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ORIGINAL ARTICLE

Beneficial Effects of *Caesalpinia digyna* Extract against acid Aspiration-Induced Acute Lung Injury in Mice

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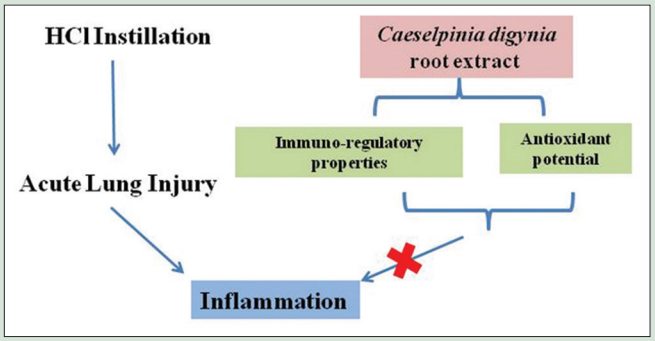
ABSTRACT

Objective: *Caesalpinia digyna* belongs to the genus *Caesalpinia*, which is known since ancient times for its medicinal properties. The present work was designed to evaluate the beneficial potential of hydroalcoholic extract of the roots of *C. dignea* against hydrochloric acid (HCl)-induced acute lung injury in mice. **Materials and Methods:** Ethanolic extract of *C. dignea* roots at a dose of 50, 100, or 200 mg/kg boy weight was given once orally 90 min before HCl administration. Mice were then analyzed for infiltration of inflammatory cells in bronchoalveolar lavage fluid (BALF) and oxidative stress markers in the lung tissue. Further, the effects of the extract were compared with bergenin isolated from the extract. **Results:** Our results showed that an oral administration of the extract 90 min before HCl instillation reduced the infiltration of neutrophils in the lungs in a dose-dependent manner. Reduction in lung inflammation was associated with decline in pulmonary edema as the total protein content in the BALF was found to be decreased substantially. The drug also restored the redox balance in the lungs toward normal on HCl treatment as assessed by measuring the levels of reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), and catalase activity. Bergenin, isolated from the plant, was able to suppress the neutrophils but increased the macrophage number in BALF when administered before HCl instillation, suggesting immunoregulatory properties of the key constituent of the extract. **Conclusion:** Our data suggest that hydroalcoholic extract of *Caesalpinia digyna* roots constitute the phytochemicals that can protect against HCl-induced acute lung injury in mice.

Key words: Acute lung injury, *Cesalpinia digyna*, hydrochloric acid, redox status, reactive oxygen species

SUMMARY

- Hydroalcoholic extract of *Caesalpenia dignea* roots provides protection against hydrochloric acid (HCl)-induced acute lung injury
- The HCl-induced redox imbalance was restored toward normal by the extract as reflected by the levels of reactive oxygen species, malondialdehyde, glutathione, and catalase activity in the lung tissues
- The hydroalcoholic extract provides better protection than bergenin alone (a key component of the extract) against acute lung injury.



Abbreviations Used: ALI: Acute lung injury, BALF: Bronchoalveolar lavage fluid, MDA: Malondialdehyde, ROS: Reactive oxygen species, DCF-DA: 2′ 7′-dichlorofluorescein diacetate

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INTRODUCTION

Acute lung injury (ALI) and its severe form acute respiratory distress syndrome (ARDS) are lung disorders, which are characterized by acute hypoxemic respiratory failure, severe inflammatory response, and damage of exchange activity of the lungs.[1-3] The cellular changes that characterize ALI include excessive transepithelial neutrophils migration, pro-inflammatory cytokine release, and loss of integrity of alveolar capillary membrane.[4,5] Plasma levels of interleukin 8 (IL-8), IL-6, IL-2, IL-1 β, and tumor necrosis factor-α (TNF-α) are persistently elevated in ALI, which are strong predictors of mortality.[6] Apart from this, altered activity of transcriptional factors such as nuclear factor kappa B (NF-κB) and AP-1 is also associated with the diseases pathogenesis.[7,8]

ALI can be triggered by various direct or indirect factors. Direct factors include pneumonia, aspiration of gastric contents, pulmonary contusion, or inhalation of toxic gases. Indirect factors include multiple trauma, sepsis, blood transfusion, or pancreatic inflammation.[3,4] Gastric aspiration is a frequently reported event in patients with impaired consciousness due to general anesthesia, head trauma, and gastrointestinal/esophageal abnormalities.[9]

ALI being an inflammatory lung disease leads to the generation of reactive oxygen species (ROS) such as superoxide anion, hydroxyl anion, or hydrogen peroxide (H_2O_2), produced primarily by the neutrophils recruited at the site of inflammation.[10,11] Several studies have shown that oxidant/antioxidant imbalance has injurious effects on alveolar epithelial cells.[11,12] Levels of H_2O_2 were found to be higher in the exhaled breath of patients with ALI.[13,14] Further, the levels of reduced glutathione (GSH), an antioxidant, declines in the ALI patients.[3] Accordingly, it is believed that the restoration of oxidant/antioxidant balance can limit the lung injury.[3,11]

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Since various types of insults to lungs result in the development of ALI, different types of animal models are used to carry out preclinical studies, namely, lipopolysaccharide (LPS)-induced ALI model and hydrochloric acid (HCl)-induced ALI model. HCl-induced ALI is a well-established model and is being used for preclinical research by various groups.[15-17] The model mimics the lung injury caused due to aspiration of gastric contents, and various pathological features of the diseases, such as neutrophilic inflammation, airway and alveolar epithelium damage, and pulmonary edema, are produced.[18] Owing to the aforementioned reasons, the model was used in the present study to evaluate the anti-inflammatory potential of *Caesalpinia digyna* extract.

Plants are being used for medicinal purposes since ancient times. Recent studies carried out by various research groups have shown that plants possess anti-inflammatory, antipyretic, and antiseptic properties that could be utilized for the treatment of various disorders.[19-21] Members of the genus *Caesalpinia* such as *Caesalpinia sappan, Caesalpinia bonducella,* and *Caesalpinia pulcherrima* are long known for their therapeutic properties.[19,22-24] *C. digyna* is a large perennial prickly shrub or climber with a height of 10 m, found in the areas of Sri Lanka, India and Malay Peninsula. In India, it is growing wild in the scrub forests of the Eastern Himalayas, Assam, West Bengal, Madhya Pradesh and the Eastern Ghats of Andhra Pradesh.[25] Its common name is Teri pod and Hindi name is Vakerimul. The roots of *C. digyna* are medicinally useful. The methanolic root extract of *C. digyna* has shown beneficial effects in amelioration of hypertension and diabetes.[19,26,27] It also possesses anti-inflammatory, antipyretic activities, and inhibits the growth of *Mycobacterium tuberculosis*.[19,28] In a recent study, we have reported that ethanolic extract of the plant roots exhibits anti-anxiety activity in mice.[29] Considering the above-mentioned reports, showing the anti-oxidant and anti-inflammatory potential *C. digyna*, we hypothesized that hydroalcoholic extract of plant roots might protect against acid aspiration-induced ALI.

MATERIALS AND METHODS

Animals

Laca female mice, weighing 25–30 g and 6–8-week-old, were obtained from the Central Animal House of Panjab University, Chandigarh. They were kept in polypropylene cages and were supplied with proper diet and water. During the experiments, the animals were handled with proper care according to the guidelines approved by University Ethics Committee (Ethical approval number: PU/IAEC/S/16/04).

Plant material

Dried roots of *C. digyna* were procured from Manilal Lallubhai and Co., Mumbai, India. The identity of the roots was confirmed through the Head, Raw Materials, Herbarium and Museum at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India vide letter number NISCAIR/RHMD/Consult/2013/2351-131-3.

Chemicals

Chemicals used for the present work were purchased from Millipore, Merck, Empula, HiMedia, and Fisher scientific. HCl used for the induction of ALI was purchased from Sigma.

Preparation of extracts

Moderately coarse powder of the *C. digyna* roots (100 g) was refluxed with ethanol: Water (70:30) for 3 h. The extract was filtered and the filtrate was dried using Eyela N 1100 rotary vacuum evaporator and was preserved in a vacuum desiccator containing anhydrous silica gel blue. Tween 80 (5%) in aqueous of the test substance was prepared by suspending appropriate quantities in the vehicle to administer 0.2 ml of extract per mouse through oral route.

Isolation and characterization of bergenin

Bergenin was isolated from the ethanolic extract of *C. digyna* roots using column chromatography, and was characterized based on melting point, Rf value, Ultraviolet (UV), Infrared (IR), Proton Nuclear Magnetic Resonance (^{1}H NMR), Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR), and Mass spectra; as explained earlier.[30] Further purity of bergenin was verified by High performance liquid chromatography (HPLC).[30]

Experimental design

The mice were divided randomly into four groups and were given treatment as below

Control group

Mice were provided with standard diet and were not given any other treatment.

Acute lung injury group

Mice were administered 60 µl of 0.1N HCl (per mice) intratracheally under anesthesia to mimic acid aspiration-induced ALI.

Acute lung injury + extract

Mice were divided into subgroups and were orally given extract at a dose of 50, 100, or 200 mg/kg body weight 90 min before HCl administration.

Acute lung injury + bergenin

Mice were treated with bergenin at a dose of 50 mg/kg body weight 90 before HCl administration.

After 24 h of HCl administration, mice were sacrificed with overdose of diethyl ether. The mice were subjected to procurement of bronchoalveolar lavage fluid (BALF) and lung tissue. Total and differential cell analysis was done using BALF.[31] Lung tissue homogenate was prepared for performing various biochemical assays.

Analysis of biochemical parameters

The total protein content was assayed by method of Lowry.[32] ROS levels in the lung tissues were measured using 2' 7'-dichlorofluorescein diacetate (DCF-DA) dye. Briefly discussing, homogenates were prepared from freshly procured lung tissues. The homogenates were then incubated in reaction buffer containing 10 µM DCF-DA and 10 µM sodium succinate for 30 min at 37°C. DCF-DA was oxidized by ROS into 2,7-dichlorofluorescin, a highly fluorescent compound, which was then measured at excitation 485 nm and emission 530 nm.[33] Malondialdehyde (MDA) as a marker of lipid peroxidation was assayed by the method of Ohkawa *et al.*, 1979.[34] Total GSH and reduced GSH were measured according to the Cleland and Ellman protocols, respectively.[35,36] The catalase activity was measured by the method of Aebi.[37]

Statistical analysis

Results are depicted as mean ± standard error of mean. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons using GraphPad prism software (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered as statistically significant.

RESULTS

Oral administration of *Caesalpinia digyna* extract before hydrochloric acid treatment reduced the inflammatory cells in bronchoalveolar lavage fluid

As stated earlier neutrophils play an important role in the pathogenesis of ALI and they tend to accumulate within the lung structures in case of any lung insult such as injury, trauma, or infection.[38] Therefore, we quantified the total as well as neutrophil number in BALF procured from different groups of mice. Figure 1a depicts the total cell count in the BALF procured from mice given different treatments. Data indeed show that HCl treatment results in tremendous increase in the total cell number as compared to the control group ($P < 0.001$). Interestingly, prior treatment with hydroalcoholic extract of *C. digyna* reverted the total cell count toward normal in a dose-dependent manner. The maximum protection was observed at a dose of 200 mg/kg ($P < 0.001$). Next, we conducted the differential analysis of cells in the BALF, and our data corroborate earlier findings showing that majority of the cells recruited to the lungs were found to be neutrophils on HCl mediated ALI [Figure 1b]. Number of neutrophils were found to be increased significantly on intratracheal administration of HCl when compared to control ($P < 0.001$). The pretreatment with different doses of extract reduced the number of neutrophils in a dose-dependent manner. Extract at a dose of 200 mg/kg reduced the number of neutrophils maximally ($P < 0.001$).

The pathogenesis of ALI includes injury of endothelial and epithelial barriers leading to protein-rich edema, and inflammation induced by cytokines and chemokines released from inflammatory cells, lung epithelial cells, or fibroblasts. Weakening of the endothelial barrier enhances the transendothelial diapedesis of leukocytes into lung tissues further contributing to pulmonary dysfunction. To analyze the extent of pulmonary edema, the protein content was measured in BALF of mice [Figure 1c]. It was observed that BALF protein content increased significantly ($P < 0.001$) on administration of HCl while pretreatment of mice with extract lowered such increase in a dose-dependent manner. Normalization of BALF protein content by extract was found to be significant at a dose of 100 ($P < 0.05$) and of 200 mg/kg ($P < 0.001$). Overall, the data strongly suggest the beneficial effects of the extract in modulating acid aspiration-induced lung inflammation as well as pulmonary edema.

Caesalpinia digyna extract normalize the levels of reactive oxygen species and lipid peroxidation in lungs on hydrochloric acid treatment

It is well established that overproduction of ROS during ALI lead to redox imbalance, which ultimately causes damage to DNA, proteins, and membrane lipids. One of the products of lipid peroxidation, MDA is routinely assayed to assess oxidative stress.[39] Accordingly, we have measured the levels of ROS and MDA in lung tissue of mice subjected to different treatments. Figure 2a depicts the ROS levels in lung tissue of mice given different treatments. Intratracheal administration of HCl led to a steep rise in ROS level ($P < 0.001$). Oral administration of *C. digyna* extracts before HCl treatment decreased the ROS levels in a dose-dependent manner with 200 mg/kg dose showing the maximal effect ($P < 0.001$). Figure 2b depicts the effects of the extract on the MDA content in lungs of mice from different groups. The MDA concentration

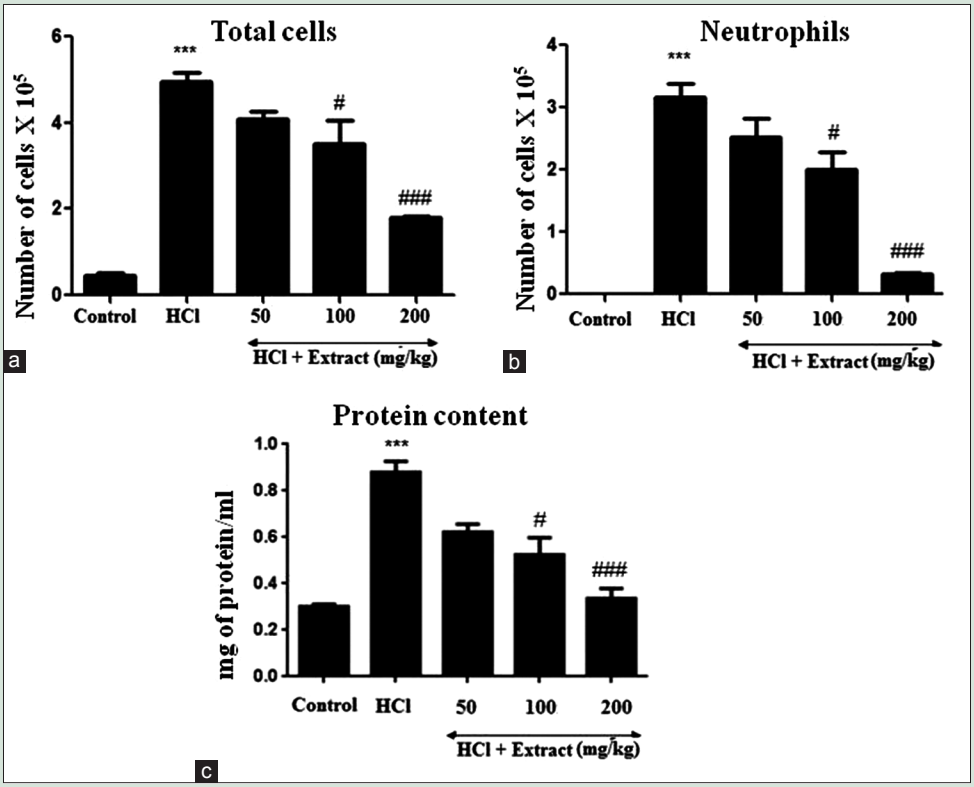

Figure 1: Oral administration of *Caesalpinia digyna* extract before hydrochloric acid treatment reduced the number of total inflammatory cells as well as neutrophils in bronchoalveolar lavage fluid. Mice treated as explained in materials and methods section were subjected to bronchoalveolar lavage and cells obtained were differentially stained for counting total cells (a) and neutrophils (b). Supernatant of bronchoalveolar lavage was assessed for total protein content (c). Results are depicted as Mean ± standard error of mean. ***significant with respect to control, $P < 0.001$; ###significant with respect to hydrochloric acid, $P < 0.001$; #significant with respect to hydrochloric acid, $P < 0.05$

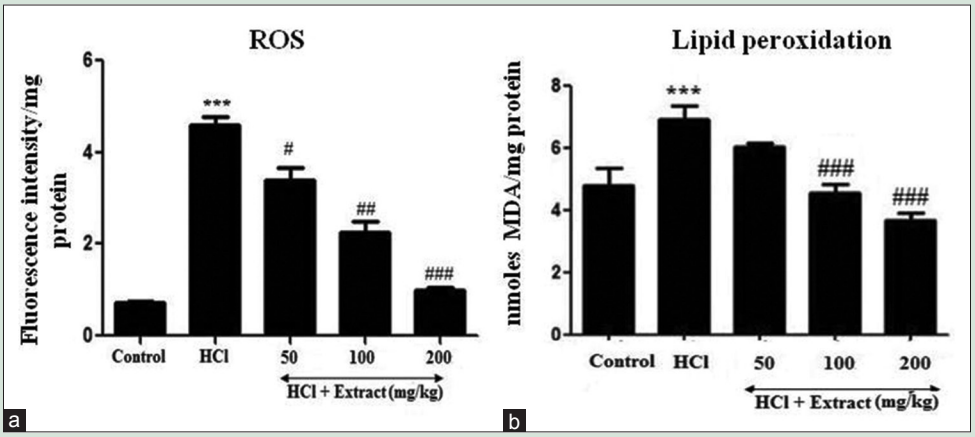

Figure 2: *Caesalpinia digyna* extract normalize the levels of reactive oxygen species and lipid peroxidation in lungs on hydrochloric acid treatment. Mice were treated with hydrochloric acid and/or extract as explained earlier. Lung tissue homogenate was analyzed for reactive oxygen species (a), Malondialdehyde (b). Results are depicted as mean ± standard error of mean. ***significant with respect to control, $P < 0.001$; ###significant with respect to hydrochloric acid, $P < 0.001$; ##significant with respect to hydrochloric acid, $P < 0.01$; #significant with respect to hydrochloric acid, $P < 0.05$

increased significantly ($P < 0.001$) in lung homogenate on HCl treatment when compared with control group. The pretreatment with different doses of the extract resulted in restoration of MDA content toward normal in a dose-dependent manner. The maximum restoration was observed at dose of 200 mg/kg ($P < 0.001$).

Caesalpinia digyna restores the glutathione content and catalase activity in lungs on hydrochloric acid treatment

During periods of oxidative stress, reduced GSH is oxidized to Glutathione disulfide (GSSG), and its restoration back to reduced form become defective. Accordingly, a fall in GSH is considered as indicator of oxidative stress. Catalase is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2.[40,41] Therefore, GSH content and catalase activity were measured to evaluate the potential of extract in restoring these parameters following HCl administration. Figure 3a depicts that GSH level declined significantly on administration of HCl ($P < 0.001$). Treatment with 50 mg/kg dose of extract did not modulate GSH imbalance. However, increasing the dose of extract further to 100 mg/kg and 200 mg/kg restored the GSH content back toward normal significantly ($P < 0.001$).

Figure 3b depicts the activity of catalase enzyme in lung homogenate derived from different groups of mice. The catalase activity decreases significantly on intratracheal administration of HCl ($P < 0.001$). The prior treatment with different doses of extract reverted the catalase activity toward normal significantly at a dose of 200 mg/kg ($P < 0.001$). Lower doses of extract did not alter the catalase activity significantly when compared with HCl-treated group.

Bergenin isolated from *Caesalpinia digyna* decreased the number of neutrophils but conversely increased the macrophage count in bronchoalveolar lavage fluid on hydrochloric acid treatment without altering the levels of reduced glutathione

We have recently reported that ethanolic extract of *C. digyna* roots constitute bergenin as a major component and have shown excellent anti-anxiety activity.[30] In addition, bergenin ameliorate LPS-induced ALI.[42] Therefore, we decided to examine the anti-inflammatory action of the drug in HCl mediated ALI. Surprisingly, administration of bergenin before HCl treatment did not cause any major change in number of total cells in BALF [Figure 4a]. However, qualitative analysis of data [Figure 4b] revealed that bergrnin reduced the HCl-induced neutrophil number in BALF significantly ($P < 0.001$). The protection provided by the purified bergenin at a dose of 50 mg/kg body weight (39.48%) is almost to the similar extent as exhibited by the 100 mg/kg crude extract (34.26%). Interestingly, bergenin increased the number of macrophages in BALF as compared to HCl treated mice [Figure 4c], which may explain the inability of the begenin to reduce the total BALF cells on HCl treatment. As bergenin is believed to possess antioxidant potential, we then analyzed the GSH content in the lung tissue. Our data showed that bergenin alone could not restore the levels of reduced GSH toward normal on HCl treatment [Figure 4d]. It appears that purified bergenin reduced the number of neutrophils in lungs independent of its antioxidant potential in our model.

DISCUSSION

Several studies have demonstrated the association between HCl aspiration and subsequent development of the ALI.[9,18,43,44,45] Intratracheal administration of HCl is a potent inducer of inflammatory responses in different animal models of ALI.[4,38,46,47] A dose-dependent reduction in the BALF neutrophil count by hydroalcoholic extract of *C. digyna* clearly suggests the anti-inflammatory properties of the extract.

Alveolar epithelium and endothelium barrier of the pulmonary microvasculature play a key role in the ability of the lung to perform gas exchange. When such barrier is disrupted during ALI, interstitial, or alveolar edema may develop through leakage of plasma proteins and other substances.[1,48] Reduction in BALF protein content by the extract further reflects its protective efficacy against pulmonary edema during ALI. As several neutrophil chemotactic chemokine/cytokines are known to play a critical role in the acid-induced lung injury, it would be interesting to know if the down-regulation of lung inflammation by *C. digyna* in our model is due to general suppression in the cytokines, or it specifically target any individual cytokine/chemokine such as IL-6, IL-1 β, and TNF-α.[49,50]

ROS plays a critical role in the diseases associated with pulmonary vascular abnormalities such as ALI as they enhance neutrophil-dependent

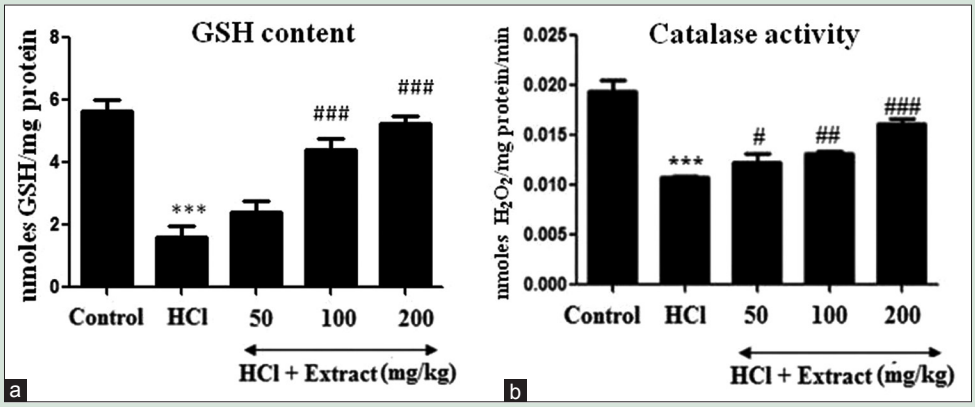

Figure 3: *Caesalpinia digyna* restores the glutathione content and catalase activity in lungs on hydrochloric acid treatment. Total lung homogenate prepared from different group of mice were assessed for glutathione content (a) and Catalase activity (b). Results are depicted as mean ± standard error of mean. ***significant with respect to control, $P < 0.001$; ###significant with respect to hydrochloric acid, $P < 0.001$; ##significant with respect to hydrochloric acid, $P < 0.01$; #significant with respect to hydrochloric acid, $P < 0.05$.

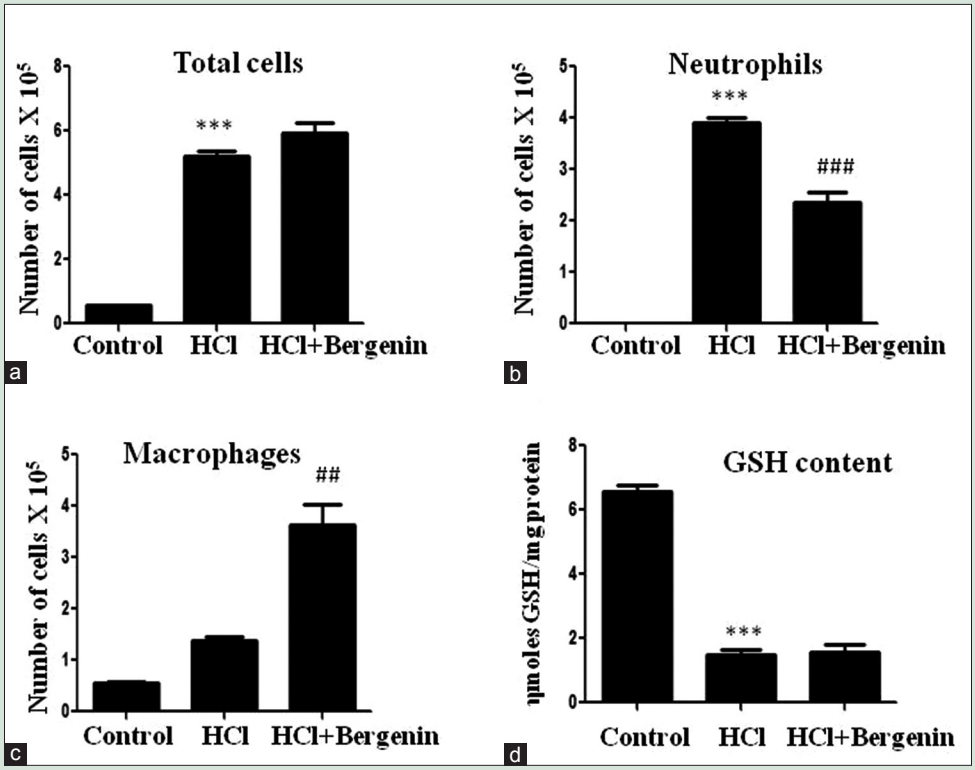

Figure 4: Bergenin protects against lung neutrophilia without affecting total number of leukocytes on hydrochloric acid treatment. Mice were treated with hydrochloric acid and/or bergenin as explained earlier. Bronchoalveolar lavage fluid or lung tissue homogenate was analyzed for total cells (a), neutrophils (b), macrophages (c), and glutathione (d). Results are depicted as mean ± standard error of mean. ***significant with respect to control, $P < 0.001$; ###significant with respect to hydrochloric acid, $P < 0.001$; ##significant with respect to hydrochloric acid, $P < 0.01$

endothelial lung injury.[14,46] Moreover, ROS may also indirectly contribute to the development of ALI by depleting antioxidants leading to imbalance in oxidant-antioxidant equilibrium. In view of the fact that *C. digyna* is traditionally used for the treatment of inflammatory diseases due to its free radical scavenging effect and thus antioxidant activity,[23] we contemplate that the reduction in lung injury by *C. digyna* extract may be through normalization of redox status within the tissue. Indeed our results confirmed the antioxidant action of the extract as reflected by restoration of levels of ROS, MDA, GSH, and catalase activity towards normal. Elevated levels of antioxidant enzyme systems such as SOD or catalase, remove or inactivate formed ROS, thereby hindering lipid peroxidation and normalizing MDA levels in lungs.[51]

A profile of principal constituents of the extract remains to be analyzed to identify key phytochemicals, which may ameliorate lung inflammation. *C. digyna* is known to contain several polyphenols through which it can exert its antioxidant activity. It is already known that bergenin is the major polyphenolic constituent of *C. digyna* extract.[23] We have recently confirmed that bergenin is the major constituent of the ethanolic extract

of the plant roots.[29] Our data that bergenin reduced the neutrophil numbers but increased the number of macrophages in the mice lungs on HCl administration, indicate that isolated compound has the ability to potentiate immune defense mechanism. Indeed, enhanced clonal expansion and activation of macrophages have been reported for the antimicrobial activity of bergenin in murine model of *Mycobacterium tuberculosis* infection.[52] Interestingly, studies have indicated that ethanolic and methanolic extracts of *C. digyna* containing bergenin, exhibit superior-free radical scavenging ability and antioxidant activity than bergenin alone[25] and therefore can lead to synergistic effect and thereby increase the efficacy of the antioxidant mixture. It is quite possible that bergenin alone ameliorates the lung injury by potentiating macrophage activity independent of its antioxidant potential, as drug alone failed to restore the HCl mediated reduction in GSH. On the other hand, the extract exerts its anti-inflammatory action possibly through improved antioxidant activity due to the presence of mixture of phytochemicals in it, as reported by Singh *et al.*[25] It may also be possible that when whole plant extract is used, it will exhibit improved bioavailability, as compared to the single chemical entity as observed by us. Nevertheless, our studies suggest a potent anti-inflammatory effect of hydroalcoholic extract of *C. digyna* roots on acid aspiration-induced ALI in mice.

CONCLUSION

Overall, our data show that hydroalcoholic extract of *Caesalpenia dignea* roots has potential to ameliorate HCl-induced ALI. Further, it was found that the extract provides a better protection than bergenin alone against ALI.

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Conflicts of interest

There are no conflicts of interest.

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