Effect of Clerodendron colebrookianum Walp. aqueous leaf extract on liver oxidative stress and the immune system of mice exposed to cold restraint stress

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Abstract

To investigate the effect of Clerodendron colebrookianum aqueous leaf extract on oxidative parameters in liver and the immune system of mice exposed to cold restraint stress (CRS). Three groups of mice were employed namely control, CRS and C. colebrookianum leaf extract+CRS. Mice were restraint by keeping them in polyvinyl chloride (PVC) pipe restrainers and were exposed to cold at 4°C for 5 consecutive days (3 hrs/day). CRS mice received extract (100 mg/kg b.w.) orally for 12 days (7 days prior to stress and 5 days during stress treatment). For oxidative parameters, experiments such as catalase activity, lipid peroxidation, reduced glutathione level, superoxide dismutase activity in liver tissue were performed and splenocyte count, blood lymphocyte subsets count, hemagglutination antibody titre, bone marrow megakaryocyte count, bleeding time, phagocytic index were analysed for immune system study. The above studied parameters were significantly altered in CRS group of mice compared to the control group. The CRS induced liver oxidative changes and immune changes were significantly ameliorated by pre-treatment with aqueous leaf extract. The results indicate that C. colebrookianum aqueous leaf extract exert protective effects against oxidative stress in liver and immune changes under CRS in mice which may be responsible for its anti-stress property.

Keywords: anti-stress; cold restraint stress; Clerodendron colebrookianum; immune system; oxidative stress

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Introduction

Stress may be defined as a state of disharmony where the homeostasis of a biological system is altered resulting in a series of events eliciting a physiological response. Physiological response to stress is an organism’s response to a stressor which ranges from physical to psychological factors including cold, heat, infection, toxins etc. (Selye, 1973). Laboratory animals have been indispensable in their roles as models in the study of stress, and consequently in shedding lights on our understanding of what stress is about by using different types of acute and chronic stressors including forced swim-test, inescapable tailshock, immobilization, cold and ether stress (Sutanto and deKloet, 1994). Reports have shown that immobilisation stress accelerated by cold (a combination of two potent stressors) can disrupt the balance in an oxidant/antioxidant system and cause oxidative damage to several tissues by altering the
enzymatic and non-enzymatic antioxidant status, protein oxidation and lipid peroxidation (Sahin and Gümüşlü, 2007). The result of these stressors are reported to modify the immunological response in the organism by suppressing macrophage phagocytosis (Lu et al., 1998), inhibiting T cell proliferation (Iwakabe et al., 1998), affecting the type-1/type-2 T cell (Th1/Th2) balance (Iwakabe et al., 1998), impair platelet function (Hiroshi et al., 1992) and modulating antibody production (Komori et al., 1996; Fukui et al., 1997). Stress has also been associated with alterations in leukocyte whole blood profiles and increased susceptibility to infection and disease (Zieziulewicz et al., 2013).

There is an ever-increasing demand for plant-based therapeutics that are capable of alleviating the effect of stress due to a growing recognition that they are natural products, non-narcotic and, in most cases, easily available at affordable prices; they also have no side effects (Pandey et al., 2011). Moreover, a number of plants such as Asparagus racemosu, Ocimum sanctum, Nithamla somnifera, Panax ginseng, Hypericum perforatum and Ginkgo biloba have been shown to possess anti-stress properties (Grover et al., 1995; Bhattacharya and Ghosal, 2000; Ellis and Reddy, 2002). Clerodendron colebrookianum Walp. (Family: Verbenaceae) is a shrub, nontoxic, potent hypotensive plant with antiperoxidative and lipid lowering activity (Kotoky et al., 2005; Devi et al., 2011). Our laboratory have published paper previously on the effect of C. colebrookianum leaf extract on cold restraint stress (CRS) in mice on parameters such as WBC count, differential count, blood glucose level, serum alanine amino transferase activity, plasma corticosterone level, liver DNA fragmentation level, transmission microscopic study of liver and spleen (Majaw et al., 2008). Alterations in all the above parameters under CRS condition were prevented by the extract administration. The phytochemical analysis of the C. colebrookianum leaf has also been performed in our laboratory (Majaw and Moirangthem, 2009). Although several studies have investigated the effects of CRS on the antioxidant system and induction of lipid peroxidation in several tissues (Dekanski et al., 2011), to date, no information is available regarding the effect of C. colebrookianum aqueous leaf extract on antioxidant balance in liver and immune system under CRS condition. The influence of stress on the liver is also of interest from the clinical point of view, because stress plays a potential role in aggravating liver diseases in general and hepatic inflammation in particular, probably through the generation of reactive oxygen species (ROS) (Dekanski et al., 2011). Thus, the effect of C. colebrookianum aqueous leaf extract on oxidative stress in liver and on the immune system of mice exposed to CRS will be investigated with a purpose to further widen the scope of using this plant extract for its anti-stress property.

Materials and Methods

Collection of plant material and extract preparation

C. colebrookianum leaves were collected twice a year from Ri-Bholi District, Meghalaya, India. The specimens were submitted and identified by herbarium curator, Dr. P.B. Gurung, Department of Botany, North-Eastern Hill University, Meghalaya.
Suktilang et al., 2016

India (voucher no. 6786). Plant material were thoroughly washed with water and dried in oven (40°C) for 4 days. They were then ground into coarse powder form and dissolved in distilled water (1:10) and then filtered. The filtrate was evaporated using rotary evaporator (Stuart RE300, UK) at 40°C, and lyophilized (HetoLyolab-3000, Denmark) at -50°C which was used for further analysis (Majaw et al., 2008).

Animal model and treatment

Healthy Swiss albino male mice (25-30 g) were reared at 24±2°C on a 12 hrs light/dark cycle in specific pathogen free conditions in the animal room and had free access to water and standard pellet diet. 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: (i) Control (ii) CRS, (iii) C. colebrookianum leaf extract (100 mg/kg b.w.) + CRS. Mice were restraint by keeping them in polyvinyl chloride (PVC) pipe restrainers (Brehe and Way, 2008) and were exposed to cold at 4°C for 5 consecutive days (3 h/day) (Bharihoke et al., 2000). Extract was administered orally for 12 days (7 days prior to stress and 5 days during stress treatment).

Oxidative parameters in liver

Liver tissue from each group was removed and homogenised. The homogenised liver was used for estimation of catalase activity expressed as U/mg protein (Aebi, 1984), lipid peroxidation expressed as nmol MDA/min/mg protein (Placer et al., 1966), reduced glutathione (GSH) expressed as μmol/mg protein) (Sedlak and Lindsay, 1968), superoxide dismutase (SOD) expressed as U/mg protein) (Marklund and Marklund, 1974). Protein was estimated following the method of Bradford (1976).

Splenocyte count

The dissected spleen was teased on a tissue grinder using 1xphosphate buffered saline (PBS, pH 7.2) and the suspension was centrifuged at 1,000 rpm for 5 min. The pellet containing splenocytes were suspended in hypotonic erythrocyte lysing solution containing 0.85% of ammonium chloride (NH₄Cl). After 7 min incubation followed by centrifugation, cells were resuspended in PBS, counted through a haemocytometer after staining the cells with 0.4% trypan blue in the ratio of 1:1.

Blood lymphocyte subsets count

Blood samples were collected from mice of all the groups via the retro-orbital route and immediately processed for separation of B- and T-lymphocytes using a sterilized nylon wool fiber column employing the standard experimental protocol (Yadava and Mukherjee, 2006). The cells were then counted in a haemocytometer.

Hemagglutination antibody titre

All the three groups of mice were immunized with 7.3 x 10⁷ sheep red blood cells (SRBC) intraperitoneally before the first day of stress treatment. After five days of immunization, blood was collected in heparin and serum was separated. The antibody level was determined by the hemagglutination method (Moudgil and Singh, 2006). The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre. Antibody titre was expressed as log₂X where X is the reciprocal dilutions.
Bone marrow megakaryocyte count

The marrow of each femur removed from mice was gently flushed out with 1 ml of WBC diluting fluid into a tube. The megakaryocytes were then counted in haemocytometer. Megakaryocyte counts were expressed as total cell counts per femur (Taeko et al., 1988).

Bleeding time

Bleeding time was measured according to the tail transaction technique (Taeko et al., 1988). Bleeding time was defined as the time from the moment the cut was made until complete cessation of bleeding without any re-bleeding within 30 sec.

Phagocytic index

Water was warmed up to 30ºC and the mice tail was dipped in it. Carefully 0.1 ml of carbon ink (Rotring, Germany) was injected into the tail vein. Blood was collected at time intervals of 0, 10 and 15 min (Razdan and Roy, 2008). Phagocytic index was calculated using the following formula:

\[
\text{Phagocytic index (K)} = \frac{\text{t}_{15} - \text{t}_{10}}{(\log_{10} \text{OD}_{10} - \log_{10} \text{OD}_{15})}
\]

Where, OD: optical density and t= time

Statistical analysis

Values were presented as mean ± SEM and for comparison between two groups student’s t test was used. Differences within the data were considered statistically significant when \(p<0.05\), \(p<0.01\) and \(p<0.001\) level.

Results

The effect of C. colebrookianum aqueous leaf extract on the oxidative parameters in liver of mice exposed to CRS is shown in table 1. There was a significant decrease in catalase activity by 34.23\% \((p<0.05)\) in liver of CRS group from that of the control group. Administration of extract to CRS group, significantly increased the catalase activity by 46.40 \%(p<0.05) which was found to be comparable to the control group. There was significant increase in lipid peroxidation by 330.28\%(p<0.001) in liver of CRS group from that of the control group. After administration of extract, lipid peroxidation was found to be significantly lowered by 57.23\%(p<0.001) in liver from that of the CRS group. The level of GSH was significantly increased by 199.08\%(p<0.001) in liver of CRS group from that of the control group. GSH level was also significantly lowered by 45.03\%(p<0.01) in liver of CRS group treated with extract. SOD activity has also significantly increased by 132.94\%(p<0.01) in liver of CRS group from that of control group. SOD activity was decreased by 28.53\%(p<0.05) in CRS group on treatment with leaf extract. The effect of C. colebrookianum aqueous leaf extract on the immune system of mice exposed to CRS is given in Table 2. There was a substantial decrease in the splenocyte count of the mice subjected to CRS by 47.11\%(p<0.05) from that of the control group, however, extract treatment increased the splenocyte count by 44.34\%(p<0.01) from that of CRS group. B and T lymphocytes count were markedly decreased by 57.14\%(p<0.01) and 47.46\%(p<0.01) in CRS group from that of the control group. However, this suppression in blood lymphocyte subsets was shown to stabilise when the animals were administered with the extract. The production of antibody in mice under CRS condition was found to decrease by 25.91\%(p<0.001) from that of the control group. The
Table 1. Effect of *C. colebrookianum* aqueous leaf extract (100 mg/kg b.w.) on catalase activity (U/mg protein), lipid peroxidation (nmol MDA/min/mg protein), GSH (µmol/mg protein), SOD (U/mg protein) in liver of cold restraint stress group of mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group</th>
<th>CRS group</th>
<th>Leaf extract + CRS group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (U/mg protein)</td>
<td>0.3751± 0.032</td>
<td>0.2467 ± 0.011*</td>
<td>0.3612± 0.034#</td>
</tr>
<tr>
<td>Lipid peroxidation (nmol MDA/min/mg protein)</td>
<td>2.1840 ± 0.197</td>
<td>9.3975 ± 0.060**</td>
<td>4.0207 ± 0.289###</td>
</tr>
<tr>
<td>GSH (µmol/mg protein)</td>
<td>0.0974 ± 0.010</td>
<td>0.2914 ± 0.016</td>
<td>0.1602 ± 0.017##</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>0.2588 ± 0.010</td>
<td>0.6029 ± 0.076**</td>
<td>0.4309 ± 0.040#</td>
</tr>
</tbody>
</table>

The values are represented as mean ± SEM and the number of sample taken was three where CRS is cold restraint stress, GSH is reduced glutathione, SOD is superoxide dismutase (Statistical significance: *,#: p<0.05, **,##: p<0.01, ***,####: p<0.001, *compared with control group; # compared with cold restraint stress group)

Table 2. Effect of *C. colebrookianum* aqueous leaf extract (100 mg/kg b.w.) on the immune system in mice exposed to cold restraint stress (CRS)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group</th>
<th>CRS group</th>
<th>Leaf extract + CRS group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenocyte count (x 10⁴/ml)</td>
<td>55.703 ± 6.958</td>
<td>29.463 ± 1.261*</td>
<td>42.527 ± 1.546**</td>
</tr>
<tr>
<td>Blood lymphocyte subset counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-lymphocytes (x10⁴/ml)</td>
<td>72.33 ± 4.842</td>
<td>**</td>
<td>72 ± 2.309###</td>
</tr>
<tr>
<td>T-lymphocytes (x10⁴/ml)</td>
<td>210 ± 1.0</td>
<td>110.33 ± 13.67**</td>
<td>194 ± 4.509##</td>
</tr>
<tr>
<td>Hemagglutination antibody titre (Log₂X)</td>
<td>13.12 ± 0.200</td>
<td>9.72 ± 0.245***</td>
<td>12.72 ± 0.245##</td>
</tr>
<tr>
<td>Megakaryocyte count (x10⁴/femur)</td>
<td>5.00 ± 0.456</td>
<td>1.50 ± 0.204**</td>
<td>4.62 ± 0.315##</td>
</tr>
<tr>
<td>Bleeding time (min)</td>
<td>8.673 ± 1.254</td>
<td>3.697 ± 0.367*</td>
<td>7.03 ± 0.597#</td>
</tr>
<tr>
<td>Phagocytic index (K)</td>
<td>0.0016 ± 0.000</td>
<td>.0069 ± 0.001***</td>
<td>0.0034 ± 0.000##</td>
</tr>
</tbody>
</table>

The values are represented as mean ± SEM and the number of sample taken was three except for hemagglutination antibody titre with 5 samples (Statistical significance: *,#; p<0.05, **,##; p<0.01, ***,####; p<0.001, *compared with control; # compared with cold-restraint stress)
extract seem to increase the production of antibody considerably by 30.86% ($p<0.001$) from that of the CRS group. In CRS group, bone marrow megakaryocyte count per femur was found to decrease by 70% ($p<0.01$) from that of the control group and the megakaryocyte count was brought closer to the control by the administration of the extract thus, showing a neutralizing effect of the plant on stress. There was a 57.37% ($p<0.05$) reduction in the bleeding time of CRS group, while the phagocytic index was markedly increased by 334.15% ($p<0.001$) from that of the control group. However, administration of leaf extract increased the bleeding time by 90.47% ($p<0.05$) in CRS group bringing it closer to the control group, and the phagocytic index was brought down closer to control by 50.43% ($p<0.01$) from that of the CRS group.

**Discussion**

The present study was aimed to determine the effect of *C. colebrookianum* aqueous leaf extract on the oxidative parameters in liver and the immune system of mice under CRS condition. The experimental data indicated that CRS in mice significantly increased the lipid peroxidation level in liver. The observed increase in lipid peroxidation is in agreement with previous studies (Atif et al., 2008; Devaki et al., 2011; Ahmad et al., 2012). In our study, we found increase in SOD activity and decrease in catalase activity in the liver of CRS group. Increase in SOD activity suggests that cold stress may increase the rate of O$^\cdot$ generation and subsequently H$_2$O$_2$ formation (Liu et al., 2000). The SOD elevation may provide mainly protection against stress induced liver injury. The decreased catalase activity leads to the accumulation of H$_2$O$_2$, which may be the cause of oxidative stress (Ahmad et al., 2012; Sarumathi and Saravanan, 2012). In this study, increased levels of GSH measured in liver tissue of CRS group were probably the consequence of an increased need for antioxidant defense to meet the increased peroxidative challenge due to increased pro-oxidant activity. GSH plays a multifunctional role in antioxidant protection, maintaining other reductants and protein sulphhydryl groups in their reduced state, acting as a cofactor for a number of antioxidant enzymes and directly scavenging ROS and peroxides (Sahoo and Kara, 2014).

In this study, the splenocyte count and the peripheral blood lymphocyte subsets count (both B cells and T cells) had shown significant decrease under CRS condition, this resulted in decreased antibody production against novel antigen, SRBC, which was seen as a low hemagglutination antibody titre. These results were consistent with the findings of other laboratories (Cheng et al., 1990; Goundasheva et al., 1994). Further, previous studies have shown that stressors of acute duration of several days following immunization with SRBC can suppress the number of B cells producing antibody (Zalcman and Anisman, 1993; Bhatnagar et al., 1996).

In this study, the bone marrow megakaryocyte count per femur had reduced during the stress condition in mice while the bleeding time of mice had decreased significantly. The bone marrow megakaryocyte count is an important parameter reflecting platelet producibility (Kuter, 1998). It is known that platelets account for the
recovery of blood vessel injury and help in stopping of bleeding by coagulation, thus decreasing the bleeding time. The number of platelets formed by a single megakaryocyte is proportional to the amount of the cytoplasm (Harper, 1974). The augmented megakaryocyte count in stressed mice is thought to indicate enhanced production of platelets, although other parameters such as cell size and ploidy should also be taken into consideration. Further studies is required to investigate the relation of megakaryocyte count and bleeding time, it may be inferred that stress induces the cytoplasmic size of megakaryocytes to increase although the count decreases and perhaps the reason for increased production of platelets which account to the decrease in bleeding time (Taeko et al., 1988).

Phagocytic index which is an indicator of \textit{in vivo} macrophage function was found to increase largely in our study. Previous reports have shown that intense acute stressor (swimming to exhaustion) increased phagocytic activity in peritoneal macrophages (Ortega et al., 1992). Likewise, cold swim stress results in the activation of peritoneal macrophages (Kizaki et al., 1996). Shilov et al. obtained similar results with Quindos et al. (1986), indicated that restraint stress increased phagocytic activity of blood cells after 6 hrs (Shilov and Orlova, 2003). Our results also showed increased phagocytic index in CRS group of mice. The observed changes in the oxidative parameters in liver and the immune system due to CRS were prevented or were minimized in mice that were administered with the leaf extract of \textit{C. colebrookianum}. The efficacy of most herbal remedies is attributed to various active principles, in combination. Results of phytochemical screening showed the presence of lipid, flavanoid, alkaloid, saponin, tannin, terpenoid, steroid in the leaf (Majaw and Moirangthem, 2009). It is therefore probable that the components that are present in abundance in the extracts might contribute in part to the observed anti-stress effect. In conclusion, the experimental studies reveal that the aqueous leaf extract of \textit{Clerodendron colebrookianum} exhibit protective effects against oxidative stress in liver and immune changes in mice exposed to CRS. Studying these effects from a different perspective has added new elements in understanding the anti-stress property of this plant extract.

\textbf{References}


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