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Silibinin extenuates arsenic instigated oxidative pulmonary damage and fibrosis in rats

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Abstract

Arsenic (As) and its compounds were widely used as a medicine in the past years for the treatment of such diseases as diabetes, psoriasis, syphilis, skin ulcers and joint diseases. Long-term exposure to arsenic from drinking-water and food can cause cancer and skin lesions. It has also been associated with cardiovascular, lung diseases and diabetes. Its exposure could cause severe oxidative stress and fibrotic injuries in lung tissue. Due to the antioxidant and anti-inflammatory properties of siibinin (SB), the present study investigated its effects on As-induced pulmonary toxicity. For the experimental study, twenty four male rats were randomly categorized into four groups of six. Initially, the first and fourth groups were treated intragastrically with normal saline and SB (80 mg/kg) for 28 consecutive days, respectively. The second and third groups were treated with As (5mg/kg BW) and As along with SB (80 mg/kg BW) for 28 consecutive days, respectively, At the end of the experimental tenure, the animals were anesthetized with ketamine and xylazine, and lung tissue samples were collected for biochemical and histological examinations. The results showed that As significantly increased hydroxyproline (HP) and lipid peroxidation (LPO) and decreased the lung tissue antioxidant capacity. In addition, myeloperoxidase (MPO) activity increased significantly, while glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) activity declined substantially. The administration of therapeutic doses of SB could prevent the oxidative, fibrotic, and inflammatory effects of As-induced lung toxicity, and these changes were consistent with histological observations. In conclusion, SB may improve the antioxidantdefense of lung tissue and prevent the spread of inflammation and the development of As-induced fibrotic injuries by enhancing antioxidant enzymes and preventing inflammatory cell infiltration.

Keywords: Arsenic**;** Silibinin; Oxidative stress; Pulmonary toxicity; Antioxidant

1 Introduction

Environmental or occupational or even accidental exposure to certain heavy metals can cause generation of reactive oxygen species (ROS) which are major causes of various diseases. Arsenic (Ar), a heavy metal is considered to be an oldest environmental toxin and recognized as a king of poison. It is also a renowned occupational toxin. The toxicity of As has been well documented in experimental animals and also in human beings (Bencko and Foong, 2017; Rahaman et al, 2013). This element is associated with a lot of adverse health effects. Studies revealed that As exposure causes generation of ROS and modification of antioxidant defense systems in animals and occupationally exposed workers. Production of highly reactive oxygen species (ROS), such as superoxide radicals ($O_{2^{-}}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-}) and lipid peroxides mediated by As ions are known to damage various cellular components including proteins, membrane lipids and nucleic acids (Sarkar et al, 2020). As enters into the body through various routes and through circulation it is carried to various organs and gets deposited mainly in the soft organs and bones. Oxidative stress involves an imbalance between oxidants and antioxidants (Lichtenberg and Pinchuk 2015) and resides

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in the high concentrations of oxidants which can damage cellular components including proteins, lipids, etc. It also causes severe physiological damage associated with abnormal cell function and even cell death (Raeeszadeh et al. 2022)

In recent years, lung has gained increasing attention as it is a uniquely susceptible target organ for orally ingested As. Several epidemiological studies reported the association between chronic As exposure and nonmalignant respiratory effects such as impaired lung function chronic obstructive pulmonary disease, pneumonia, bronchiectasis and so on (Wei et al., 2018). As directly induces the production of reactive oxygen species (ROS) leading to lipid peroxidation (LPO), causing oxidative damage (Manna et al., 2007; Rashid et al., 2013). People drinking As polluted water for a long time, increased MDA levels in the lung along with a decrease in glutathione (GSH) and catalase (CAT) activity, causing a striking imbalance of antioxidants pro-oxidants ratio and also induced DNA strand break, ultimately causing impaired lung functions (Yamanaka et al., 1989). Oral As ingestion significantly altered DNA methylation, causing expression in the modification of pulmonary genes (Andrew et al., 2007; Boellmann et al., 2010).

In recent years, plant bioactive compounds have received more attention as protective molecules in the field of pathophysiology. Stilbenoids are the major group of natural bioactive antioxidants due to their ability of inducing antioxidant enzymes gene expression as well as possessing various antioxidant properties like free radical scavenging, hydrogen donating, and so on (Muthumani and Miltonprabu,2012b). Silibinin has been shown to have immune-modulatory, anti-inflammatory, antiapoptotic and antioxidant activity. Several investigations revealed that SB can potentially modulate functions of organs and/or the immune system by regulating the NF-kB DNA binding activity in the therein. As induces oxidative stress through the generation of free radicals and SB have an antioxidant potential (Awanish et al, 2022). Present investigation is aimed at studying the protective effects of SB on As induced oxidative stress in the lung tissue of rats. Silibinin can be considered as a potent pyhtopharmaceutical entity for future drug development against heavy metal induced lung toxicity for individuals who get regularly exposed to toxic heavy metals like lead occupationally or environmentally.

2 Material and method

2.1 Animals

This study used 24 healthy adult male albino Wistar rats weighing 210 ±10 g. The animals were purchased from the Central Animal House, Faculty of Medicine, Annamalai University, Annamalai Nagar, Tamil Nādu, India. They were kept in well ventilated plastic cages under standard conditions, which included temperature (25±2°C), humidity (45-55%), and a 12 h light/12 h dark cycle and allowed food and tap water ad libitum in accordance with the Principles of Laboratory Animal Care approved by Annamalai University. All animal experiments were conducted in accordance with the Ethical Norms on Animal Care and use approved by the Institutional Animal Ethical Committee (IAEC) of Rajah Muthiah Medical College and Hospital (Reg. No.684/2010/CPCSEA), Annamalai University, India.

2.2 Chemical

Sodium arsenite (Na_2AsO_3), silibinin ($C_{25}H_{22}O_{10}$) were procured from sigma, MO, USA. All other fine chemicals, reagents and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

2.3 Experimental groups

Animals were acclimated to the laboratory environment for seven days prior to the study, and then twenty-four rats were randomly classified into four groups of six as follows:

- **Group 1:** animals received 1 ml/kg/day of normal saline for 28 days.
- **Group 2:** animals received sodium arsenite (5 mg/kg/day, PO) for 28 consecutive days.
- **Group 3:** animals received sodium arsenate (5 mg/kg/day, PO) and SB (80 mg/kg, PO) for 28 days.
- **Group 4:** animals received SB (80 mg/kg, PO) for 28 days.

Animals were anesthetized with ketamine and xylazine and underwent laparotomy at the end of the study. After separating and weighing the lung tissue, the right lobe was placed in 10% neutral buffered formalin (NBF) for histopathology studies. The left lung lobes were transferred to a -80°C freezer following quick freezing in a nitrogen tank.

2.4 Collection and analysis of BALF

The alveolar lavage fluid was collected as follows. First, the thorax was opened and after collection of the blood from the heart, the intact lung tissue was carefully stripped. The upper end of the trachea and the right bronchus were ligated with surgical thread, and the left lung of each rat was lavaged with 2 mL precooled saline, three times. BALF was collected and placed in a 10 mL centrifuge tube. It was then centrifuged at 4 ◦C and 1500 rpm for 10 min, and the acellular supernatant was collected, packed separately, and stored at − 80 ◦C. TP (Total protein), LDH (lactate dehydrogenases), ACP (acid phosphatase), and AKP (alkaline phosphatase) kits (Sigma,Mumbai,India) were used to detect protein contents and enzyme activity.

2.5 Lung tissue preparation

To prepare 10% (w/v) tissue homogenate, 100 mg of lung tissue was homogenized in 0.1 M phosphate buffer solution, and after centrifugation (14000 rpm for 10 min), the supernatant was collected and aliquoted in amounts of 0.5 ml to minimize intermittent freeze-thaw cycles and stored in a freezer at -80°C (30).

2.6 Hydroxyproline assay

Hydroxyproline (HP) content in the lung tissue was determined based on Woessner et al. method with some modifications (31). Briefly, 0.5 ml of each homogenous sample was acid hydrolyzed for 18 h in 3 ml of 3 M hydrochloric acid at 110 °C. Adding 1 ml of Chloramine T (0.5 M) reagent, the sample was incubated for 20 min at 37 °C. Afterward, 1 ml of 3.15 M perchloric acid and 1 ml of para-dimethylaminobenzaldehyde were added to each sample, followed by 10 min of incubation at 80 °C. Finally, absorbance was read at 550 nm using a microplate reader (Synergy HTX, Biotek, USA). Lung HP content was expressed as µg/g tissue.

2.7 Myeloperoxidase activity

Myeloperoxidase (MPO) activity in lung tissue was measured using a kit purchased from Sigma Company, Mumbai. This experiment is based on the production of hypochlorite and its reaction with taurine. After reacting with TNB chromogen to produce DTNB, its absorbance intensity was measured at 405 nm against a standard curve. Finally, the data were reported as enzyme unit/mg of protein.

2.8 Lipid peroxidation assay

The lipid peroxidation (LPO) assay is based on the reaction of active aldehydes with thiobarbituric acid (TBA), creating a pink complex (32). To prepare the TBA reagent (0.2%), 40 mg of thiobarbituric acid was dissolved with 20 ml of sulfuric acid (0.05 M). For 30 min, 50 µl of lung tissue homogenate was incubated at 90°C with 30 µl of TBA reagent. The light absorbance of the samples was measured at 532 nm against the standard curve prepared from different concentrations of 1,1,3,3-Tetramethoxypropane. The results were reported as nM/g of lung tissue.

2.9 Total antioxidant capacity

Total antioxidant capacity (TAC) was determined using the FRAP (ferric reducingantioxidant power) technique (33). This method is based on determining the ability of lung tissue to reduce Fe3+ to Fe2+ ions in the presence of TPTZ. To prepare the FRAP reagent, 25 ml of sodium acetate buffer (0.3 mM; pH 3.6) was mixed with 2.5 ml of 20 mM ferric chloride and 2.5 ml of 10 mM TPTZ. The solution was then incubated for 30 min at 37°C. The changes in absorbance of the blue complex caused by the interaction of Fe2+ and TPTZ were measured at 593 nm against the standard curve prepared from different concentrations of iron sulfate ($FeSO_4.7H_2O$). The data were expressed as nmole/mg of protein.

2.10 Glutathione peroxidase activity

The glutathione peroxidase (GPx) activity in lung tissue was measured using a kit purchased from Sigma Company, Mumbai. In this method, GSH is oxidized to GSSG by the GPX enzyme. Then, GSSG is reduced to GSH by glutathione reductase with NADPH consumption. The change of NADPH absorption at 340 nm was used to indicate GPx activity in this reaction. Finally, the results were expressed as enzyme unit/mg of protein.

2.11 Catalase activity

Lung tissue catalase activity was assayed by measuring the initial rate of hydrogen peroxide (H_2O_2) disappearance as explained by Shangari et al. (34). Briefly, 50 µl of homogenous lung tissue was mixed with 550 µl of phosphate buffer (0.05 M) and 400 µl of hydrogen peroxide (30 mM) at ambient temperature, and its absorbance changes were measured at 240 nm. Finally, the catalase activity was reported as enzyme unit/mg of protein.

2.12 Superoxide dismutase activity

The superoxide dismutase (SOD) activity in lung tissue was measured using a kit purchased from Sigma Company, Mumbai. The reduction of nitroblue tetrazolium by superoxide radicals produced in xanthine/xanthine oxidase systems and forming a Formazan red color were used to measure SOD activity. Afterward, the light absorbance was measured at 570 nm, and the results were reported as enzyme unit/mg of protein.

2.13 Protein assay

The Bradford method was used to determine the total protein content in supernatant prepared from lung tissue. This method measures the binding of protein molecules to Coomassie blue dye in acidic conditions. The sample absorbance was read at 595 nm against the standard curve made from various concentrations of bovine serum albumin (BSA).

2.14 Histological examination

After fixing the samples in 10% formalin, paraffin blocks were prepared and sliced to a thickness of 5-6 µm using a microtome. The tissue sections were stained with hematoxylin and eosin (H&E) and evaluated with an optical microscope (Olympus CX41) fitted with a digital camera (DP25, Olympus).

2.15 Statistical Analysis

The data were expressed as Mean ± SD. A one-way analysis of variance (ANOVA) test was conducted using SPSS software to analyze the data. Comparison among the groups was performed via Duncan's multiple range comparison test with Tukey's post hog analysis. P< 0.05 was considered statistically significant.

3 Results

3.1 Arsenic-induced cytotoxicity in lung tissue

The activities of LDH, AKP, and ACP in BALF are important indicators of cytotoxicity. Compared with the control group, the activities of LDH, AKP, and ACP in the BALF of arsenic poisoned rats were significantly increased. In addition, the concentration of TP was also increased only in the BALF of the As treated group, indicating bronchial epithelial cell injury and/or alveolitis (Table.1).

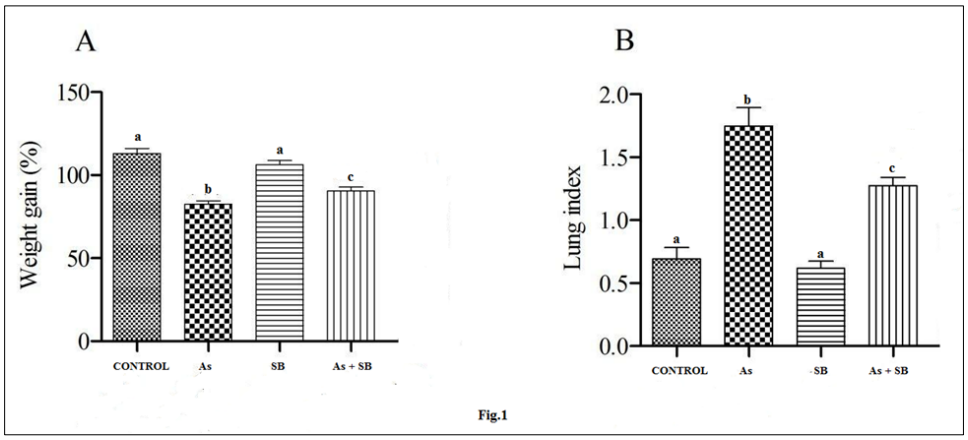
Table 1 Effect of SB treatment on lung cytotoxicity markers in As-treated rats

Groups	Control	Arsenic	Arsenic + Silibinin	Silibinin
LDH (IU/L)	1.42 ± 0.12^{a}	3.41 ± 0.27^{b}	1.34 ± 0.17^{c}	1.44 ± 0.11^{a}
AKP (IU/L)	1.96 ± 0.18^{a}	3.92 ± 0.32^{b}	2.14 ± 0.21^{c}	2.01 ± 0.16^{a}
ACP (IU/L)	1.78 ± 0.16^{a}	3.57 ± 0.39^{b}	1.89 ± 0.12^{c}	1.72 ± 0.14^{a}
TP (g/mL)	0.24 ± 0.06^{a}	0.37 ± 0.09^{b}	0.29 ± 0.04^{c}	0.25 ± 0.05^{a}

Values are expressed mean ± SD for six rats in each group; Superscript letters in a row not sharing a common superscript letters ($^{a, b, \text{and } c}$) differs significantly at $p < 0.05$ (DMRT).

3.2 SB effect on weight and pulmonary index

As shown in Figure 1A, the group receiving As experienced significantly less weight gain than the control group ($p<0.05$). The administration of SB at doses of 80 mg/kg ($p<0.05$) could considerably prevent the weight loss caused by As toxicity. As depicted in Figure 1B, the pulmonary index increased significantly in the As group compared to the control group ($p<0.05$). The administration of 80 mg/kg of SB considerably prevented the increased pulmonary index caused by As toxicity ($p<0.05$).

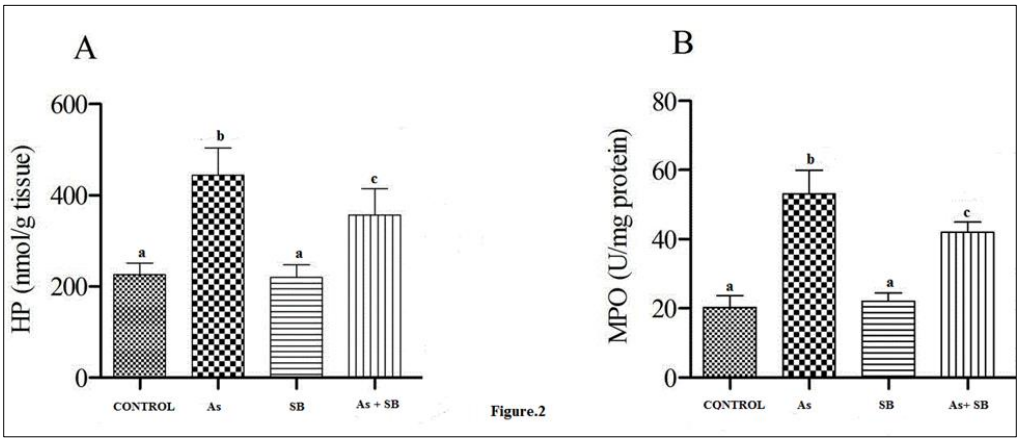


Data are expressed as mean ± standard deviation (SD) of six rats in each group. Statistical analysis used one-way ANOVA with Tukey's post hoc test (DMRT). Bars with different superscript letters differs significantly at P<0.05.

Figure 1 Effect of SB on weight gain and lung index in As-induced toxicity

3.3 SB effect on the pulmonary HP and MPO activity

As could increase HP ($p<0.05$) and pulmonary MPO activity ($p<0.05$) significantly compared to the control groups (Figures 2A and 2B, respectively). After administration of 80 mg/kg of SB, HP ($p<0.05$) and MPO ($p<0.05$) levels in the lungs decreased substantially compared to the As group. Our findings indicated that 80 mg of SB might be more effective in improving lung MPO activity and HP content.

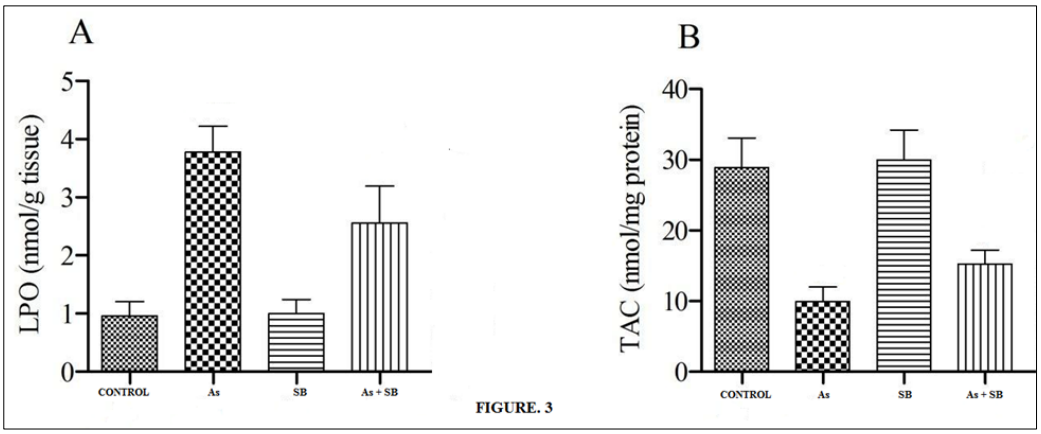


Data are expressed as mean ± standard deviation (SD) of six rats in each group. Statistical analysis used one-way ANOVA with Tukey's post hoc test (DMRT). Bars with different superscript letters differs significantly at P<0.05.

Figure 2 Effect of SB on pulmonary HP content and MPO activity in As -induced pulmonary toxicity

3.4 SB effect on the LPO and TAC levels in lung tissue

Compared to the control group, there was a significant increase in LPO (Figure 3A) and a decrease in TAC (Figure 3B) in the lung tissue of the As group ($p<0.05$). Administration of 80 mg/kg of SB were able to prevent the increase in LPO ($p<0.05$) and decrease in TAC ($p<0.05$) caused by As toxicity.

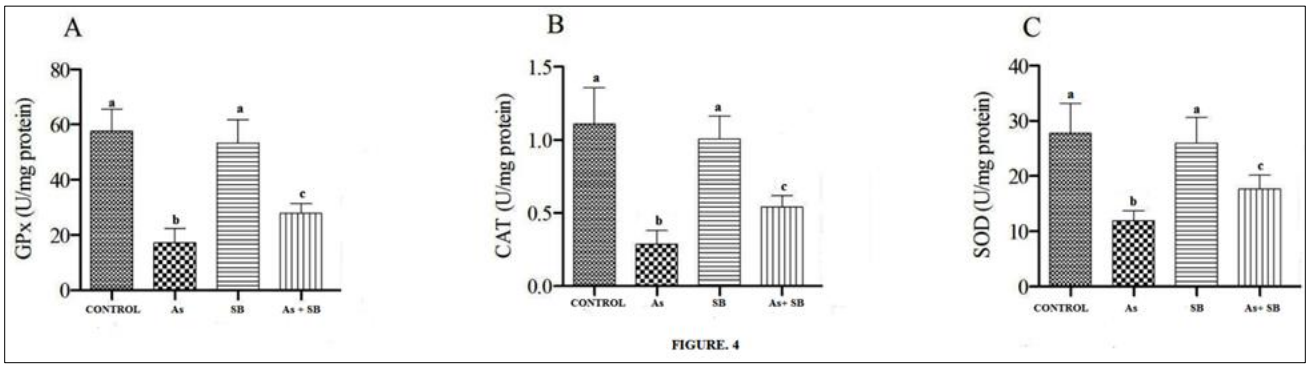


Data are expressed as mean ± standard deviation (SD) of six rats in each group. Statistical analysis used one-way ANOVA with Tukey's post hoc test (DMRT). Bars with different superscript letters differs significantly at P<0.05.

Figure 3 Effect of SB on pulmonary oxidative stress markers in As-induced toxicity

3.5 SB effect on antioxidant enzyme activity in lung tissue

As depicted in Figure 4, AS significantly reduced the enzyme activities of GPx ($p<0.05$), catalase ($p<0.05$), and SOD ($p<0.05$). Administration of 80 mg/kg of SB could prevent the inhibitory effects of As on the activity of cellular antioxidant enzymes.



Data are expressed as mean ± standard deviation (SD) of six rats in each group. Statistical analysis used one-way ANOVA with Tukey's post hoc test (DMRT). Bars with different superscript letters differs significantly at P<0.05.

Figure 4 Effect of SB on pulmonary antioxidant enzymes status in As-induced pulmonary toxicity

3.6 SB effect on lung tissue changes

Figures 5A show that the alveolar walls were thin, the alveolar space was large and completely normal, and most of the lungs in the control and SB groups consisted of alveolar spaces. There was no indication of fibrotic damage in either group (Figures 5D). In the group receiving As, lung tissue injury manifested as inflammation, increased interstitial connective tissue volume and alveolar wall thickness, and a decrease in the ratio of alveolar space to total lung volume and the estimated degree of fibrotic injury was 7-8 (Figure 5B). After administration of SB (80 mg/kg), inflammation and the thickness of the alveolar wall decreased significantly compared to the As group (Figure 5C), and the estimated degree of fibrotic injury was 3-4 (Figure 5C).

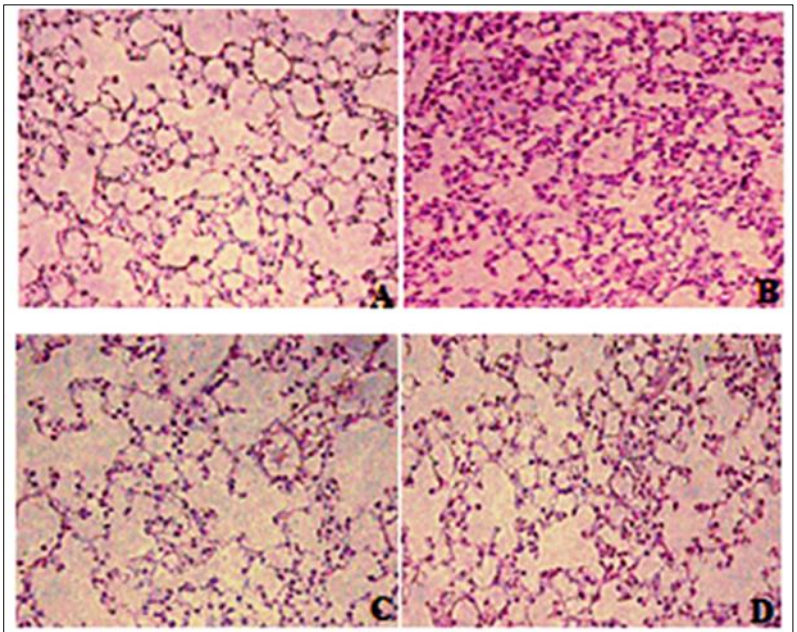


Figure. 5

The lung samples were stained using hematoxylin and eosin (20x, H&E). Group (A)&(D) showing the normal Alveoli, Group (B) As treated rats showing the ruptured alveolar wall, fibrotic areas with collagen fibers. Group (C) As+SB treated rats showing almost normal appearance of alveolar structure without any degenerative and inflammatory changes.

Figure 5 Light Photomicrographs of lung tissue in control and treated groups

4 Discussion

In the present investigation, administration of As resulted in oxidative and fibrotic lung tissue damage, and SB efficiently prevented lung injury caused by As toxicity. Body weight change is regarded as an essential indicator of toxicity in chronic toxicant exposure. Several studies demonstrated the relation between lung injury and an increase in the pulmonary index (Sanchez et al., 2016). In our experiment, increased weight ratio in the lung of As intoxicated rats indicated inflammation and increased water permeability. Analyzing H&E stained histological tissue sections; we found there were significant structural alterations in the As intoxicated lung like wide areas of lung collapse, increased thickness alveolar septum, increased inflammatory cell infiltration and hyperemia in the alveoli. In our study, As significantly decreased the body weight gain and increased pulmonary index in rats, which is in line with findings reported by Liu et al. (2020). Moreover, administration of SB can effectively prevent weight and pulmonary index changes caused by As toxicity.

Hydroxy proline (HP) is considered as an essential marker of fibrotic index and rises following the accumulation of collagen in the lung tissue. In the present study, pulmonary HP content was significantly increased due to As toxicity, corroborating with the previous findings (Mahalanobish et al, 2018; Liu et al 2020). Owing to high oxidative toxicity nature of As and the occurrence of inflammatory responses in the lung tissue, the proliferation of fibroblast cells increased, which led to an increase in the collagen synthesis process, expansion, and the accumulation of extracellular matrix proteins that ultimately lead to the development of fibrotic damages. The pulmonary HP content of animals exposed to As decreased significantly after the administration of SB, confirming with our histological findings. SB antifibrotic effects may be linked to the inhibition of inflammatory signaling pathways (Muthumani and Miltonprabu, 2012d&2014).

MPO is a marker of inflammatory processes, and its release and activity elevated by increasing the infiltration and proliferation of neutrophils and macrophages in tissues. In our study, the activity of pulmonary MPO increased significantly after chronic exposure to As, which is consistent with the findings of Mahalanobish et al, 2019. Increased activity of MPO leads to an elevated levels of hypochlorous acid that might play an imperative role in fibrosis progression by increasing the activation of growth factors such as TGF-β and matrix metalloproteinases (Ravichandran et al, 2011). The administration of SB could effectively prevent increased pulmonary MPO activity. Tian et al (2017) reported that SB suppressed the lung injury caused by LPS through lowering the levels of NLRP3 and NF-kB, followed by lowering the infiltration of immune cells such as neutrophils into the alveoli. Furthermore, SB might prevent the inflammatory

reactions by inhibiting the release of inflammatory cytokines such as TNF-α, IL-6, and IL1β, as well as inhibiting cyclooxygenase and preventing eicosanoids production.

In biological systems, oxidative stress is demarcated as an imbalance between free radical production and antioxidant defense, which can lead to the depletion of antioxidant reserves and destruction of cellular macromolecules. In this repute, free radicals lead to lipid peroxidation (LPO) and produce active aldehydes such as malondialdehyde. These by-products are used as efficient diagnostic biomarkers to assess the severity of oxidative damage (Muthumani and Miltonprabu, 2014b). In the present study, the administration of As significantly increased the LPO level and decreased the antioxidant capacity (Lowered CAT, SOD & GPx activity) of the lung tissue, confirming the findings of Wang et al. (2020). It has already been reported that As exposure caused the generation of excess ROS. This high oxygen pressure in the lung tissue caused the cationic form of As to transfer its unpaired electron to molecular O2 and produce superoxide radicals. These reactive oxygen radicals react with unsaturated fatty acids and destruct lung cell membranes by creating fatty acid hydroperoxides (Muthumani and Miltonprabu, 2012a). The administration of SB was able to prevent the increase in LPO and decrease in total antioxidant capacity (TAC) of lung tissue in rats exposed to As. It appears that a portion of the antioxidant properties of SB can be attributed to the hydroxyl groups present in its molecular structure. According to the evidence, SB inhibits cell oxidation by losing hydrogens and forming phenoxyl radicals (Misra et al, 2022, Surai,2015).

SOD, catalase, and GPx are the three crucial antioxidant enzymes for preventing oxidative damage resulting from reactive oxygen species. After an increase in superoxide anions, the SOD enzyme transforms these radicals into hydrogen peroxide. A portion of hydrogen peroxide is eliminated from the body via glutathione and GPx activity, and another is eliminated via catalase activity (Muthumani and Miltonprabu, 2012c). In the present study, there was a marked reduction in the activity of CAT, GPx, and SOD after the exposure of As. Administration of SB could increase the activity of SOD, catalase and GPx enzymes in in animals exposed to As. SB effects may be attributable to its direct influence on reducing the production of superoxide radicals. In addition, Guo et al (2020) reported that SB could enhance intracellular antioxidant defense by increasing the mRNA expression of Nrf2 mediated antioxidant signaling pathway and through which it might activated the antioxidant enzymes expression.

5 Conclusion

Our findings indicate that SB could prevent oxidative and fibrotic injuries induced by As in lung tissue by increasing the activity of antioxidant enzymes such as GPx, CAT and SOD. Therefore, clinical studies are recommended to confirm the use of SB as a therapeutic supplement in patients poisoned with Arsenic.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper

Statement of ethical approval

All animal experiments in the present study were conducted in accordance with the Ethical Norms on Animal Care and use, approved by the Institutional Animal Ethical Committee (IAEC) of Rajah Muthiah Medical College and Hospital (Reg. No.684/2010/CPCSEA), Annamalai University, India.

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