Biochemical and ultrastructural alterations in the brain of mice induced by aqueous leaf extract of a medicinal plant, *Lantana camara* L. and its amelioration by nimodipine and flunarizine

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**ABSTRACT**

*Lantana camara* L., a plant used as an ethnomedicine, is considered to be toxic. In this context, evaluation of neurotoxic effect of *L. camara* was studied. Here, experimental mice were orally administered with an aqueous leaf extract of *L. camara* (AQLC) (500 mg/kg body weight) for 14 days. Biochemical assays, acetylcholinesterase (ACHE) activity as well as microscopic studies were performed to assess its neurotoxic effects. Administration (per oral) of AQLC in mice induced significant oxidative damage in the brain cortex and cerebellum as evidenced by increased lipid peroxidation, protein carbonylation, and also the activity of ACHE. This was associated with declining of the endogenous antioxidants such as superoxide dismutase and glutathione peroxidase. Co-treatment of calcium channel blockers, nimodipine (20 mg/kg body weight) and flunarizine (10 mg/kg body weight) along with the aqueous extract of *L. camara* were seemed to ameliorate the neurotoxic effect caused by AQLC. Furthermore, disintegration in the ultrastructure of brain cortex induced by *L. camara* was restored by treating calcium channel blockers. Altogether, the findings of this study concluded that the AQLC has potential neurotoxic effect in mice.

**1. INTRODUCTION**

*Lantana camara* L. belonging to the Family – Verbenaceae is a well-known traditional medicine used by indigenous people all over the world. It is commonly known as wild sage or Lantana. Despite its medicinal properties, *L. camara* is also considered as a noxious weed and is reported to be toxic [1,2]. The presence of two triterpenic acids, lantadene A and lantadene B in *L. camara* induces intra hepatic cholestasis [3-6]. It has been reported that ingesting foliage of *L. camara* by cattle caused hepatotoxicity [7]. It is also reported that the phytochemical studies of different species of *Lantana* showed the presence of alkaloids, steroids and terpenoids. Kazmi et al., 2012 reported that a terpenoid compound, ursolic acid stearoyl glucoside possess anticonvulsant and depressant like effect [8]. Terpenoids affect the intracellular calcium ions and facilitated the inhibition of calcium ion (Ca²⁺) influx through L-type Ca²⁺ channels [9,10].

Oxidative stress is caused due to the disturbance between the reactive oxygen species (ROS) and reactive nitrogen species which leads to oxidative cellular damage. Brain is sensitive to oxidative damage thereby interfering with the normal function of neuron leading to cell death [11]. Free radicals produced by oxidative damage cause disintegration of plasma membrane and due to which ionic gradients get disbalanced eventually leading to excess ions in the cell which includes calcium. Calcium ion is an important second messenger in the central nervous system (CNS) and its signaling pathway takes part in the survival and functional of neurons. Accumulation of calcium intracellularly is thought to be the main factor of cellular death [12].

Regulation of calcium homeostasis can be regulated by voltage-gated calcium channels which are categorized into groups such as high-voltage activated calcium channels (L-type) and low-voltage activated calcium channels (T-type). Both L-type and T-type calcium channels are found in CNS and have been developed to treat various neurological diseases [13-15]. Nimodipine (NIM), an L-type calcium channel blocker inhibits the calcium influx through voltage sensitive L-type calcium channels and promotes the cerebral flow. It is lipophilic in nature enabling it to cross the blood brain barrier and enter the brain. It has been reported that it improves the neurologic recovery after global ischemia, reducing damage caused by brain edema [16]. On one hand, flunarizine (FLU) blocks T-type calcium channels. In the previous studies, it has been shown to attenuate the neurotoxic effects of iron and display anticonvulsant effect in maximum electroshock-induced seizure mice [17].

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In the present study, we aim to determine the neurotoxic effects of aqueous extract of *L. camara* (500 mg) in mice by performing biochemical assays, AChE activity as well as examining ultrastructure by transmission electron microscope (TEM). The study also evaluates the amelioration of the effects of two calcium channel blockers, NIM and FLU in mice.

2. METHODOLOGY

2.1. Drugs and Chemicals

Ketamine hydrochloride, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), and di-nitrophenyl-hydrazine (DNPH) were obtained from Sigma Aldrich, India. NIM, flurazepam, pyrogallol, glutathione reductase, and hydrogen peroxide were obtained from Hi-media Ltd., India. All other reagents and chemicals were of highest analytical grades (SRL, Merck: India).

2.2. Plant Samples

Fresh leaves of *L. camara* were collected from Cachar district, Assam, India in its respective season. The plant was subjected to identification and authentication at the Botanical Survey of India, Shillong, Meghalaya, India bearing number BSI/ERC/2015/Plant Identification/669 and voucher specimen bearing number Ph.D./AUS/ASLC/19 to the Department of Life Science and Bioinformatics, Assam University, Silchar.

2.3. Extraction of Plant

The plants collected were dried under the shade and ground to powder form. Thirty grams of leaf parts of the plant were cold percolated with 300 ml of distilled water for 72 h. The obtained extract was made to concentrate and evaporated at 50°C to get crude extract. The dried extract was stored in air tight container at 4°C for further use [18].

2.4. Animals

Experiments were conducted using thirty adult Swiss albino mice weighing (8–9 weeks; 25±5 g; n = 5). The animals were housed in a hygienic condition and were maintained at appropriate temperature. The mice received a standard diet and water *ad libitum* and were acclimatized for at least a week before the beginning of experiment. The care and use of experimental animals were processed according to the Assam University Animal Ethics Committee (Regn. No. –AUS/IAEC/2017/PC/07).

2.5. Acute Toxicity and Experimental Design

The median lethal dose of *L. camara* was established according to OECD 423 guideline. Mice were fasted for 16 h and randomly divided into six groups with four mice per group. The first group is the control receiving saline water and the rest groups were graded doses of the plant extract was stored in air tight container at 4ºC for further use [18].

2.6. Preparation of Brain Tissues

At the end of exposure day, animals were induced anesthesia by injecting a mixture of ketamine hydrochloride (15 mg/kg) and xylazine (10 mg/ml). Animals were sacrificed by cervical decapitation and the brain was immediately taken out, placed in chilled isotonic saline. Cerebrum and cerebellum were dissected out and minced into pieces and homogenized in a potassium phosphate buffer (50 mM, pH 7.4). They are then divided into three portions: The first was centrifuged at ×700 g for 20 min for the estimation of lipid peroxidation (LPO) and protein carbonyl content (PC); the second portion was centrifuged at ×8500 g for 30 min to determine the activity of antioxidant parameters such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx); and the remaining portion was for the determination of acetylcholinesterase (AChE) activity and protein concentrations.

2.7. Biochemical Parameters

2.7.1. Assay of malondialdehyde (MDA) levels (EC 1.11.1.7)

The levels of LPO in cerebrum and cerebellum were measured by Ohkawa et al. [22] by measuring MDA formation. Here, about 100 µl of the tissue homogenate, 2 ml of TCA, and 2 ml of TBA reagent were added and mixed. It was then incubated in boiling water bath for 15 min, let it cooled and then centrifuged at 35000 rpm for 10 min at room temperature. In this process, TBA reacts with LPO and results in the pink colored product, MDA and was estimated at 535 nm. The lipid concentration was expressed in terms of nanomoles of MDA per mg of protein.

2.7.2. Assay of superoxide dismutase, SOD (EC 1.15.1.1) activity

The SOD activity was assessed by the method of Marklund and Marklund [23] with slight modifications. Along with tissue homogenate, the mixture contained 0.2 mM of pyrogallol, 1 mM of EDTA, and 50 mM of Tris-HCl buffer, pH 8.2 were added. The SOD activity was performed by following the auto-oxidation of pyrogallol at 420 nm for 3 min and optical density was noted. One unit of SOD was defined as the 50% inhibition in pyrogallol auto oxidation/mg protein/min.

2.7.3. Assay of catalase, CAT (EC 1.11.1.6) activity

Brain CAT activity was assayed by the method of Aebi [24]. Here, the decomposition rate of *H₂O₂* was measured at 240 nm. Briefly, 50 mM phosphate buffer (pH 7.0), 10 mM *H₂O₂* and homogenates of cerebrum and cerebellum were allowed to react and the rate of *H₂O₂* was noted for 30 s. Results are represented as change in absorbance/min/mg protein.

2.7.4. Assay of glutathione peroxidase, GPx (EC 1.11.1.9) activity

The activity of GPx was assessed by the method of Rotruck et al. [25]. Briefly, the reaction mixture contained 0.2 ml each of EDTA, sodium azide, 0.2 ml of 4 mM reduced glutathione, *H₂O₂*; 0.4 ml of phosphate buffer, 0.1 ml of homogenate and it was then incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of 10% TCA, and tubes were subjected to centrifugation at 2400 rpm 10 min. The supernatant (0.2 ml) was then added with 4 ml disodium hydrogen phosphate and 0.5 ml of DTNB. The developed color was recorded at
420 nm. The GPx activity was expressed in terms of μg of glutathione utilized/min/mg protein.

2.7.5. Acetylcholinesterase, AChE (EC 3.1.1.7) activity
The activity of AChE was assessed according to the procedure of Ellman et al. [26] in the cerebrum and cerebellum using acetylthiocholine iodide as substrate. Here, 10% brain (cerebrum and cerebellum) homogenates (w/v) were added to the test tubes containing 3 ml of phosphate buffer, 0.02 ml of acetylthiocholine solution and 0.1 ml of DTNB. Acetylthiocholine was hydrolyzed by AChE to acetic acid and thiocholine. The catalytic activity was measured by following the increase of yellow anion, 5-thio-2-nitrobenzoate, produced from thiocholine when it reacted with DTNB at 412 nm and the results are expressed as nmol/min/mg protein.

2.7.6. Protein carbonyl, PC
The PC content of the cerebrum and cerebellum of mice brain was determined by the procedure as described by Levine et al. [27] using DNPH as a substrate. About 200 μl of supernatant was added to 800 μl of 10 mM DNPH in HCl and incubated for 1 h in the dark room at room temperature. One milliliter of 20% TCA was then added for precipitation for 5 min and then centrifuged at ×10,000 g for 10 min. The pellets formed were washed off 3 times with 1:1 ethanol:ethyl acetate to remove excess DNPH. The pellets were then redissolved in 500 μl of SDS solution. The amount of carbonyl compounds were measured at 370 nm and calculated using a molar extinction coefficient (ε) of 22,000/M/cm after subtracting blank absorbance. The carbonyl content was expressed in nmol/mg protein.

2.7.7. Estimation of protein content
Protein content of tissue homogenates was assayed by Folin-Phenol reaction as described by Lowry et al. [28] using bovine serum albumin as a standard.

2.8. TEM Study
Mice were anesthetized with a mixture of ketamine hydrochloride (15 mg/kg) and xylazine (10 mg/ml) and perfused transcardially with physiological saline and then fixed with a fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer [29].

2.9. Statistical Analysis
All statistical analyses were performed using SPSS version 19.0 software. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test with Bonferroni correction for multiple comparisons. The data were expressed as mean ± SEM from five animals per treatment. The level of significance was set at P < 0.05. All biochemical assays were repeated at least 4 times.

3. RESULTS
3.1. Acute Toxicity
The median lethal dose of the aqueous extract of L. camara L. was found out to be 2480 mg/kg body weight. The dose selected for this study was below the median lethal dose, that is, 500 mg/kg.

3.2. Effects of L. camara on Antioxidants in Cerebrum and Cerebellum
Antioxidant enzymes are the first cellular molecules required for defense against ROS generation. The first enzyme of the enzymatic antioxidative pathway is SOD which converts superoxide anions into peroxides and then to water by CAT and GPx. Thus, in the present work, increased oxidative stress was evident by significant decreased GPx (P < 0.001) [Figure 1] and SOD (P < 0.001) [Figure 2] levels in cerebrum and cerebellum as compared to those of controls. Furthermore, there is a significant alteration in CAT level (P < 0.001), [Figure 3] in comparison to control groups. These make the agreement with the fact that L. camara reduced the total antioxidant levels enhancing imbalance between pro-oxidant and antioxidant potentials. These changes were significantly attenuated when co-treated with NIM and FLU.

3.3. Effect of L. camara Induced Mice on LPO Activity
In Figure 4, it demonstrated that the level of MDA in the cerebrum and cerebellum of the L. camara (500 mg/kg) induced mice was significantly elevated (P < 0.001) than that of the control group. This raise of LPO indicates an increase oxidative stress in the brain of L. camara treated mice. Interestingly, calcium antagonists NIM and FLU could attenuate the elevation.

3.4. Effect of L. camara Treatment on AchE Activity
The administration of L. camara in mice produced a significant decrease in the activity of AchE, an enzyme involve in the metabolism of acetylcholine in cerebrum (P < 0.001) and cerebellum (P < 0.05) as compared to control mice [Figure 5]. Simultaneously when NIM and FLU were treated singly, significant decrease of AchE activity was seen in comparison to control. However, the combined treatment of L. camara with NIM and FLU clearly improved the alteration in AchE activity when compared to L. camara treated group.

3.5. Effect of L. camara Treatment on PC Content
Administration of L. camara significantly increased protein oxidation in both cerebrum (P < 0.001) and cerebellum (P < 0.01) in comparison to the control [Figure 6]. The increase in PC was more pronounced in cerebrum than in cerebellum. Mice co-administered with NIM (P < 0.01) and FLU (P < 0.01, P < 0.05) showed significant decrease among the treated group.

3.6. TEM Morphological Changes in the Cerebrum
In Figure 7, aqueous extract of L. camara (500 mg/kg) administration was found to cause neuronal damage such as dense and clumpy chromatin; swollen mitochondria with disappeared cristae. Ruptured of axonal myelin sheath was also observed when compared with control. These changes were found to be reduced when treated combined with NIM (20 mg/kg).

4. DISCUSSION
In this study, we demonstrated that the administration of 500 mg/kg (orally) L. camara for 14 consecutive days was able to induce neurotoxicity in mice. The results from the study indicate that the aqueous extract of L. camara causes disturbances in cholinergic neurotransmission characterized by increased levels of acetylcholine associated with depletion of endogenous antioxidants (SOD, GPx) levels and increased levels of CAT and LPO in both cerebral cortex and cerebellum whereas co-administration of calcium channel blockers, NIM, and FLU with L. camara revealed a significantly better effect. So far, there is no study regarding the neurotoxic effect of L. camara in vivo on mice models. As a result, no reference could be provided to compare our results with the previous works. Studies have shown that CNS consumes 20% of the total body oxygen; hence, it is much more
Figure 1: Effect of Lantana camara (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (20 mg/kg) on GPx activity (U/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with control group; #p<0.05 as compared with L. camara (500 mg/kg) group.

Figure 2: Effect of Lantana camara (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (10 mg/kg) on SOD activity (U/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. **P < 0.01, ***P < 0.001 when compared with control group; ##P < 0.01 as compared with L. camara (500 mg/kg) group.

Figure 3: Effect of Lantana camara (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (10 mg/kg) on catalase level (nmol H₂O₂/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. ***P < 0.001 when compared with control group; ###P < 0.001 as compared with L. camara (500 mg/kg) group.
Figure 4: Effect of *Lantana camara* (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (10 mg/kg) on malondialdehyde concentration (nmol/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. ***$P < 0.001$ when compared with control group; ###$P < 0.01$; ####$P < 0.001$ as compared with *L. camara* (500 mg/kg) group.

Figure 5: Effect of *Lantana camara* (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (10 mg/kg) on acetylcholinesterase activity (nmol/ min/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. *$P < 0.05$, ***$P < 0.001$ when compared with control group; # $P < 0.05$, ##$P < 0.01$ as compared with *L. camara* (500 mg/kg) group.

Figure 6: Effect of *Lantana camara* (500 mg/kg) along with nimodipine (20 mg/kg) and flunarizine (10 mg/kg) on protein carbonyl content (nmol/mg protein) in cerebrum and cerebellum after 14 days of exposure. Values are given as mean ± SEM of five mice per group. **$P < 0.01$, ***$P < 0.001$ when compared with control group; # $P < 0.05$, ##$P < 0.01$ as compared with *L. camara* (500 mg/kg) group.
vulnerable to oxidative damage caused by free radicals [11]. Calcium channel blockers, NIM, and FLU when treated alone has shown some adverse effects which indicated that they are not beneficial without oxidative stress.

High lipid content causes damaged to the brain leading to the activation of dihydropyridine-sensitive calcium currents and increases intracellular calcium levels in neurons [30]. It is seen that there was a significant increased MDA levels, an indicator of LPO, in both cerebrum and cerebellum when treated with the crude aqueous extract of L. camara as compared to control group which signs the efficiency of L. camara in releasing lipid products.

SOD is the enzyme which catalyzes dismutation of superoxide radical and also use as a substrate. Generation of SOD produces hydroxyl group as well as hydrogen peroxide, which induce LPO of cell membrane and the later got neutralized by the enzymes CAT and GPx. In our case, we noticed decreased SOD levels in cerebrum and cerebellum of L. camara treated mice compared to those found in controls. Antioxidant enzymes play the role of defense mechanism against ROS generation. Therefore, in the current work, rise in oxidative stress was evident by the depletion of GPx activity, elevation of CAT level in the cerebrum and cerebellum.

Proteins in the brain tissue are prone to ROS which has been hypothesized by several studies showing that PC groups got elevated by oxidative stress, eventually leads to upregulation of toxic by-products in brain [31,32]. In the present study, we have found that the effect of L. camara on PC significantly increased as compared to control indicating the presence of oxidative stress and later attenuates by pre-treatment of calcium channel blockers, NIM, and FLU. Similar results are seen in the studies performed by Domijan et al., [33] Łukaszewicz-Hussain [34] which confirm that oxidative stress is the cause for the protein damage.

AChE is a protease that hydrolyses the neurotransmitter, acetylcholine and takes important part in conducting nerve action potential at the neuromuscular junctions and motor function. Alterations in the level of Ach or AChE activity may affect the cholinergic transmission process and leads to learning deficits. A significant increase in the Ach level in mice following L. camara treatment has been observed in the present study, Tair et al. [35] reported the same disturbances of AChE in brain when treated with aluminum.

There is a continuous production of free radicals in the process of oxidation and reduction reactions in the cell. When these free radicals exceed their threshold level, they begin to bind the unsaturated bonds.
of fatty acids and cholesterol leading to membrane peroxidation. This further disbalances the ionic concentrations and cations, especially calcium ions [36]. Rise in intracellular calcium levels in the brain mediates oxidative neuronal death. Oxidative stress induction also affects the calcium concentration in mitochondria [37,38].

Earlier studies conducted have shown that *L. camara* Linn. possess toxic compounds, Lantadene A and Lantadene B [39]. The possible mechanism to the toxic properties of the compounds is that the former compound triggers the gene BCL 2L1 which regulate the cell death by binding to the anion channel and control the caspase activator, CYC1 found in the mitochondrial membrane. Furthermore, it promotes apoptosis when interacts with clusterin protein, CLU which releases and activates apoptosis regulator BAX [40]. The later targets the gene, NOS2 which produces nitric oxide mediating tumor and synthesizing pro-inflammatory cytokines such as IL6 and IL8 [41].

The toxic principle of the alcoholic crude extract of *L. camara* shown the depressive properties on CNS and has anxiolytic activity [42,43].

It is reported that calcium channel blockers serve as membrane protecting agents by inhibiting the peroxidation of the lipid bilayer. Both L and T type of calcium channel blockers can protect by inhibiting calcium influx [44]. In the present study, both NIM and FLU were administered orally where NIM and FLU being treated before *L. camara* induction resulting in attenuation of excitotoxic damage in mice by preventing intracellular increase of calcium ions which induced anxiety. Furthermore, it was observed that combination of NIM with *L. camara* was significantly more effective than FLU in ameliorating oxidative damage in neurons.

The examination of TEM images of the brain tissue further confirmed that *L. camara* caused abnormal ultrastructural changes in the cerebrum including neuroapoptosis; mitochondrial changes such as loss of cristae, swollen, and elongated mitochondria as well as nuclear alterations; coarse and clumpy chromatin, non-myelination of axons. These changes were similar to the findings of [45,46].

5. CONCLUSION

The potentiality of neurotoxic effect of *L. camara* may be considered. The present data show *L. camara* elevates the LPO level, protein oxidation, and AchE activity which eventually decrease the antioxidant levels. These markers are further proved by the disorientation of ultrastructural images caused by *L. camara*. Amelioration of induced neurotoxicity is done by the pre-administration of the calcium channel blockers, NIM, and FLU. Furthermore, the protective effect was found to be *L. camara* concurrent with NIM. Taking into account the above findings, it can be concluded that the plant, *L. camara* Linn., is neurotoxic to mice at the given dose and hence, serious concern should be taken for the utilization of the plant when used as medicinal purpose.

6. ACKNOWLEDGMENT

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7. AUTHORS’ CONTRIBUTIONS

Singha, HA. contributed to design and drafted manuscript; Sengupta, M. and Bawari, M. critically revised manuscript. All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

8. DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTARY MATERIAL

Power analysis of the study

To avoid Type-II error, power analysis is conducted for independent samples.

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Total number of samples per experiment=30

In the above table, it is clearly indicated that the observed power are more than 80% which inclined that the null hypothesis are correctly rejected. Therefore, the sample size is suitable to detect the effect of a given test at the desired level of significance.