statistical significance: *P < 0.05. a: Group II compared with Group I. b: Group II compared with Group III. NS- not significant. Group IV compared with Group I.

activity when compared to control as it might undergo rapid changes which is an essential feature as a part of buffering system to avoid fluctuations in ATP/ADP ratios.[42] Such an enhanced activity was almost towards the normal value on *PA* treatment again depicting attenuation of stress on *PA* administration.

Figure 7 shows that 5HT$_{2A}$ expression showed a decreased expression in stress induced depression when compared to that of control group and drug treated group. Regulation of 5-HT release in the context of stressful and arousing stimuli via mPFC seems to be an important mechanism for effectively dealing with a stressor and is associated with the cessation of fear-related behaviour.[45] Studies show down regulation of 5HT$_{2A}$ receptors

in prefrontal cortex of depressed patients and suicide victims. During the stress condition, 5-HT$_{2A}$ receptor mediates excitatory effects of 5-HT release on mPFC projection neurons that, in turn, facilitate regulation of amygdala reactivity and associated emotional behaviors.

It was noted that a decrease in total cholesterol as observed in the stress induced group II may induce a relative increase in brain cell membrane fluidity with increased presynaptic 5-HT reuptake and decreased postsynaptic 5-HT function, resulting in an antiaggressive, depression-promoting effect.[46] The observed decrease in mRNA level in 5HT$_{2A}$ in the suggests that the drug might have a positive impact on coping-up mechanisms
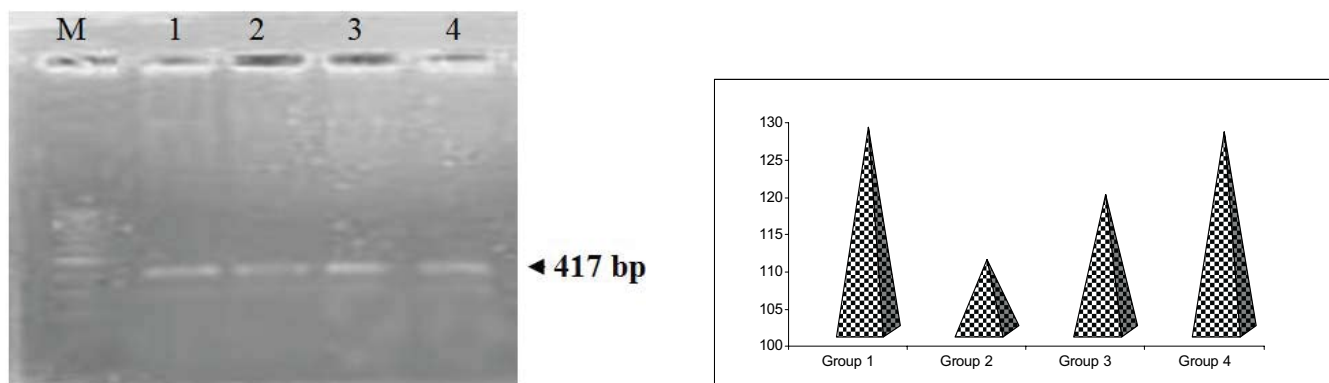
Fig. 7: RT-PCR analysis of the effect of PA on the expression levels of $5HT_{2A}$ in control and experimental groups of rats. and their corresponding densitometric analysis. Lane 1: control (group 1); Lane 2: stress induced (group 2); Lane 3: drug treated (group 3); Lane 4: drug control (group 4).

against stressors as 5-HT is involved in regulation of stress-related behaviour.

PA seems to establish a protective strategy against light induced stress in rats by virtue of its ability to alter biochemical, histopathological and $5HT_{2A}$ mRNA expression as observed in the current study.

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