

Hydroalcoholic Extract of *Argyrea speciosa* Roots Ameliorates HCl-mediated Acute Lung Injury in Mice

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ABSTRACT

Objective: *Argyrea speciosa* is a popular Indian medicinal plant. It has long been used in the traditional Ayurvedic Indian medicine for various diseases. The present work was designed to evaluate anti-inflammatory potential of hydroalcoholic extract of *A. speciosa* roots using mouse model of HCl-induced acute lung injury (ALI) by conducting a dose–response studies.

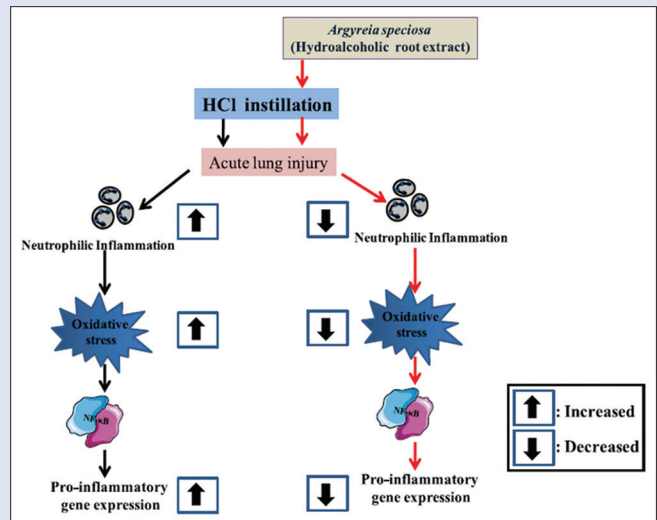
Materials and Methods: The extract was given once orally at a dose of 50, 100, or 200 mg/kg b.wt. 90 min before HCl administration. Lung tissue of different group of mice was analyzed for the recruitment of inflammatory cells, redox status, and mRNA expression of pro-inflammatory genes to evaluate the anti-inflammatory effects of the extract. **Results:** Our results showed that the extract reduced HCl-induced lung inflammation in a dose-dependent manner. The neutrophil numbers in bronchoalveolar lavage fluid (BALF) were almost completely abrogated at a dose of 100 mg/kg b.wt. The severe reduction in neutrophils was accompanied with reduced pulmonary edema as the total protein content in BALF was found to be decreased substantially. In addition, the extract prevented the HCl-mediated oxidative stress in lungs as reflected by the normalization of levels of reactive oxygen species, malondialdehyde, redox status, and catalase activity. Extract seems to blunt the activity of redox-sensitive transcription factor nuclear factor-kappa B (NF-κB) as it suppressed the gene expression of ALI-linked pro-inflammatory cytokines, namely, interleukin-1 β and tumor necrosis factor-alpha. **Conclusion:** Overall, our data provide evidence that cocktail of natural phytochemicals present in hydroalcoholic extract of *A. speciosa* protects against HCl-induced ALI in mice potentially by curbing oxidative stress and consequent activation of NF-κB in the tissue.

Key words: Acute lung injury, *Argyrea speciosa*, HCl, nuclear factor-kappa B, oxidative stress

SUMMARY

- Hydroalcoholic extract of *A. speciosa* ameliorates the HCl induced lung inflammation and edema in a dose dependent manner.
- Prior administration of the extract restored the HCl induced redox balance in the lungs towards normal reflecting its anti-oxidant potential.
- The HCl induced expression of the NF-κB dependent pro-inflammatory genes

was suppressed effectively by the extract which may partly be responsible for its anti-inflammatory action.



Abbreviations used: ALI: Acute lung injury, BALF: Bronchoalveolar lavage fluid, IL-1: Interleukin-1, TNF-α: Tumor necrosis factor-alpha, ROS: Reactive oxygen species, MDA: Malondialdehyde

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INTRODUCTION

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), result in substantial morbidity and mortality.^[1,2] ALI/ARDS is characterized by severe hypoxemia, hypercapnia, diffuse infiltrates on chest radiography, and substantial reduction in pulmonary compliance. Physiologically, the ratio of arterial oxygen pressure and the inspiratory oxygen concentration is $\text{PaO}_2/\text{FiO}_2 \leq 300$ mmHg for ALI and ≤ 200 mmHg for ARDS.^[3,4] Several direct (pneumonia, gastric contents aspiration, and inhalation injury) and indirect (sepsis, severe trauma with shock and multiple transfusions, burns, and acute pancreatitis) insults lead to acute inflammatory response in the lungs.^[1,3,5] ALI has a high incidence rate of 78.9/100,000 persons with overall mortality rate of 35%–40% depending on the etiological and geographical variations.^[1,5] Lung injury has also been linked with damage to various organs (kidney, brain), which can lead to multiple organ failure.^[6–8]

During early stages of ALI, pulmonary edema develops due to vasoconstriction of blood vessels within the lung.^[9] The advancement of ALI is associated with enhanced production of chemokines and pro-inflammatory agents such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α), followed by recruitment of neutrophils in the lungs.^[3]

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Reactive oxygen species (ROS) production plays a central role in the progression of inflammation.^[10,11] The epithelial cells and the alveolar macrophages, resident cells of the lungs, are activated due to the increased production of ROS. This perpetuates a vicious cycle that generates a number of chemotactic molecules that recruit additional inflammatory cells such as neutrophils, monocytes, and lymphocytes into the lung, which in turn produces more cytotoxic mediators that increase the burden of oxidative stress in the lung, leading to profound injury to the alveolocapillary membrane.^[5,12]

Argyreia speciosa is a popular Indian medicinal plant and an important “Rasayana” herb used extensively as a medicine for various diseases in the Ayurvedic System of Medicine. It belongs to the family *Convolvulaceae*. It is a large climber growing throughout India. It has thin roots, thick stem, and hairy leaves. It is commonly known as Hawaiian Baby Woodrose, Elephant creeper, or Woolly morning glow in English, and in Sanskrit, it is called as *Vridhadaraka* meaning “antiaging.”^[13] Pharmacologically, the plant is used for its nootropic, aphrodisiac, immunomodulatory, hepatoprotective, antioxidant, anti-inflammatory, antihyperglycemic, antidiarrheal, antimicrobial, antiviral, nematocidal, antiulcer, anticonvulsant, analgesic, and central nervous depressant activities.^[13,14] In Hindu medicine, the root is regarded as an alternative tonic and useful in rheumatic affection and disease of the central nervous system.^[15] As *A. speciosa* extract has been shown to possess anti-inflammatory and antioxidant activities^[16,17] and the fact that inflammation and oxidative stress play a key role in ALI, we hypothesized that the extract may exert anti-inflammatory effects during ALI. Accordingly, the dose–response study was conducted to evaluate the protective effects of hydroalcoholic extract of *A. speciosa*, prepared from plant roots, on acid aspiration-induced mouse model of ALI.

MATERIALS AND METHODS

Animals

Laca female mice weighing 25–30 g, 6–8 weeks 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 experiments, animals were handled with proper care according to the guidelines approved by the University Ethics Committee (Ethical clearance number: PU/IAEC/S/16/02).

Plant material

Dried roots of *A. speciosa* (4 kg) were procured from Manilal Lallubhai and Company, Mumbai. The roots were authenticated by the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Ref. No. NISCAIR/RHMD/Consult/-2013/2351-131-1).

Chemicals

Chemicals used for this project were purchased from Sigma, Bio-Rad, Merck, Empula, HiMedia, Fisher Scientific, etc., HCl used for the induction of ALI was purchased from Sigma.

Preparation of extracts

Moderately coarse powder of the *A. speciosa* roots (100 g) was refluxed with 1 L of mixture of ethanol:water (70:30) for 3 h at 100°C. The hydroalcoholic solution dissolves almost all constituents (of varying polarities) present in plant tissues.^[18] The extract was filtered and the filtrate was dried using Eyela N 1100 rotary vacuum evaporator. The dried filtrate was preserved in a vacuum desiccator containing anhydrous silica gel blue. The extract was characterized by the presence of triterpenoids, flavonoids, phenols, alkaloids, tannins, protein, carbohydrates, steroids, and fatty acids as described previously.^[19] Tween 80 (5%) in aqueous carboxymethylcellulose (0.5% w/w) was used as a vehicle for preparing the suspension of extract. Doses of

various test substances were prepared by suspending appropriate quantities in the vehicle to administer 200 µl of extract per mouse through oral route.

Experimental design

The mice were divided randomly into the following groups:

- Group 1 (control): The mice were given standard diets. No other treatment was given so that they can perform normal functioning
- Group 2 (0.1N HCl): Intratracheal (*i.t.*) instillation of 0.1N HCl was done under anesthesia at dose of 60 µl per mouse. Mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg)/ketamine (100 mg/kg)
- Group 3 (0.1N HCl + extract): *i.t.* administration of 0.1N HCl was performed in the mice, the same as explained for Group 2. The extract was given at a dose of 50, 100, or 200 mg/kg orally 90 min before HCl administration.

The range of dose was decided based on the previous reports.^[20,21] Mice were sacrificed 24 h after administration of HCl using overdose of diethyl ether. The mice were subjected to procurement of bronchoalveolar lavage fluid (BALF) and lung tissue. BALF was used for analysis of protein content and total as well-differential leukocytes recruited into lung airways.^[22] Lung tissue was processed for the extraction of RNA or preparation of homogenate for performing various biochemical assays.

Analysis of biochemical parameters

The total protein content was assayed by method of Lowry *et al.* (1951).^[23] ROS levels were measured by the method of Wang *et al.* (1990) using the fluorescent protein 2’7’-dichlorofluorescein diacetate.^[24] The malondialdehyde (MDA) as a marker of lipid peroxides was assayed by the method of Ohkawa *et al.* (1979).^[25] Total glutathione (GSH) and GSH were measured according to Ellman’s method.^[26,27] The catalase activity was measured by the method of Aebi (1984).^[28]

Extraction of RNA from lung tissue and real-time polymerase chain reaction analysis

Lung tissues were processed for the extraction of total RNA using TRIzol reagent. The extracted RNA was reverse transcribed into cDNA using reverse transcriptase III (iScript™ cDNA Synthesis Kit, Bio-Rad) and was analyzed for the expression of IL-1 β, TNF-α, intercellular adhesion molecule-1 (ICAM-1), and β-actin using polymerase chain reaction (PCR) thermocycler.^[22] The sequence of the primers used is given below:

IL-1β: Forward-5’-GAC CTT CCA GGA TGAGGA CA-3’; Reverse-5’-AGG CCA CAG GTA TTT TGTCG-3’; TNF-α: Forward-5’-TAT GGC TCA GGG TCC AAC TC-3’; Reverse-5’-CTC CCT TTG CAG AAC TCA GG-3’; ICAM-1: Forward-5’-AGC ACC TCC CCA CCT ACT TT-3’; Reverse-5’-AGC TTG CAC GAC CCT TCT AA-3’ β-actin: Forward-5’-TACAGCTTACCACCACAGC-3’; Reverse-5’-TCTCCAGGG AGGAAGAGGAT-3’.

Statistical analysis

Results are depicted as mean ± standard error of the mean. Statistical analysis was performed by one-way ANOVA test followed by Tukey’s multiple comparison using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA). *P* < 0.05 was considered statistically significant.

RESULTS

Oral administration of *Argyreia speciosa* extract before HCl treatment reduced the inflammatory cells in bronchoalveolar lavage fluid

Figure 1a represents the effect of different doses of *A. speciosa* extract on the total number of cells present in the BALF upon *i.t.* administration

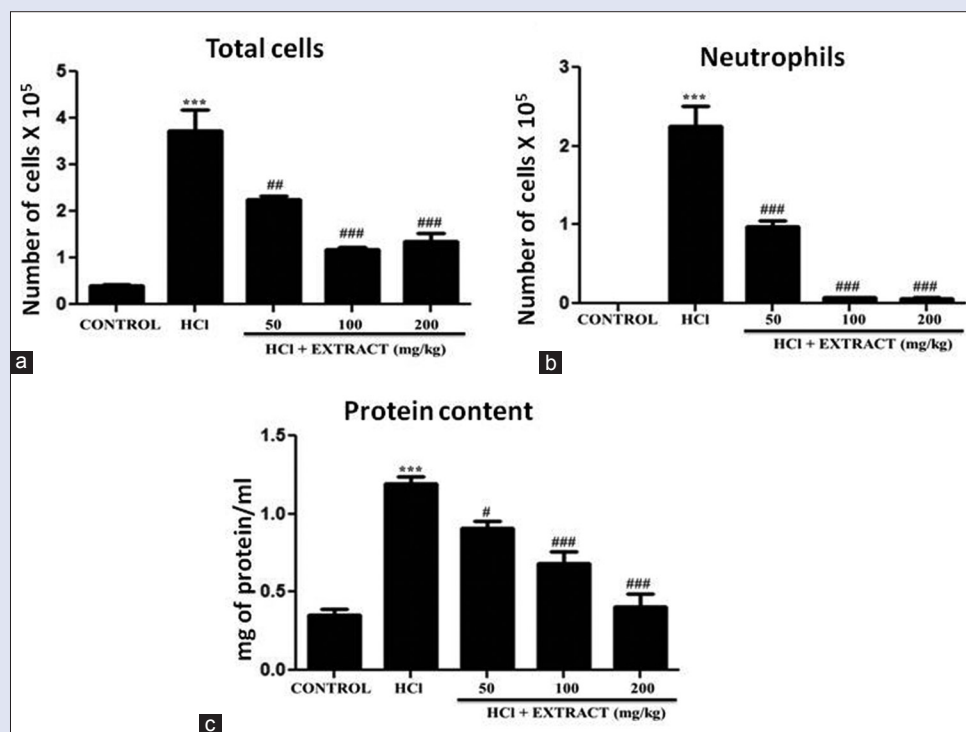


Figure 1: Oral administration of *Argyrea speciosa* extract before HCl treatment reduces the number of total inflammatory cells as well as neutrophils in bronchoalveolar lavage fluid. Mice treated as explained in materials and methods section were subjected to bronchoalveolar lavage and cells obtained 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 \pm standard error of the mean. ***significant w.r.t. control, $P < 0.001$; ###significant w.r.t. HCl, $P < 0.001$; ##significant w.r.t. HCl, $P < 0.01$; #significant w.r.t. HCl, $P < 0.05$

of 0.1N HCl in mice. HCl treatment leads to sharp increase in the number of total inflammatory cells in BALF significantly ($P < 0.001$). Different doses of the extract (50, 100, and 200 mg/kg) were used to screen its effectiveness in amelioration of HCl-induced ALI. In mice preadministered with the *A. speciosa* extract, the total number of inflammatory cells in BALF was found to be restored toward normal significantly even at dose of 50 mg/kg. The reduction in inflammatory cells by the extract seems to get saturated at dose of 100 mg/kg as number of BALF cells was found to be similar at dose of either 100 or 200 mg/kg. Thus, it appears that *A. speciosa* possess anti-inflammatory properties against HCl-induced ALI in mice.

Figure 1b represents the effect of *A. speciosa* on the number of neutrophils present in the BALF following HCl administration. An abrupt increase in the neutrophil number after the administration of 0.1N HCl can be seen when compared to the control mice. However, the administration of *A. speciosa* extracts at a dose of 50 mg/kg before 0.1N HCl administration leads to significant reduction in the number of neutrophils toward normal ($P < 0.001$). Increasing the dose of the extract to 100 or 200 mg/kg suppressed the number of neutrophil almost completely. Overall, it appears that the dose of 100 mg/kg of extract is able to suppress the recruitment of neutrophils in the lungs maximally.

Figure 1c represents the effect of *A. speciosa* extract on the protein concentration in BALF in HCl-treated mice. The protein concentration increased significantly in the mice group treated with HCl as compared to control ($P < 0.001$). The *A. speciosa* extract lowered the HCl-induced BALF protein content significantly at a dose of 50 mg/kg ($P < 0.05$), suggesting the reduction in pulmonary edema. Although the number of neutrophils

was suppressed almost completely at 100 mg/kg dose, pulmonary edema was reduced maximally at 200 mg/kg dose of the extract ($P < 0.001$).

Argyrea speciosa extract normalizes the levels of reactive oxygen species and lipid peroxidation in lungs upon HCl treatment

Figure 2a represents the effect of *A. speciosa* on ROS levels, which indicates the level of oxidative stress. A significant elevation in the ROS levels can be seen in lungs of mice treated with HCl when compared to the control group of mice ($P < 0.001$). Administration of *A. speciosa* extracts before the HCl instillation decreased the ROS level significantly ($P < 0.001$) at all doses used in the present study. A dose of 200 mg/kg of the extract seems to exert maximum potential to restore the levels of ROS.

Figure 2b represents the effect of *A. speciosa* on the oxidative stress marker MDA that is an index of lipid peroxidation. A significant elevation in the MDA levels can be seen in mice group treated with HCl when compared to the control mice group ($P < 0.001$). Administration of *A. speciosa* extract (50 mg/kg) before the HCl instillation decreased the levels of MDA significantly ($P < 0.01$). Again, a dose of 100 mg/kg of the extract seems to be the most effective dose as it ameliorates the MDA content maximally ($P < 0.001$).

Argyrea speciosa restores the redox status and catalase activity in the lungs upon HCl treatment

Figure 3a represents the effect of *A. speciosa* extracts on levels of reduced GSH in lungs upon HCl-mediated ALI in mice. A significant

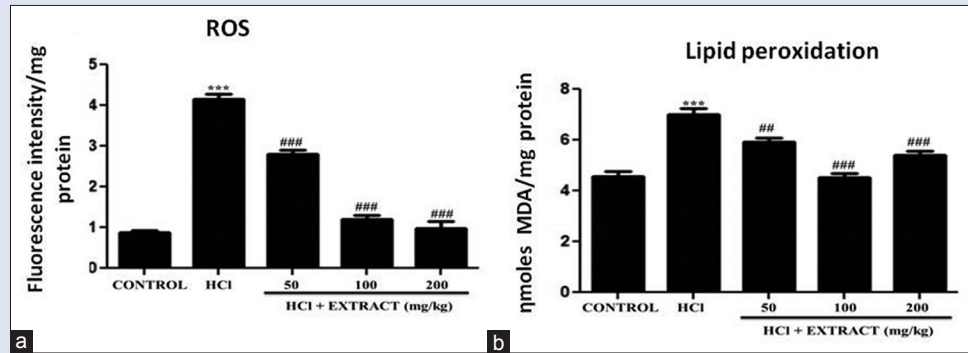


Figure 2: *Argyrea speciosa* extract normalize the levels of reactive oxygen species and lipid peroxidation in lungs upon HCl treatment. Mice were treated with HCl and/or extract as explained earlier. Lung tissue homogenate was analyzed for reactive oxygen species (a), malondialdehyde (b). Results are depicted as mean \pm standard error of the mean. ***significant w.r.t. control, $P < 0.001$; ###significant w.r.t. HCl, $P < 0.001$; ##significant w.r.t. HCl, $P < 0.01$

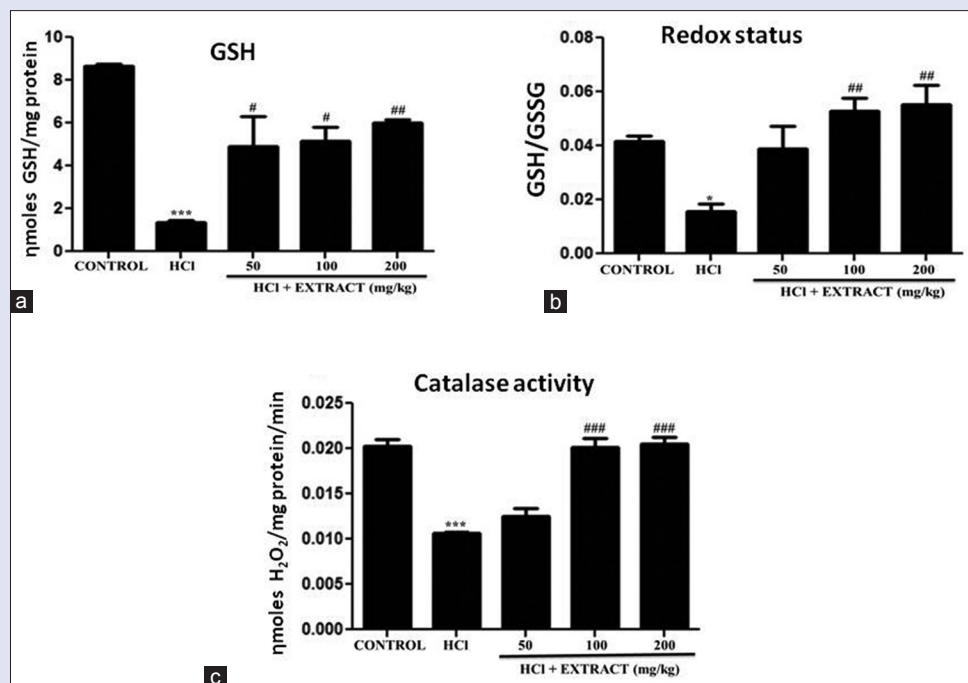


Figure 3: *Argyrea speciosa* restores the redox status and catalase activity in lungs upon HCl treatment. Total lung homogenate prepared from different group of mice and were assessed for glutathione (a), redox status (b) and catalase activity (c). Results are depicted as mean \pm standard error of the mean. ***significant w.r.t. control, $P < 0.001$; ###significant w.r.t. HCl, $P < 0.001$; ##significant w.r.t. HCl, $P < 0.01$; #significant w.r.t. HCl, $P < 0.05$

reduction in the reduced GSH levels can be seen after instillation of HCl as compared to control mice group ($P < 0.001$). The oral administration of *A. speciosa* extract in the mice before HCl treatment restored the levels of reduced GSH toward normal significantly in a dose-dependent manner.

Figure 3b represents the effect of *A. speciosa* on redox status in lung tissue upon HCl-induced ALI in mice. The ratio of reduced GSH to oxidized GSH was assessed to analyze the redox status in the lungs. A significant reduction in the redox status was observed in mice group treated with HCl when compared to control mice group ($P < 0.001$). The administration of *A. speciosa* extract before HCl instillation normalized the redox status in a dose-dependent manner.

Figure 3c represents the effect of *A. speciosa* extract on the catalase activity in ALI. A significant reduction in enzyme activity was seen after the *i.t.* instillation of HCl when compared to control group ($P < 0.001$). When *A. speciosa* extract was administered before instillation of HCl, the

activity of catalase was restored toward normal significantly at a dose of either 100 or 200 mg/kg ($P < 0.001$).

Argyrea speciosa extract downregulates gene expression of nuclear factor-kappa B-dependent pro-inflammatory factors relevant with acute lung injury

Figure 4 represents the gene expression of IL-1 β , TNF- α , and ICAM-1 whose expression is dependent on redox-sensitive transcription factor NF- κ B. We examined the expression on these pro-inflammatory factors in the lungs using real-time PCR, when the extract was given before HCl administration at dose of 100 mg/kg. Our data indeed revealed that HCl treatment leads to increased expression of IL-1 β and TNF- α , which was suppressed effectively by the extract. However, the basal expression of ICAM-1 was not altered by HCl or extract.

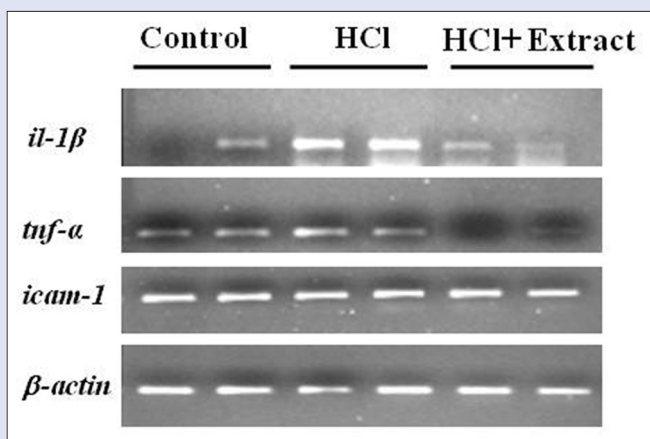


Figure 4: *Argyrea speciosa* downregulates the expression of nuclear factor-kappa B-dependent genes in the lungs upon HCl treatment. Lung tissue was processed for total RNA extraction and mRNA was transcribed to cDNA. cDNA was then analyzed by real-time polymerase chain reaction for expression of tumor necrosis factor-alpha, interleukin 1 β , and intercellular adhesion molecule-1 using specific primers. β -actin was used as loading control

Table 1: Phytochemical screening of hydroalcoholic extract

Class of phytoconstituents	Extract
Fatty acids	-
Steroids	-
Triterpenoids	+
Flavonoids	+
Phenols	+
Alkaloids	+
Tannins	+
Protein	+
Carbohydrates	+

--: Absent in the extract, +: Present in the extract

Phytochemical screening of hydroalcoholic extract of *Argyrea speciosa* roots

Since hydroalcoholic *A. speciosa* was found to possess strong anti-inflammatory potential against HCl-induced ALI, we next screened the extract for the presence of different classes of phytochemicals. Data confirmed the presence of triterpenoids, flavonoids, phenols, alkaloids, tannins, proteins, and carbohydrates in the extract. However, fatty acids and steroids were found to be absent [Table 1].

DISCUSSION

In the present study, it was found that hydroalcoholic extract of *A. speciosa* protects against HCl-mediated infiltration of the neutrophils in the lungs, thus suggesting that the components of the extract possess potent anti-inflammatory properties in the context of ALI. The various pharmacological activities of *A. speciosa* extract such as hepatoprotective, immunomodulatory, anti-inflammatory, and antioxidant have been reported previously.^[16,17] Hence, the reduction in the neutrophils by the extract in our model may possibly be due to its antioxidant and immunomodulatory properties. Pulmonary edema is the result of altered lung fluid balance due to influx of protein-rich fluid because of epithelial and endothelial injury. Restoration of ALI-associated BALF protein content toward normal upon pretreatment with root extract of *A. speciosa* confirms that constituents of the extract protect against

vascular leakage. Although the number of neutrophil was reduced almost to the same extent at dose of either 100 mg/kg or 200 mg/kg of extract, pulmonary edema was suppressed maximally at a dose of 200 mg/kg b.wt. It is possible that the number of neutrophil recruited to lungs was reduced at dose of 100 mg/kg, but vascular leakage of other pro-inflammatory factors such as cytokines/chemokines may be inhibited effectively at higher dose.

Oxidant/antioxidant level imbalance has been linked with several lung diseases including ALI/ARDS.^[12,29] Numerous studies suggest the deleterious effects of ROS in causing pulmonary vascular abnormalities that characterize ALI/ARDS. The activated inflammatory cells during ALI generate various ROS, which contributes heavily to the cellular damage. Studies have shown that MDA level is a potent marker of increased oxidative stress in the cell.^[11,30] Our data on levels of ROS and MDA indicate that extract protects against HCl-mediated ALI by curbing ROS and hence protects against lipid peroxidation. Levels of the reduced GSH, an important cellular antioxidant, indicates the severity of ALI and therefore has a central role in the maintenance of oxidative stress and controlling the pro-inflammatory process.^[31] In addition, our observations on reduced GSH levels further strengthen the notion that extract possesses antioxidant properties. Taken together with data on ROS, MDA, GSH, redox status, and catalase activity strongly indicate the ability of extract to control the oxidative stress. Altered redox balance in the cell can lead to activation of NF- κ B, a master regulator of pro-inflammatory genes.^[32] It is a well-known fact that expression of NF- κ B-dependent genes such as IL-1 β and TNF- α plays a critical role in orchestration of ALI.^[33] It is interesting to note that the extract downregulates the expression of both IL-1 β and TNF- α at the mRNA level. It is quite possible that normalization of redox balance by the extract results in subdued activation of NF- κ B and thus modulates the inflammatory process in the lungs.

Considering the fact that only, a limited number of pharmacological therapies are available against ALI/ARDS and that the steroids are generally less effective, *A. speciosa* may serve as a potential candidate for the management of the disease.^[34,35] The plant is long known for its medicinal properties (a strong antioxidant, anti-inflammatory, and antiarthritic activity) and is being consumed/used by humans without any known side effects.^[36] Recently, the beneficial effects of a polyherbal formulation (containing extract of *A. speciosa* and others) were reported in a clinical trial conducted on patients with benign prostatic hyperplasia.^[37] Furthermore, the ethanolic extract of the plant roots has also been reported to induce inhibitory effects on cafeteria diet-induced obesity in rats.^[38] Obesity is an important risk factor and has been strongly linked with ALI.^[39,40] A variety of chemicals belonging to the classes ergoline alkaloids, lipids, flavonoids, steroids, and triterpenoids have been extracted from the plant.^[14] Due to the presence of these vital constituents, *A. speciosa* is reported to possess various pharmacological activities.^[13,14,17,41] In view of the aforementioned evidence and considering the fact that the plant is already used by human without any side effects, more studies should be carried out to determine the active constituents of the preparation, which may be responsible for its anti-inflammatory action in our studies. Overall, our studies provide strong evidence that hydroalcoholic extract of roots of *A. speciosa* effectively ameliorated acid aspiration-induced ALI in mice.

CONCLUSION

The present study shows that the hydroalcoholic extract of *A. speciosa* protects against HCl induced ALI potentially by curbing of oxidative stress and consequent activation of NF- κ B in the lung tissue. Given the known medicinal properties of the plant, the extract might exert beneficial effects in the humans prone to ALL.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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