

EFFECT OF *CLERODENDRON COLEBROOKIANUM* WALP. LEAF EXTRACT ON COLD-RESTRAINT STRESS IN MICE

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Summary

This study was undertaken to assess the anti-stress properties of *Clerodendron colebrookianum* Walp. leaf extract against cold-restraint stress in Swiss albino mice. Three groups of albino mice were employed namely control, cold-restraint stress (3 h/day for 5 days at 4°C) and *C. colebrookianum* leaf extract + cold-restraint stress. The assessment was done by measuring the white blood cell (WBC) count, differential (neutrophil, eosinophil and basophil) count, blood glucose level, serum alanine amino transferase (ALT) activity, plasma corticosterone level, liver and spleen weight along with the transmission electron microscopic study and the DNA fragmentation assay of liver tissues. There was a significant reduction in the WBC count, eosinophil, basophil level and spleen weight while the level of ALT, neutrophil, blood glucose and plasma corticosterone along with the liver weight was found to increase significantly on stress treatment. The electron microscopic studies of liver tissues in cold-restraint stress group revealed the sign of apoptosis with significant change in the nucleus structure including chromatin condensation and vacuolization as compared to that of the control group. This was further confirmed by the percentage of fragmented DNA of liver tissue in which cold-restraint stress group showed increased DNA fragmentation percentage compared to the control group. Administration of *C. colebrookianum* leaf extract (100 mg/kg) significantly prevented the cold-restraint stress induced alterations in all the above parameters.

Keywords: Albino mice, *Clerodendron colebrookianum* leaf extract, cold-restraint stress, corticosterone

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Introduction

Stress is a non-specific response of the body known to alter the physiological homeostasis of the organism resulting in various neuronal, endocrine and visceral dysfunctions [1]. The concept of stress is very vague and the term is often used with different meanings. Experimental models of stress investigations are based on the forced exposure of life threatening and aversive stimuli or

situations that are normally avoided by individuals [2]. Stress particularly in the form of restraint or immobilization has been associated with a variety of pathological conditions in rodents [3, 4]. These include decrease in the total number of WBC [5], eosinophil and basophil, increase in the level of neutrophil level and blood glucose level, increase in the liver weight and decrease in the spleen weight. It has been established that stress can affect immune function through the activation of the hypothalamic-pituitary-adrenal (HPA) resulting in the production of a number of neuroendocrine mediators [6] including corticosterone or cortisol that are often used as an index of stress and any stimulus that causes an increase in HPA axis activity is identified as stressor [7]. It has been demonstrated that the effect of glucocorticoids (GCs: cortisol in humans and corticosteroids in rodents) on the immune system of mice could be measured by differential WBC count before and after confinement stress is applied. Decreased WBC including eosinopenia has been attributed to increased adrenal hormones such as the plasma corticosterone level [8, 9] which in turn increases both the longevity and rate of production of neutrophils [10, 11].

Stress like hypothermia can give rise to cell injury which is attributed to disturbances in cellular ion homeostasis and to alterations in membrane fluidity [12]. Such stress induced tissue injury, results in the release of cytosolic enzyme activities to blood plasma [13, 14] with increase in the hepatic metabolic activities and its m-RNA levels. Since more amounts of proteins are required for repair of wear and tear caused by stress, the metabolic changes are higher during stress. This results in a significant increase in the liver weight [15]. Cortisol released in response to stress also influences the mobilization of stored fat and carbohydrate reserves [16], which in turn increases the blood glucose level. This suggests that these levels are sensitive indices of stress [17]. Moreover, it was demonstrated that the hepatocyte injury provoked by hypothermia induce apoptosis [18].

Many plants have been investigated to possess anti-stress property against cold-restraint stress, which includes *Allophylus serratus* [19], *Camellia sinensis* [20], *Centella asiatica*, [21], *Butea frondosa* [22]. There are different effects of cold-restraint stress as mentioned above but the present study emphasizes more on blood parameters, enzyme assay and apoptosis at tissue level. *Clerodendron colebrookianum* Walp. is a shrub, belongs to the family Vermanaceae and is a non-toxic, potent hypotensive plant [23]. There has not been much studies on this plant related to anti-stress activities against cold-restraint stress. Thus, we study the conditions of imposing stress and stress indices for anti-stress activities of the leaves of this plant in which its physical activities are not yet known.

Methods

Plant Material and Extract Preparation

C. Colebrookianum leaves were collected twice a year from Ri-Bhoi District (Latitude 25°46' N, Longitude 91°46' E, Elevation 525 m), Meghalaya, India. The specimens were submitted and identified by herbarium curator, Department of Botany, North Eastern Hill University, Meghalaya, India (voucher no. 6786 of Department of Botany Herbarium, NEHU). The leaves were thoroughly washed with water and dried in oven (40°C) for 4 days. It was then ground into coarse powder form and dissolved in distilled water (1:10) and then filtered [24]. The filtrate was evaporated using rotary evaporator (Stuart RE300, UK) at 40°C and lyophilized at -50°C (Heto Lyolab 3000, Denmark). The lyophilized product was used for further analysis.

Animals and Treatments

Healthy adult male albino mice of Swiss strains weighing approximately 25-30 g were selected for the experiment. The animals were reared at 24 ± 2°C on a 12 h light/dark cycle in specific pathogen free conditions in the animal room and had free access to water and standard pellet diet.

The animal treatment procedures were approved by the Institutional Animal Ethical Committee. All efforts were made to minimize both the number of animals used and unwanted stress or discomfort to the animals throughout the experimental procedures. Experimental mice were divided into three groups (control, cold-restraint stress, *C. colebrookianum* leaf extract + cold-restraint stress). Mice were restrained by keeping them in a restrainer made from PVC pipe of 1.25" diameter, length of 4-5" with holes [25] for ventilation and are exposed to cold at 4°C for 5 consecutive days (3 h/day) [26]. The stress mice groups were given the leaf extract at 100 mg/kg body weight orally for 12 days (7 days prior to stress and 5 days during stress treatment).

Collection of blood and blood analysis

On the fifth day of experiment, blood sample was collected from the venous plexus behind the eye. The collected blood sample was kept overnight followed by centrifugation at 3000 rpm for 10 min to separate the supernatant containing serum. The collected serum was used for alanine amino transferase (ALT) assay according to the method of Reitman and Frankel [27] and blood glucose estimation using Dubowski method [28].

Estimation of total white blood cell

Blood was collected on each day from 1st to 5th day of post treatment and blood samples were diluted in the ratio of 1:20 using WBC diluting fluid, allowed for 10 min for the erythrocytes to lyse. Thereafter, the total WBC count was made by using the Neubauer chamber.

Estimation of differential count

A blood smear was drawn from a drop of blood collected on each day till the 5th day. The Leishman stain is made to cover the blood film and then left to stand for 2 min. Thereafter, distilled water, two times the quantity of stain is used to flood the thin film of the blood; the set up is rocked gently for 2 min and then allowed to stand for 15 min before rinsing the stain. The slide was left to dry and cells were counted under microscope.

Quantification of Corticosterone

Corticosterone level was quantified by Enzyme Linked Immunosorbent Assay (ELISA) procedure using kits from Cayman Chemical, USA, according to manufacturer's protocols. 50 µl of ELISA diluents was pipetted into the antibody coated wells, followed by 100 µl of each standard and 50 µl of test samples (plasma), shaken for 5 sec to mix the contents in the wells, covered with plate sealer and incubated for 2 h at room temperature. After incubation, contents of the wells were aspirated and washed three times with wash buffer. After complete removal of the wash buffer in the final wash, 250 µl of detection solution was added, covered with plate sealer and incubated for 60 min in dark. The wells were washed three times with wash solution and 100 µl of substrate reagent was added and incubated for 30 min in dark. The absorbance was recorded at wavelength between 405 and 420 nm using microplate reader (Bio-Rad 680, USA) and the resulting concentration of plasma corticosterone were expressed as pg/ml, using corticosterone standards prepared in different concentrations.

Weighing of Organs

On the fifth day, the mouse was dissected in the abdomen to remove the spleen and liver. Fatty tissue surrounding the organs was removed and organs were then weighed.

Electron Microscopic Studies

Primary fixation of liver tissues was made in 3 % glutaraldehyde prepared in sodium phosphate buffer (200 mM, pH 7.4) for 3 h at 4°C. Materials were washed with the same buffer and post fixed in 1 % osmium tetroxide and in sodium phosphate buffer for 1 h at 4°C. Tissue samples were then washed with the same buffer for 3 h at 4°C dehydrated in graded ethanol series and then

embedded in Araldite CY212 60-90 nm sections. Embedded tissues were cut on RMC ultramicrotome using a diamond knife and sections were mounted on a copper grid and then, stained with uranyl acetate and Reynolds lead citrate. The grids were examined under transmission electron microscope JEOL (model JEM100 CX II, Japan).

Quantitative DNA fragmentation assay

Quantitative estimation of DNA fragmentation was determined by colorimetric diphenylamine assay as described by Burton [29]. Liver samples from different groups were homogenized in chilled lysis buffer (10 mmol Tris-HCl, 20 mmol EDTA, 0.5 % Triton-X, pH 8.0). Homogenates (1ml) were centrifuged at 27,000 g for 20 min to separate intact DNA in the pellet from fragmented/damaged DNA in the supernatant fractions. Perchloric acid (to reach a final concentration of 0.5 M) was added separately to both the pellets and supernatant samples. Samples were heated at 90°C for 15 min then centrifuged at 1500 g for 10 min to remove proteins. Resulting supernatants, whether containing whole or fragmented DNA, were left to react with diphenylamine (0.088 M) for 16-20 h at room temperature, afterwards absorbance was measured at 600 nm. DNA fragmentation was expressed as a percentage of fragmented DNA to total DNA.

Statistical Analysis

Student's t-test was used for determining the level of significance between the control and the test values. Results are expressed as mean \pm SEM (where * compared with control and # compared with cold-restraint stress group). P value less than 0.05 were considered statistically significant.

Results

The level of blood glucose in control and experimental groups of mice are shown in Fig. 1. In cold-restraint stress group, the blood glucose level was increased to 74 % with respect to control. However, such enhancement in blood glucose level was reduced to control level when *C. colebrookianum* was treated to cold-restraint stress group. There was a significant elevation ($p < 0.01$) in plasma corticosterone level of mice by 92.43 % in cold-restraint stress group as compared to the control group. Administration of extract to cold-restraint stress group brought back the corticosterone level almost close to the control group (Fig. 2).

Fig. 1. INFLUENCE OF *C. COLEBROOKIANUM* LEAF EXTRACT ON BLOOD GLUCOSE LEVEL OF COLD-RESTRAINT STRESS GROUP. VALUES ARE MEAN \pm SEM OF DATA FOR 4 SUBJECTS (STATISTICAL SIGNIFICANCE: ###, ***: $P > 0.001$)

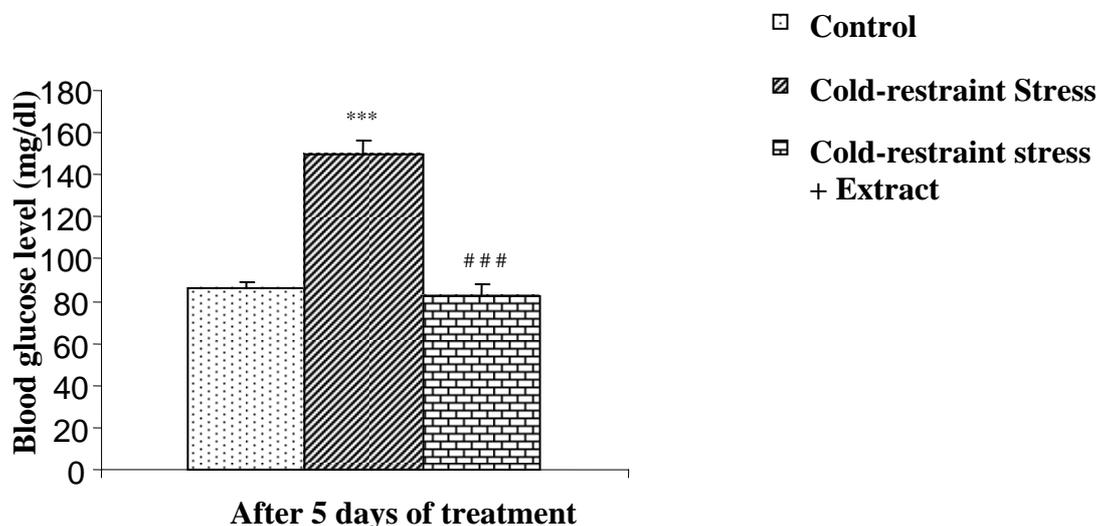
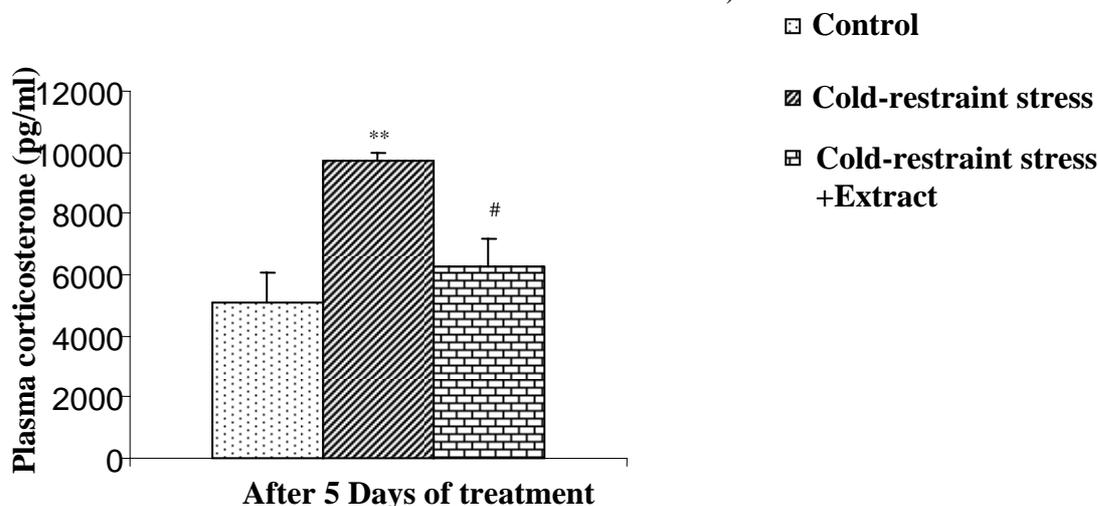


Fig. 2. EFFECT OF *C. COLEBROOKIANUM* LEAF EXTRACT ON PLASMA CORTICOSTERONE LEVEL IN COLD-RESTRAINT STRESS GROUP. VALUES ARE MEAN ± SEM OF DATA FOR 3 SUBJECTS (STATISTICAL SIGNIFICANCE: #*: P>0.05 AND **: P>0.01)



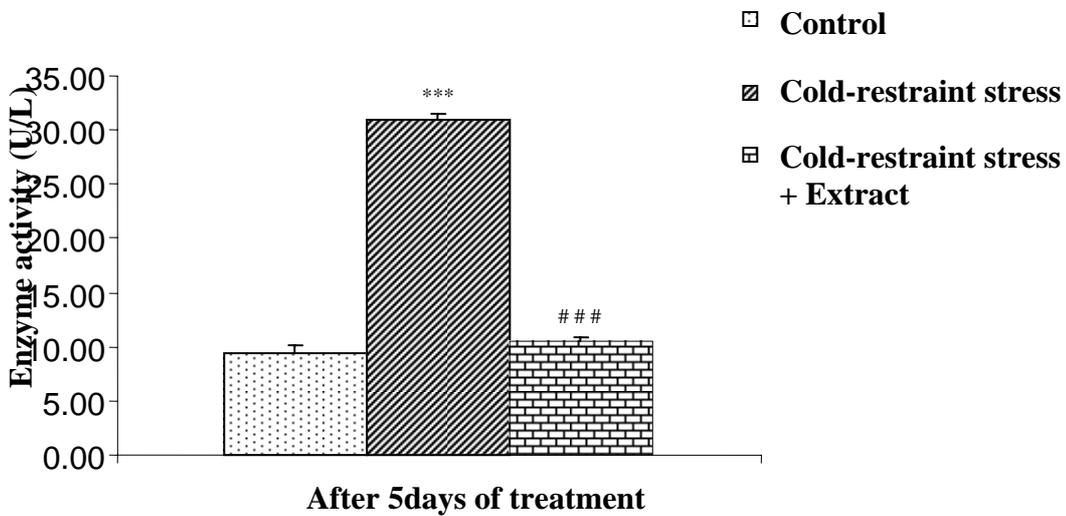
The change in the liver weight, spleen weight of control and experimental groups of mice showed significant difference [Table 1]. There was a significant increase in the liver weight of cold-restraint stress group by 13.49 % (p<0.01) compared with the control group. Upon treatment with the extract, the liver weight was improved by 11.57 % (p<0.01). Whereas there was a significant (p<0.001) decrease in the spleen weight of cold-restraint stress group compared with the control group. Treatment with *C. colebrookianum* extracts significantly restored the spleen weight by 83.26 % (p<0.01).

Table 1. EFFECT OF *C. COLEBROOKIANUM* LEAF EXTRACT ON LIVER AND SPLEEN WEIGHT IN MICE EXPOSED TO COLD-RESTRAINT STRESS. VALUES ARE EXPRESSED AS MEAN ± SEM OF SIX SUBJECTS (STATISTICAL SIGNIFICANCE: **: P>0.01 AND ***,###: P>0.001).

Parameters	Control	Cold-restraint stress	<i>C. colebrookianum</i> + Cold-restraint stress
Liver (g/ 100g body weight)	4.508 ± 0.067	5.116 ± 0.101**	4.524 ± 0.094##
Spleen Weight (g/ 100g body weight)	0.737 ± 0.034	0.378 ± 0.032***	0.693 ± 0.072##

There was a significant increase by 67.04 % (p<0.001) in the activity of serum alanine amino transferase in cold-restraint stress group as compared to the control group. Treatment with extract significantly controlled the alterations and restored the altered levels to near normalcy by 65.66 % (p<0.001) (Fig. 3).

Fig. 3. EFFECT OF *C. COLEBROOKIANUM* LEAF EXTRACT ON SERUM ALANINE AMINO TRANSFERASE ACTIVITY IN COLD-RESTRAINT STRESS GROUP. VALUES ARE MEAN ± S.E.M. OF DATA FOR 4 SUBJECTS (STATISTICAL SIGNIFICANCE: ###, *: P>0.001)**



There was a gradual and significant reduction in the WBC count of cold-restraint group. It was 55.83 %, 57.61 %, 64.43 %, 68.96 % and 74.55 % from 1st to 5th day of stress exposure. Administration of extract to cold-restraint stress group significantly prevented the reduction in the WBC count (Fig. 4).

Fig. 4. IMPACT OF *C. COLEBROOKIANUM* LEAF EXTRACT ON WHITE BLOOD CELL COUNT AFTER 3 HOURS TREATMENT FOR 1ST, 2ND, 3RD, 4TH, 5TH DAY OF COLD-RESTRAINT STRESS GROUP. VALUES ARE MEAN ± SEM OF DATA FOR 5 SUBJECTS (STATISTICAL SIGNIFICANCE: ##, ** P>0.01, ###, * P>0.001)**

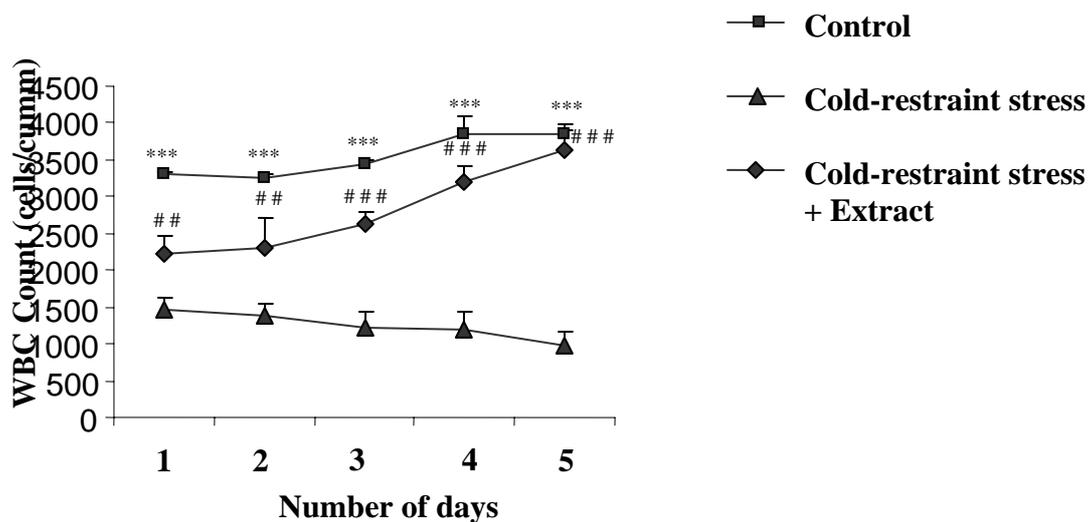
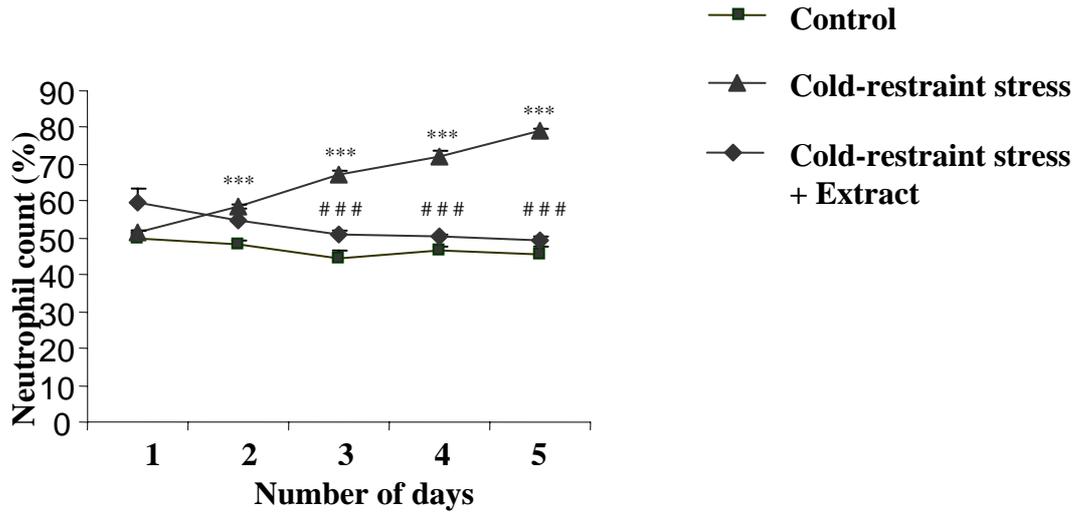
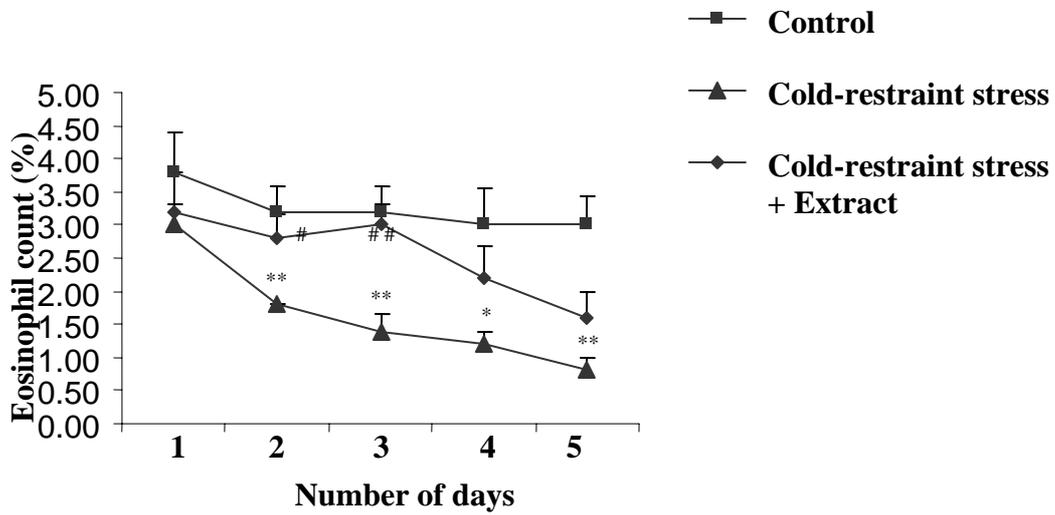


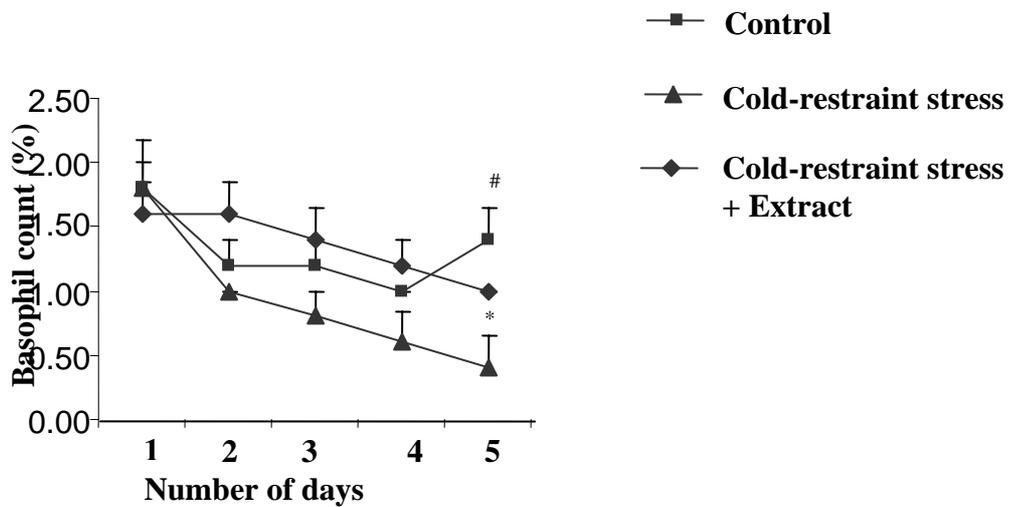
Fig. 5. NEUTROPHIL, EOSINOPHIL AND BASOPHIL COUNT (a, b, c) IN PERCENTAGE IN COLD-RESTRAINT STRESS GROUP AFTER FEEDING WITH THE *C. COLEBROOKIANUM* LEAF EXTRACT. VALUES ARE MEAN ± SEM OF DATA FOR 5 SUBJECTS. (STATISTICAL SIGNIFICANCE: #,* P>0.05, ##, ** P>0.01, ###, * P>0.001)**



a



b



c

In cold-restraint stress group, the neutrophil level increased significantly by 20.66 % ($p < 0.001$) on the 2nd day with further increased in the progressive days by 51.80 %, 55.60 %, 73.68 % on the 3rd, 4th and 5th day respectively when compared to the control group (Fig. 5 a). On extract treatment, the neutrophil level of cold-restraint stress group was significantly reduced which was similar to that of the control group. There was a significant decrease in the basophil and eosinophil level of cold-restraint group. Treatment with plant extract restored back the eosinophil and basophil level almost near to the control group (Figs. 5 b & c).

Electron microscopic studies of liver tissue of stress group showed the sign of apoptosis as there was a significant change in the nucleus structure with chromatin condensation and vacuolization when compared to that of the liver tissue of the control group. The above morphological changes were prevented on mice treated with plant extract (Figs. 6 a, b & c). This observation was further supported by the DNA fragmentation assay where the fragmentation level of hepatic DNA in control and experimental groups of mice are shown in Fig. 7.

Fig. 6 a. ELECTRON MICROGRAPH OF A NORMAL LIVER CELL WITH NUCLEUS (N) AND VACUOLE (V) IN THE CONTROL GROUP. MAGNIFICATION: $\times 5000$

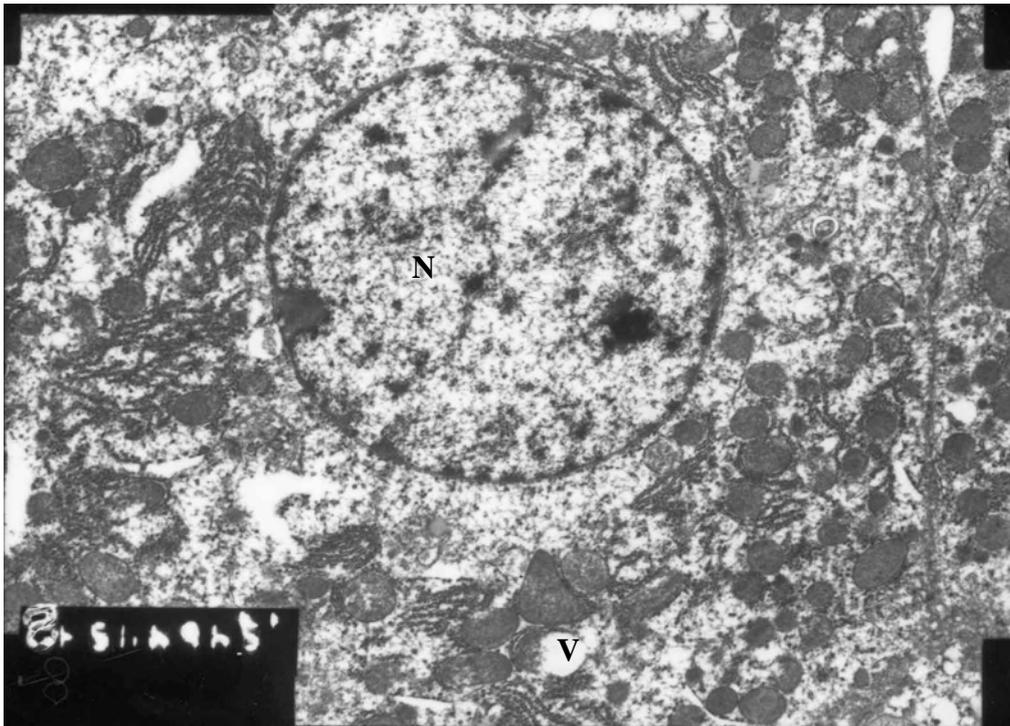


Fig. 6 b. ELECTRON MICROGRAPH OF A DISTORTED NUCLEUS (N) AND INCREASED NUMBER OF VACUOLES (V) IN THE LIVER CELL OF COLD-RESTRAINT STRESS GROUP. (MAGNIFICATION: $\times 5000$)

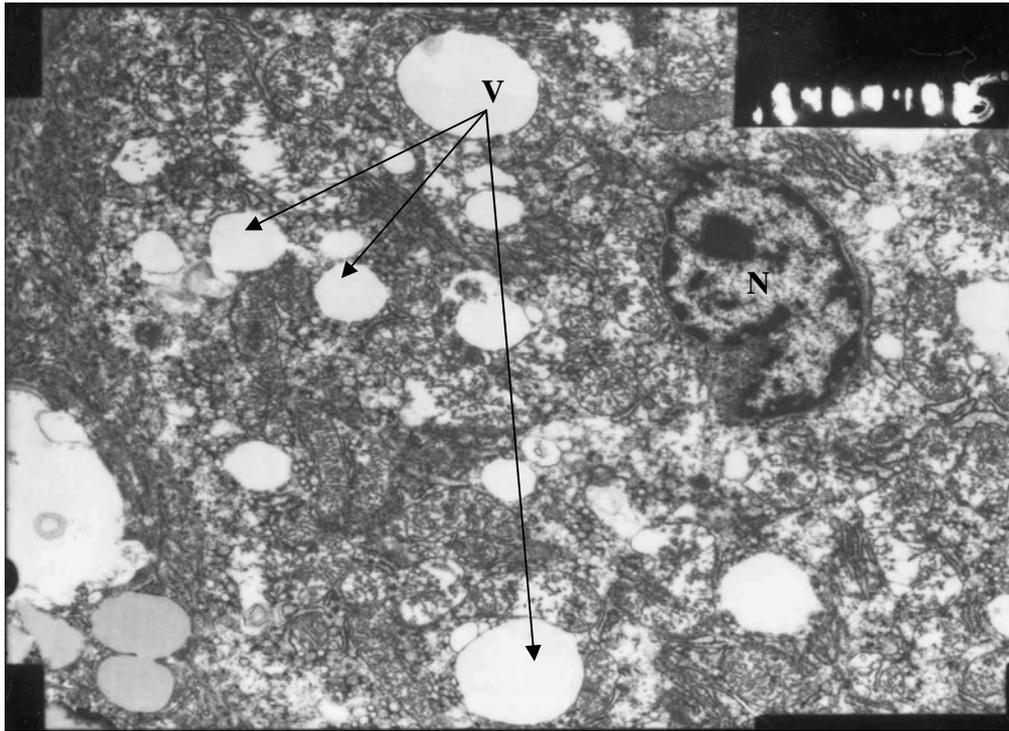


Fig. 6 c. ELECTRON MICROGRAPH OF APPARENTLY NORMAL NUCLEUS (N) WITH LESSER NUMBER OF VACUOLES (V) IN THE LIVER CELL OF *C. COLEBROOKIANUM* TREATED COLD-RESTRAINT STRESS GROUP. (MAGNIFICATION: $\times 5000$)

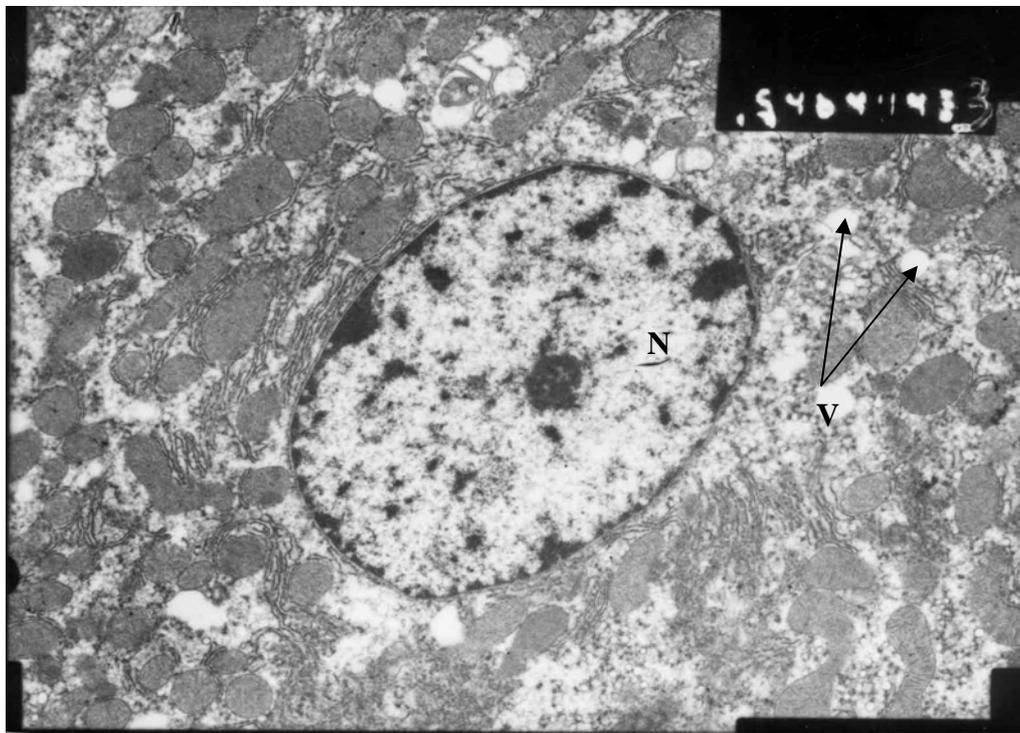
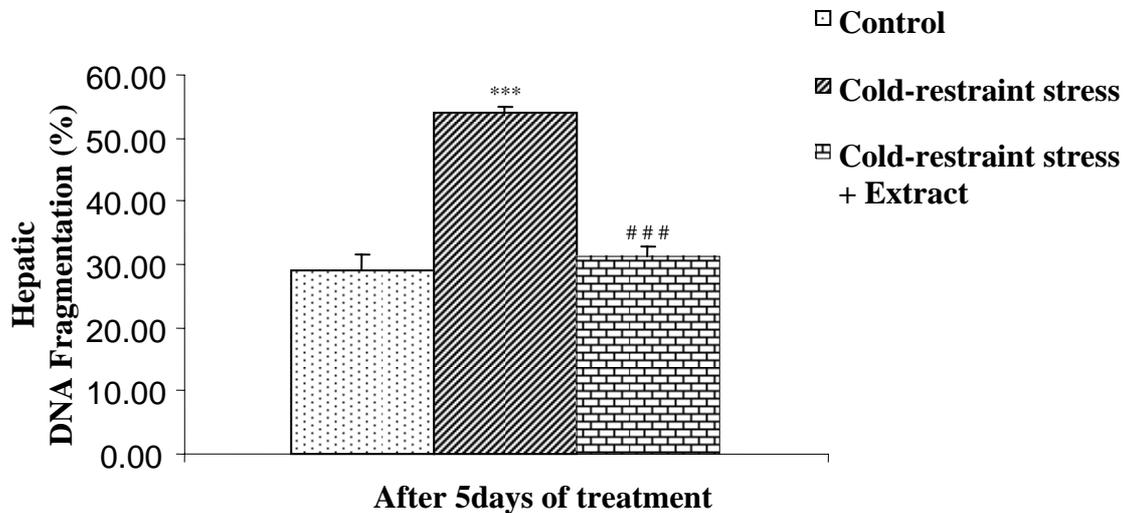


Fig. 7. EFFECT OF *C. COLEBROOKIANUM* LEAF EXTRACT ON HEPATIC DNA FRAGMENTATION IN COLD-RESTRAINT STRESS GROUP. VALUES ARE MEAN \pm S.E.M. OF DATA FOR 3 SUBJECTS (STATISTICAL SIGNIFICANCE: ###, * $P > 0.001$)**



There was a significant elevation ($p < 0.001$) in fragmented hepatic DNA level by 85.55 % in cold-restraint stress group when compared to the control group. Administration of extract, reduced the level of DNA fragmentation level by 42.53 % ($p < 0.001$).

Discussion

The present anti-stress study was done using the leaf extract of *C. colebrookianum*. The plasma corticosterone level was enhanced in cold-restraint stress group of mice which was reduced by the administration of extract.

There was a significant increase in the neutrophil level associated with decrease in the eosinophil, basophil level and WBC count of mice after cold-restraint stress. It is conjectured that increases in neutrophil number may act to counter balance some of the immunosuppressive effects of corticosterone [30]. Supplementing earlier reports, the total number of WBC in animal decreased when they are subjected to stress [5]. GCs cause marked changes in circulating leukocyte numbers. Most striking of these changes is a fall in the number of basophils, eosinophils, and monocytes to approximately 20 % of the normal circulating quantity of each of these cell types which was also reported earlier [31-33].

In the present study, it was observed that the liver was hypertrophied as the animal was exposed to stress with decreased spleen weight as it was reported earlier since higher glucocorticoid level leads to widespread lympholysis in the lymphoid organs [34]. Cold-restraint stress induced a rise in the ALT activity in mice compared to the control group and is in agreement with the reports by Fernandez *et al* [35]. Increased blood glucose level was observed in stress exposed group and this could be because of higher GCs which elevate the output of hepatic glucose by stimulating gluconeogenesis, enhancing the effects of glucagon and epinephrine on glucose availability and inhibiting the effects of insulin on energy metabolism [36].

DNA fragmentation is very typical of the apoptotic process and its measurement with the diphenylamine colorimetric assay is preferentially used here to evaluate apoptosis in cells. Distinct

chromatin condensation and vacuolization are obtained in the liver tissue of cold-restraint stress group. The possible explanation for stress-induced liver apoptosis is via the production of GCs which up-regulates Fas antigens on hepatocytes [37]. In the present study, it was shown that such cold-restraint stress induced apoptotic cell death including alterations in the leukocyte numbers, blood glucose level, ALT activity, liver and spleen weight, could be prevented by the plant extract.

The present findings reveal that *C. colebrookianum* extract might contain principle(s) that possibly exert multiple actions involving different mechanisms in exerting anti-stress effects. The results presented here would provide basic data to examine anti-stress effects of this plant and further in-depth studies could contribute in the development of functional plant materials with anti-stress activities. Currently we are carrying out investigation to understand the mechanism of action and to identify the active component(s) responsible for its anti-stress properties.

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