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Research article

EVALUATION OF THE ANTI-PROLIFERATIVE AND IMMUOMODULATORY EFFECT OF S-ALLYL CYSTEINE ON PULMONARY FIBROSIS INDUCED-RATS

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ABSTRACT: The present study aimed to evaluate the immunomodulatory effect of s-ally cysteine **(SAC)** on pulmonary fibrosis in **CCl_4**-induced rats. The animals divided into: group (I) served as control and its rats were administered olive oil. Rats of group (II) received **SAC**. Animals of group (III) were injected with **CCl_4** to induce pulmonary fibrosis. Rats of group (IV) treated with **CCl_4+SAC**. Lung tissues from **CCl_4**-injected rats revealed thickened alveoli septa, lymphatic nodules, marked increase in collagen fibres. Increase in the expression of α-smooth muscle actin (α-SMA) and glutathione S-transferase (GST). Proliferating cell nuclear antigen (PCNA) showed increase in the apoptotic activity reached 28.9%. **CCl_4** injection caused augmentation in INF-γ and serum cytokines. **SAC** administration may became a novel potential treatment for pulmonary fibrosis due to its anti-proliferative activity recorded 11.82%, which is possible mediated by induction of apoptosis, beside its inhibitory effect on GST, cytokines production, which thereby modulates collagen synthesis, and attenuated the pulmonary fibrosis.

Keywords: S-allyl sycteine, Pulmonary fibrosis, α-SMA, PCNA, INF-γ, Cytokines.

INTRODUCTION

Exposure to carbon tetrachloride **(CCl_4)** may occur by dermal contact with tap water; e.g., during bathing [1]. Surveys have found that about 99% of all groundwater supplies and 95% of all surface-water supplies contain **CCl_4** at a concentration of less than 0.5 µg/L. Exposure to **CCl_4** by ingestion may occur through consumption of contaminated drinking water or food. In a study of New Jersey tap water, the maximum monthly estimated concentration of CCl_4 was 7 µg/L [1], based on measurements by utilities [2]. Based on a typical **CCl_4** concentration of 0.5 µg/L in drinking water, daily consumption of 2 L of water by a 70 kg adult yields an estimated daily intake of about 0.01 µg/kg [1]. Exposure from contaminated food is possible, but it is not likely to be of much significance, because levels of **CCl_4** in most foods are below the limit of detection. In the U.S. Food and Drug Administration's Total Diet Study, **CCl_4** was detected in 41 of 1,331 samples (3%) of 37 food items [3]. Pulmonary fibrosis is characterized by excessive deposition of extracellular matrix proteins within the pulmonary interstitium. **CCl_4** administration has been demonstrated to cause injury to the lungs [4]. It is proven that **CCl_4** promotes injuries in these organs via oxidative stress by increasing the lipid peroxidation and lowering the endogenous antioxidant. Common diseases in the lung such as asthma, chronic obstructive pulmonary disease and cystic fibrosis had been demonstrated to share similar pathogenesis, that is, increased production of reactive oxygen species and this had been shown to correlate with the disease severity [5]. The mechanisms underlying the pathogenesis of pulmonary fibrosis involve multiple pathways, such as inflammation, oxidative stress, and developmental processes, which result in alveolar epithelial cell injury and fibroblast proliferation that consequently leads to abnormal deposition of extracellular collagen [6]. These inflammatory cells can synthesize and secrete various cytokines, chemokines, reactive oxygen species, and proteases, which can lead to aberrant fibro-proliferation and collagen production [7]. Disturbances in cytokine expression involved with the increase in free radical production. The enhanced production of free radicals and oxidative stress can also be induced by a variety of factors such as radiation or exposure to heavy metals and xenobiotics e.g., carbon tetrachloride [8]. Garlic oil has been used as medicine since the time of Hippocrates and Aristotle.

It has medical benefits and flavoring properties. Garlic, a member of allium vegetables, has been applied for medicinal uses from the ancient time [9]. Allium vegetables contained organosulfur compounds that have been found to be potentially preventive and therapeutic agents against cancers [10]. One of these compounds is S-allyl cysteine SAC, which is a water soluble compound as reported by Herman-Antosiewicz and Singh, [11]. Many studies evidenced that SAC is an anti-tumor agent against different human cancers such as breast cancer [12]. The major effects of SAC include induction of apoptosis, inhibition of proliferation and suppression of invasion and adhesion [13]. The majority of fibroblasts in alveolar septa are characterized by the presence of cytoplasmic bundles of microfilaments that contain cytoplasmic actin isoforms; these cells have been named contractile interstitial cells or V-type myofibroblasts. In the rat, they express desmin as intermediate filament protein [14]. In adult rat lung, alpha-smooth muscle actin (α-SMA) positive cells are located only at the alveolar ducts [15]. Mitchell et al. [16] hypothesize that the substantial increase in α-SMA containing cells in fibrotic regions of involved parenchyma after lung injury is responsible for the altered morphologic, biochemical, and mechanical properties. Glutathione S-transferases (GSTs) are known to function as cellular detoxifying enzymes by removing harmful metabolic byproducts that may result from a variety of biochemical reactions. As the cellular apoptotic pathway is expected to increase such byproducts, the selective enhancement of mRNA expression GSTs may be associated with the early signals of apoptosis in the regressing prostates [17]. Proliferating cell nuclear antigen (PCNA) is a 36-kDa DNA polymerase-delta auxiliary protein, which accumulates in the nucleus during S phase of the cell cycle. It is essential for cellular DNA synthesis and is also involved in DNA nucleotide excision-repair. It plays an essential role in the nucleic acid metabolism as a component of the replication and repair machinery [18]. Cytokines have been classified on the basis of their biological responses into pro- or anti-inflammatory cytokines, depending on their effects on immunocytes [19]. Cytokines act in networks or cascades. Major cytokines include the ILs (Interleukins), Growth Hormone, IFN (Interferons) and TNFs (Tumor Necrosis Factors-Alpha and Beta). Many of the cytokines act locally like autocrine hormones and their targets are cells of the same or similar type as the cytokine-producing cell. A characteristic that significantly differentiates some of the cytokines from hormones is the coupling of their activity to cell-cell interactions. The function of some cytokines such as IL-1, Il-2, IL-4, IL-5, IL-6 and IL-10 is closely associated with the interactions between B-cells and T-cells [20]. IL-2 is a critical linker between the innate immunity and adaptive immunity, capable of TH1 (T Helper type-1) differentiation and IFN-gamma release by T-cells and NK cells [21]. IFN-α, IFN-β and IFN-γ are produced in the area of infection during the early phase of immune response. IFN-α and IFN-β induce proliferation of NK-cells and stimulate innate and adaptive immune responses [22], that are specifically targeted to virus infections. Upon activation NK cells release IFN-γ, which activates macrophages to secrete cytokines that help to activate T-cells and promote the initiation of T-cell responses [23]. The present study aimed to evaluate the immunomodulatory mechanism of SAC on pulmonary fibrosis, induced by CCl_4 by investigating the histopathological picture of lung, determining α-actin smooth muscle, GST and PCNA in lung tissue and finally, measuring the levels of pro-inflammatory cytokines in blood samples.

MATERIAL AND METHODS

Reagents and Experimental Doses

CCl_4 and S-allyl-L-cysteine **(SAC)** were purchased from Sigma-Aldrich, USA. Pulmonary injury was induced experimentally by i.p. injection with 2.5% **CCl_4** dissolved in olive oil at a dose of 1 mg kg−1 Ganie et al., [24]. S-allyl-L-cysteine (≥98%) soluble in H_2O: >10 mg mL−1 according to the instructions. The dose of S-allyl cysteine (200 mg kg−1), was used in this study according to the Mizuguchi et al., [25]. Highly sensitive commercially available rat ELISA kits assay were purchased from R&D Systems (Minneapolis, MN, USA). Rat Quantikine IFN-γ (Cat. No. RIF00), IL-6 (Cat. No. PR6000B) and IL-2 (Cat. No. R2000).

Animals

Adult male albino rats of Wistar strain weighing 180-200 ±10 g used throughout this study were obtained from the Serum & Antigen Laboratories at Helwan, Egypt. The animals had access to food and water ad libitum. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 hr light and dark cycle.

Experimental Animals and Induction of Pulmonary Fibrosis

Rats were divided into four groups each containing six rats.

Group (I) Rats from this group received olive oil vehicle only at 1 ml/kg/day.

Group (II) Animals of this group received S-allyl cysteine **(SAC)**.

Group (III) Rats belonging to this group injected with **CCl_4** in olive oil vehicle only.

Group (IV) Animals of this group treated with **CCl_4+SAC** for fifteen days.

Histopathological Examinations

Lung tissue were collected from all dissected animals; lungs were perfused intratracheally with 10% neutral buffered formalin (NBF) and immersed in 10% NFB for fixation for histopathological studies and stained with haematoxylin and eosin and Masson trichrome stain for the demonstration of the fine collagen fibres.

Immunohistochemical Studies

10% neutral buffered formalin fixed, paraffin wax embedded blocks were selected from each group for immunohistochemical studies. Slides were deparaffinized, submerged in H_2O_2 for 10 min, then in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 10 min. Lung sections were incubated with mouse anti-α-SMA, GST and PCNA (Thermo, 1:50) primary antibody according to standard procedures for 30 min at room temperature. They were rinse with PBS, 3 times for 2 min. and incubated with rabbit anti-mouse IgG1-conjugated biotinylated secondary antibody for 20 min. They were further rinsed with PBS, and incubated with streptavidin-peroxidase for 10 min and washed. The color was developed using an AEC kit according to the manufacturer's directions (Zymed).

Counting PCNA-Positive Nuclei

Five sections of each experimental group were randomly selected. Five fields were randomly selected from each slide where ~ 400 nuclei were counted [26]. Every stained nucleus was considered positive, irrespective of intensity. Counting were independently scored by two observers. The percentage of positive stained cells was recorded as the PCNA labeling index (LI). Positive and negative cells were counted and the labeling index of cells immunostained for PCNA (PCNA-LI) was calculated using the following equation:

PCNA-LI = (+ nuclei/ total nuclei)*100.

Where: + nuclei are the no. of nuclei labeled with PCNA and total nuclei are the sum of the immunostained and non-immunostained within the same field.

Measurement of INF-γ, IL-6 and IL-2 Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples allowed clotting for 2 hours at room temperature before centrifuging for 20 minutes. Serum was removed and assessed immediately or aliquoted and stored samples at ≤ -20° C. The assay employs the quantitative sandwich enzyme immunoassay technique. An affinity purified polyclonal antibody specific for rat INF-γ, IL-6 and IL-2 has been pre-coated onto a microplate. Standards, Control, and samples are pipette into the wells and any rat INF-γ, IL-6 and IL-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat INF-γ, IL-6 and IL-2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat INF-γ, IL-6 and IL-2 bound in the initial step. The sample values are then read off the standard curve. The optical density was determined with a microplate reader at a wavelength of 450 nm. The values of plasma cytokines concentration were expressed as pg/ml.

Statistical Analysis

The values expressed as mean ± standard error (SE). The results were evaluated by using the SPSS, version 16.0 (SPSS, Chicago, IL), for windows and evaluated by one-way ANOVA followed by LSD comparisons were performed to assess the significance of the difference among various treated groups, with the significance level of $P<0.05$.

RESULTS

Histopathological Investigations:

Lung sections from control rat showed normal histological feature of pulmonary tissue, with normal thin septa and normal distribution of collagen fibers (Figures 1&2). Sections of lung from rat treated with **SAC** also revealed normal appearance of the cells and septa of the pulmonary tissue and normal distribution of collagen fibers (Figures 3&4). Lung sections from rats injected with **CCl_4** revealed many histopathological damage such as thickened alveoli septa containing inflammatory cells partially obliterating the air spaces, and increase in the mitotic activity (Figure 5), alveolar septa containing fibroblastic cells and thickened wall of blood vessel containing RBCs (Figure 6), thickened walls of pulmonary bronchiole, the pulmonary tissue stuffed with inflammatory cells predominantly lymphocytes as shown in figure (7). Thickened wall of the bronchiole and its lumen filled with cell debris (Figure 8), bronchiole with detached epithelium layer from the underlying muscle layer, alveolar macrophages, and RBCs (Figure 9).

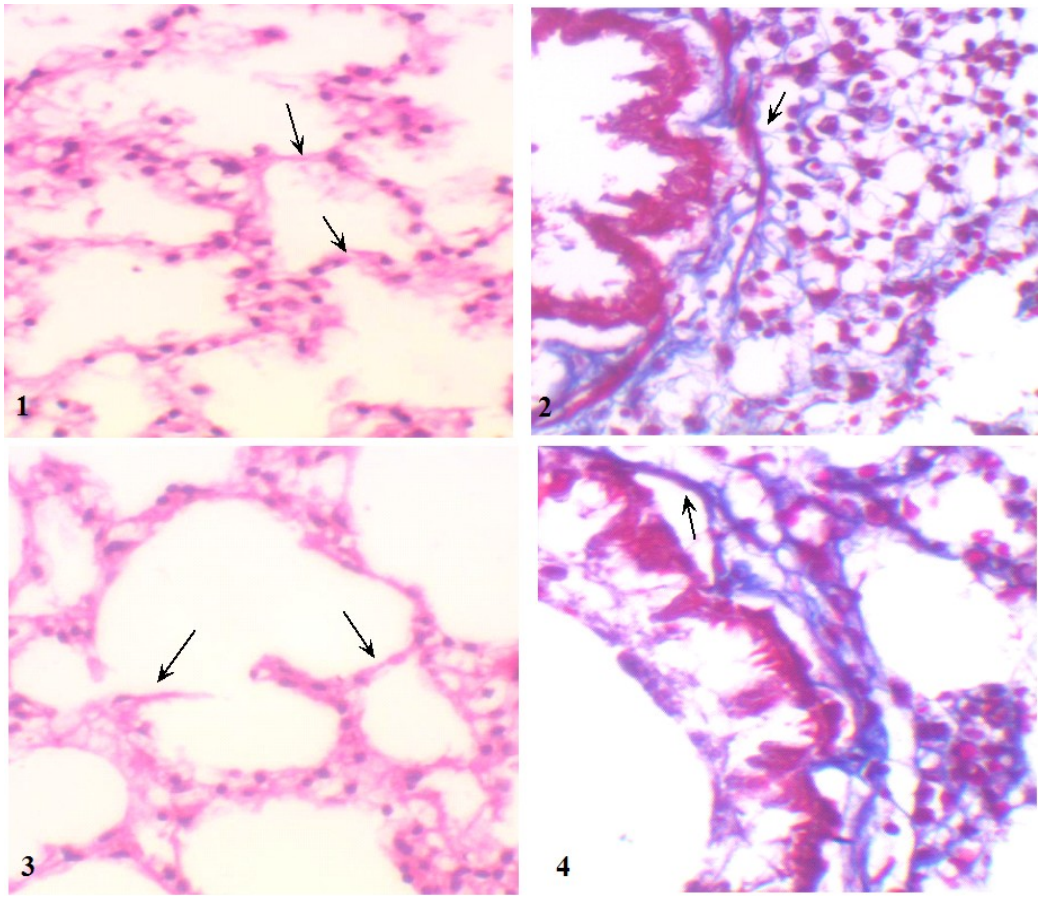

Figure 1: Photomicrograph of lung section from control rat showing normal histological structure of the pulmonary tissue with normal thin septa (arrows) (H-E, X400).
Figure 2: Photomicrograph of lung section from control rat showing normal distribution of collagen fibers (arrow) in pulmonary tissue around the bronchiole (Masson trichrome stain, X400).
Figure 3: Photomicrograph of lung section from rat treated with SAC showing normal appearance of the pulmonary tissue cells and septa (arrows) (H-E, X400).
Figure 4: Photomicrograph of lung section from SAC treated rat showing normal distribution of collagen fibers (arrow) in pulmonary tissue (Masson trichrome stain, X400).

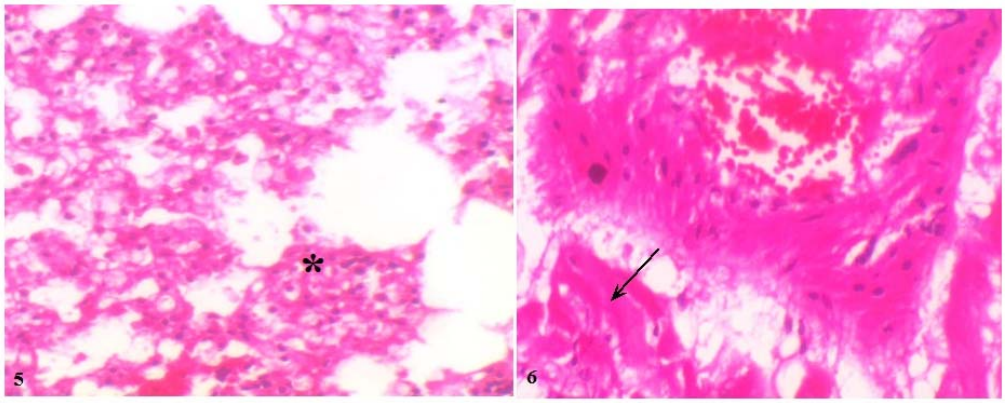

Figure 5: Photomicrograph of lung section from rat injected with CCl_4 showing alveolar septa became thickened, containing inflammatory cells; partially obliterating the air spaces (*) and increase in the mitotic activity. (H-E, X400).
Figure 6: Photomicrograph of lung section from CCl_4 injected rat showing alveolar septa containing fibroblastic cells (arrow), and thickened wall of blood vessel containing RBCs (H-E, X400).

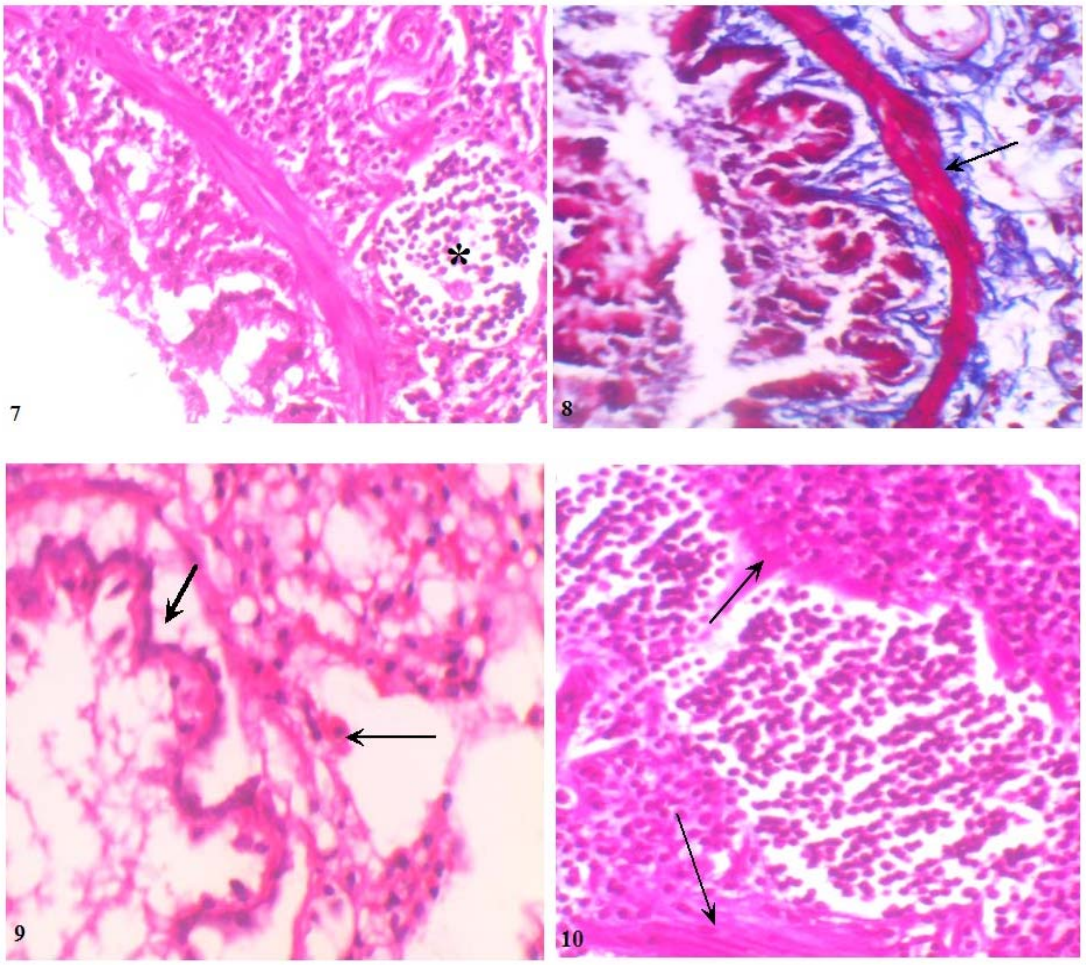

Figure 7: Photomicrograph from rat lung section after CCl_4 injection showing thickened walls of pulmonary bronchiole, the pulmonary tissue stuffed with inflammatory cells (*) predominantly lymphocytes. (H-E, X400).
Figure 8: Photomicrograph of lung section from CCl_4 injected rat showing thickened wall (arrow) of the bronchiole and its lumen filled with cell debris. (Masson trichrome stain, X400).
Figure 9: Photomicrograph of lung section from CCl_4 injected rat showing bronchiole with detached epithelium layer (thick arrow) from the underlying muscle layer, alveolar macrophages (thin arrows), and RBCs were visible. (H-E, X400).
Figure 10: Photomicrograph from rat injected with CCl_4 showing lymphatic nodules surrounded with collagen fibres (arrows). (H-E, X400).

Injection of **CCl_4** to rats caused the formation of lymphatic nodules in the pulmonary tissue surrounded with collagen fibres (Figure 10), fibrosis inside alveolar space which obscure the alveoli and increase in inflammatory cells (Figure 11), in addition to the increase in the content of collagen fibres deposited in the alveoli (Figure 12), and marked augmentation in collagen fibres deposited in the pulmonary tissue and dilated blood vessel which its lumen stuffed with RBCs (Figure 13), fibres distributed in the alveoli and Dilated blood vessel filled with RBCs and increase in the elastic fibres in the walls of pulmonary vein (Figure 14). The administration of SAC to CCl_4 injected rats ameliorate the diverse effects of **CCl_4**. Rat lung sections revealed near to normal alveoli with slight inflammatory cells appeared in the alveoli (Figure 15) and well defined bronchiole (Figure 16).

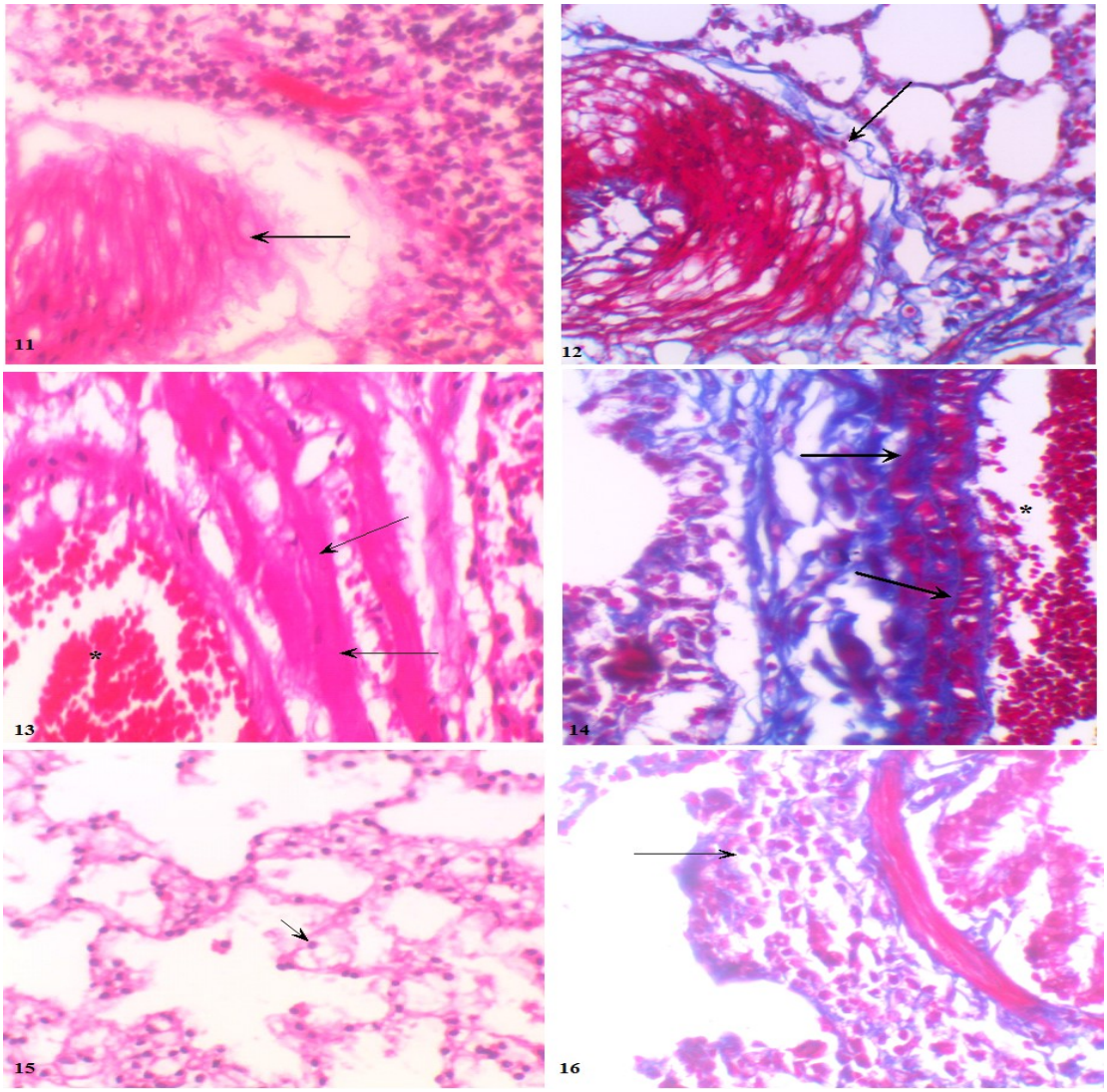

Figure 11: Photomicrograph of lung section from CCl_4 injected rat showing fibrosis (arrow) inside alveolar space which obscure the alveoli and increase in inflammatory cells (H-E, X400).

Figure 12: Photomicrograph from rat lung section injected with CCl_4 showing increase in the content of collagen fibres (arrow) deposited in the alveoli. (Masson trichrome stain, X400).

Figure 13: Photomicrograph of lung section from rat injected with CCl_4 showing marked augmentation in collagen fibres (arrows) deposited in the pulmonary tissue and dilated blood vessel which its lumen stuffed with RBCs (*). (H-E, X400).

Figure 14: Photomicrograph of rat lung section treated with CCl_4 showing collagen fibres distributed in the alveoli and Dilated blood vessel filled with RBCs (*) and increase in the elastic fibres in the walls of pulmonary vein (arrows) (Masson trichrome stain, X400).

Figure 15: Photomicrograph of rat lung section treated with CCl_4+SAC showing near to normal alveoli with few numbers of inflammatory cells (arrow) appeared in the alveoli. (H-E, X400).

Figure 16: Photomicrograph from rat lung section treated with CCl_4+SAC showing well defined bronchiole and decreased number of lymphocytes (arrow) still appeared. (Masson trichrome stain, X400).

Immunohistochemical Investigation

Lung sections from CCl_4 injected rats revealed an increase in the α-SMA expression in the pulmonary tissue as appeared around the bronchiole and alveolar thickened septa as shown in figures (19 & 20) as compared with normal control in figure (17) and SAC treated rat (Figure 18), which expressed only in the bronchial musculature. On the other hand, pulmonary tissue sections from CCl_4 injected rat treated with SAC showed inhibition in the expression of α-SMA (Figure 21).

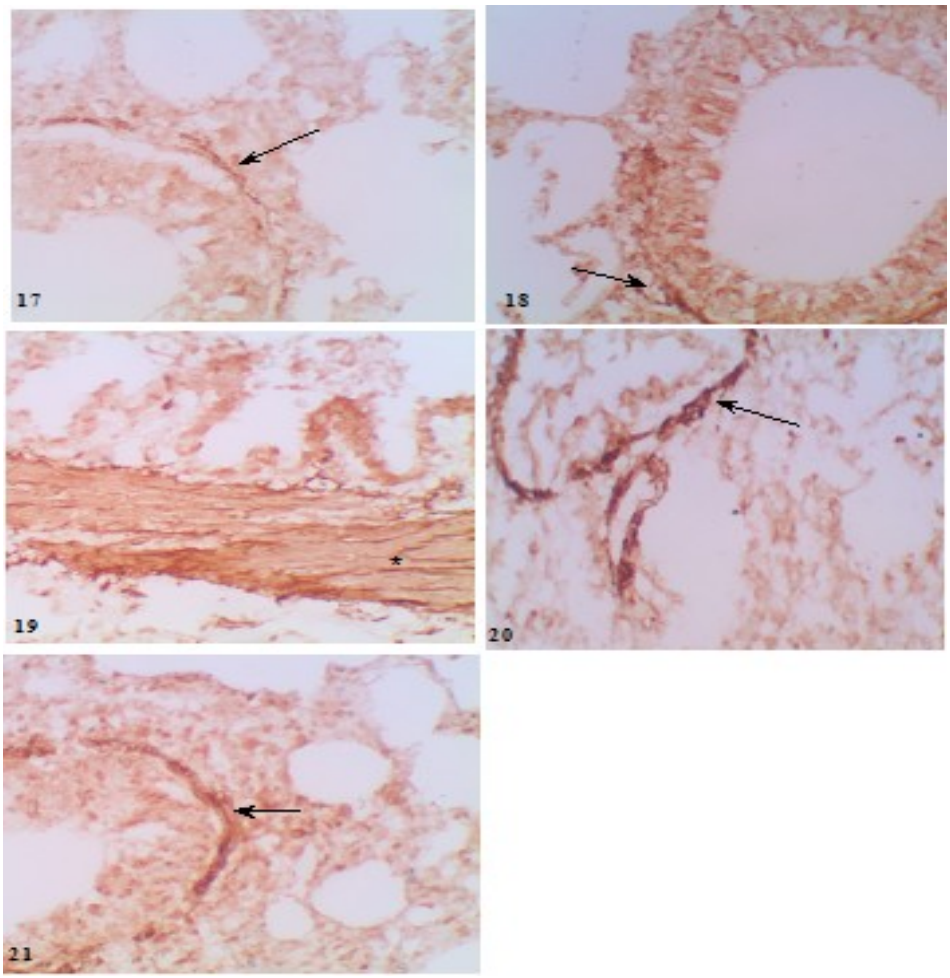

Figure 17: Photomicrograph of lung section from control rat showing expression of α-SMA only in the bronchial musculature (arrow) (Immunohistochemical stain, X400).
Figure 18: Photomicrograph of lung section from rat treated with SAC showing expression of α-SMA in the bronchial musculature (arrow) (Immunohistochemical stain, X400).
Figure 19: Photomicrograph of lung section from CCl_4 injected rat showing increase in the expression of α-SMA positive fibroblasts (*) in pulmonary tissue (Immunohistochemical stain, X400).
Figure 20: Photomicrograph of lung section from CCl_4 injected rat showing the thickened septa (arrow) with many α-SMA positive cells rich in α-SMA positive cells (Immunohistochemical stain, X400).
Figure 21: Photomicrograph of lung section from CCl_4 injected rat treated with SAC showing decrease in α-SMA expression (arrow) as compared with pulmonary fibrotic rat lung (Immunohistochemical stain, X400).

Lung sections from control rat and SAC treated rat revealed the normal cytoplasmic expression of GST in the pulmonary tissue (Figures 22 & 23). Lung sections from rat injected with CCl_4 showed decrease in the expression of GST in the bronchiole and thickened alveoli (Figure 24).

Interestingly the present study found that the supplementation with SAC for 15 days were able to maintain the GST expression towards the normal value, which revealed slight decrease in GST expression in the cells of the bronchiole and alveoli as compared with CCl_4 injected rat (Figure 25). Determination of PCNA positive cells in lung section from both control and SAC treated rats showed the normal levels of PCNA positive cells in brown of bronchiole and alveoli cells (Figures 26 & 27). Lung section from rat injected with CCl_4 delineated an increase in apoptotic activity (Figure 28). While, lung section from rat injected with CCl_4 and treated with SAC showing decrease in PCNA positive cells as compared with CCl_4 injected rat (Figure 29).

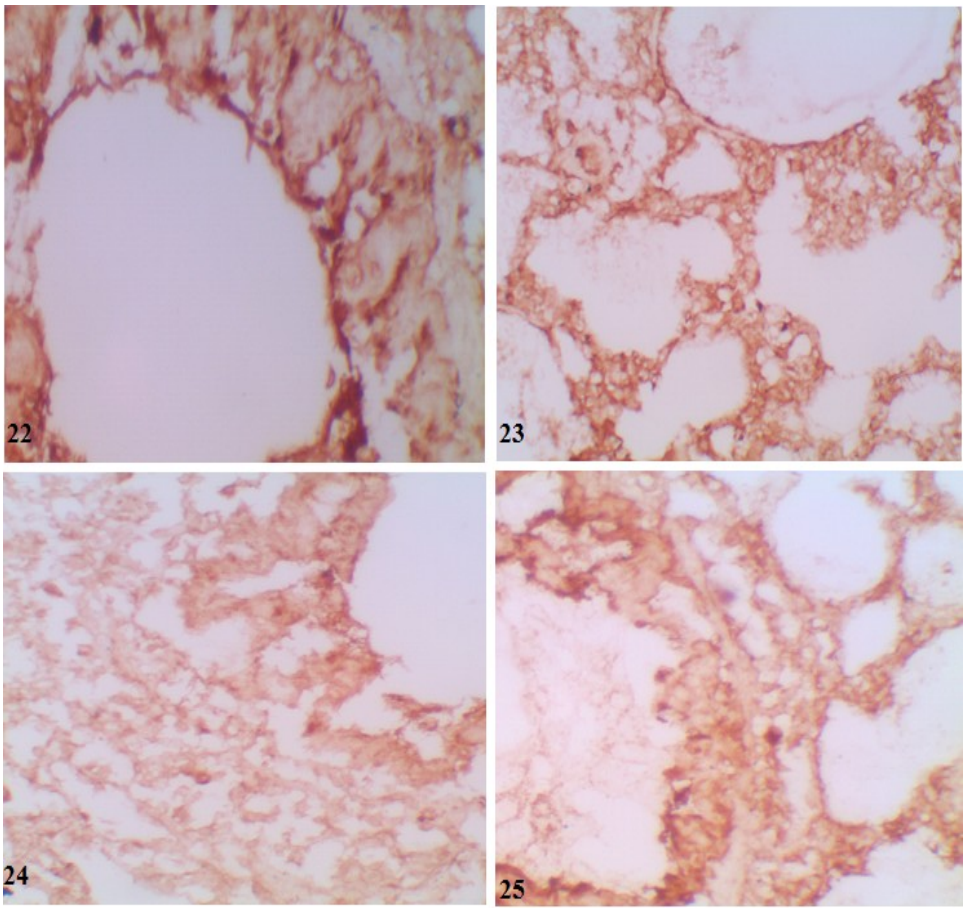

Figure 22: Photomicrograph of lung sections from control rat showing the normal cytoplasmic expression of GST in the pulmonary tissue (Immunohistochemical stain, X400).
Figure 23: Photomicrograph of lung sections from rat treated with SAC showing the normal cytoplasmic expression of GST in the pulmonary tissue (Immunohistochemical stain, X400).
Figure 24: Photomicrograph of lung sections from rat injected with CCl_4 showing decrease in the expression of GST in the bronchiole and thickened alveoli (Immunohistochemical stain, X400).
Figure 25: Photomicrograph of lung section from rat treated with CCl_4+SAC showing slight decrease in GST expression in the cells of bronchiole and alveoli as compared with CCl_4 injected rat (Immunohistochemical stain, X400).

The increased apoptotic activity of pulmonary tissue cells reported in CCl_4 injected rats, reached 28.9% as compared with control (4.95%) and SAC treated rats (5.2%). Decrease in PCNA-LI recorded (11.82%) in the pulmonary tissue from CCl_4 injected rats treated with SAC as shown in table (1) as compared with **CCl_4** injected group.

Table 1: Averages of PCNA-LI in Different Experimental Groups

Experimental Groups	C	SAC	CCl_4	CCl_4+SAC
Mean (+ve nuclei/~400 nuclei)	19.8±2.9	20.1±3.0	115.6 ± 7.8^{ab}	47.3 ± 4.9^{abc}
PCNA-LI (%)	4.95	5.2	28.9	11.82

Values are mean±SE. Superscript letters denote the significant difference at ($P<0.05$).

a: values are significantly different from control group. b: values are significantly different from **SAC** group.
c: values are significantly different from **CCl_4** group.

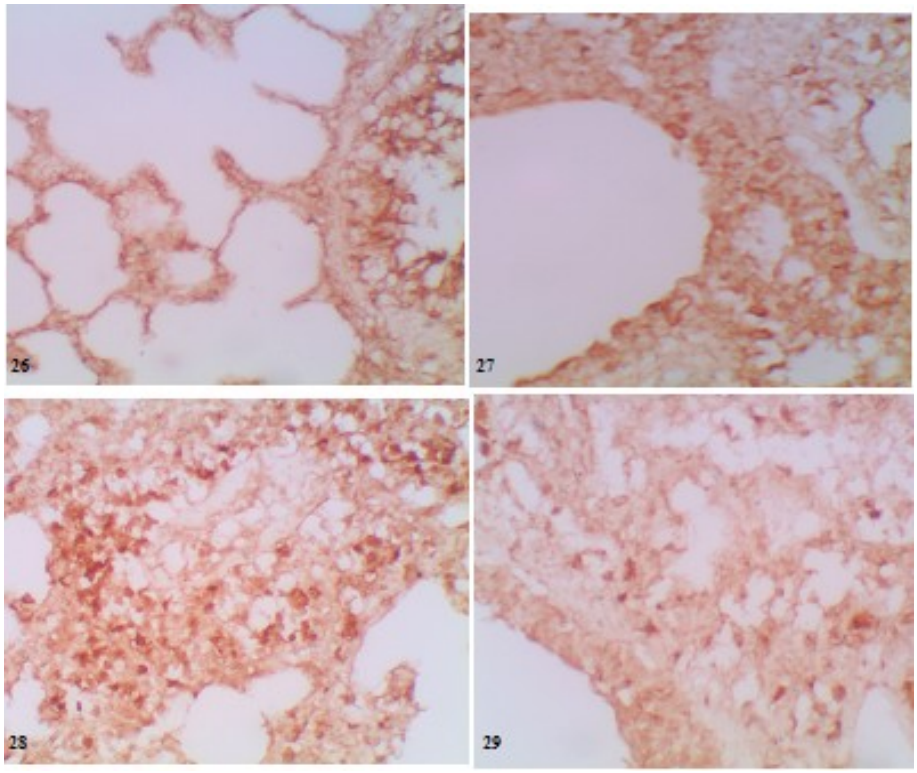

Figure 26: Photomicrograph of lung section from control rat showing the normal levels of PCNA positive cells in brown (Immunohistochemical stain, X400).
Figure 27: Photomicrograph of lung section from rat treated with SAC showing the normal levels of PCNA positive cells. The cells of bronchiole and alveoli showing slight immunopositive for PCNA. (Immunohistochemical stain, X400).
Figure 28: Photomicrograph of lung section from rat injected with CCl_4 showing increase in apoptotic activity as much numbers of cells stained in brown are appeared (Immunohistochemical stain, X400).
Figure 29: Photomicrograph of lung section from rat injected with CCl_4 and treated with SAC showing decrease in PCNA positive cells as compared with CCl_4 injected rat (Immunohistochemical stain, X400).

Cytokine Investigations

Inflammation is commonly associated with pulmonary fibrosis during exposure to CCl_4. To estimate the immunomodulatory effect of SAC and explore its mechanism in suppressing inflammation, the present study concerned about the investigation of INF- γ, IL-6 and IL-2 pro-inflammatory cytokines in serum. Detection of the cytokine INF- γ, IL-6 and IL-2 by ELISA, revealed that CCl_4 exposure elevated their concentrations relative to control group reached 177.1, 164.8 and 93.1 pg/ml respectively as recorded in table 2. SAC administration counteracted **CCl_4** effect on cytokine production where these levels recorded 133.6, 102.8 and 54.8 pg/ml for INF- γ, IL-6 and IL-2 respectively.

Table 2: Averages of Serum Cytokines in Different Experimental Groups

Experimental Groups	C	SAC	CCl_4	CCl_4+SAC
INF- γ	112.3 ± 6.8	111.8 ± 4.7	177.1 ± 9.6^{ab}	133.6 ± 6.1^{abc}
IL-6	80.0 ± 2.6	72.8 ± 2.3	164.8 ± 3.6^{ab}	102.8 ± 4.3^{abc}
IL-2	42.3 ± 2.4	42.6 ± 3.1	93.1 ± 3.6^{ab}	54.8 ± 2.1^{abc}

Values are mean±SE. Superscript letters denote the significant difference at ($P<0.05$).

a: values are significantly different from control group. b: values are significantly different from **SAC** group.
c: values are significantly different from **CCl_4** group.

DISCUSSION

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders characterized by the excessive deposition of extracellular matrix proteins within the pulmonary interstitium. There is good evidence that this process is driven by growth factors and cytokines produced by activated inflammatory and immune cells, which induce fibroblasts to proliferate or produce excess collagen [27]. The injection of CCl_4 to rats caused its distribution and deposition to organs such as the liver, lung, brain, kidney and heart [28]. The toxicity of CCl_4 probably depends on formation of the trichloromethyl radical (CCl_3•), which in the presence of oxygen interacts with it to form the more toxic trichloromethyl peroxyl radical (CCl_3O_2•) from the metabolic conversion of CCl_4 by cytochrome P-450as reported by [29]. These free radicals initiate the peroxidation of membrane poly unsaturated fatty acids, cell necrosis, GSH depletion, membrane damage and loss of antioxidant enzyme activity. The increased production of reactive oxygen species (ROS) that may be critical in producing pro-inflammatory eicosanoids that lead to pulmonary toxicity, and may eventually lead to lung fibrosis [30]. In recent literature, the presence of several ROS has been found in clinical cases of idiopathic pulmonary fibrosis [31], and decreased production of ROS has been shown to protect mice against pulmonary fibrosis.

The present study reported that CCl_4 caused many deleterious effects on the pulmonary tissue. This come in accordance with Pääkkö et al., [32] who reported that injection of CCl_4 caused lung injuries in rats such as acute haemorrhagic interstitial pneumonia; chronic interstitial pneumonia, and residua of injury or advanced chronic interstitial pneumonia and also exhibited features for diffuse alveolar damage and both interstitial and intra-alveolar fibrosis. The authors delineated that hydroxyproline content and the activities of prolyl hydroxylase and galactosyl hydroxylysyl glucosul transferase were elevated, which is the cause of an early onset of pulmonary fibrosis. CCl_4 is mainly metabolized by hepatic cytochrome P450, CCl_4 induced systemic inflammation and some organ fibrosis [25]. The extent of initial damage caused by free radicals is further amplified by Fenton reaction generated hydroxyl radicals in the presence of superoxide and hydrogen peroxide. Thus, the redox state and concentration of iron ions in the cellular milieu plays a crucial role in amplification of damage as they interact with membranes to generate alkoxyl and peroxyl radicals, thereby inflicting further damage to the cellular system [33].

On the other hand, SAC administration to CCl_4 injected rats revealed an improved histological picture in the pulmonary tissue. Ho et al. [34] reported the antioxidant properties of a garlic compound, SAC, and its ability to inhibit damage caused by oxidative stress. SAC exhibited dose-dependent inhibition of H_2O_2 formation. SAC exhibited dose-dependent inhibition of NF-kappaβ activation. Our data suggest that SAC may act via antioxidant mechanisms to inhibit the atherogenic process.

Again, Helen et al. [35] showed that administration of SAC to nicotine treated rats showed decreased concentrations of thiobarbituric acid reactive substances and hydroperoxides in liver, lungs, and heart as compared with the values found in rats treated with nicotine alone. The activities of catalase and superoxide dismutase increased. The levels of the antioxidants like vitamins A, C, and E in the liver and glutathione in all tissues increased significantly in SAC-treated fed rats. SAC significantly attenuated CCl_4-induced systemic inflammation and fibrosis of lung [25], by inhibiting the decrease of thiol levels, the increase of inducible nitric oxide synthase expression, the infiltration of leukocytes, and the generation of reactive oxygen species in lungs. These results indicated that SAC is more effective than other cysteine compounds in reducing CCl_4-induced lung injury, and might be useful in prevention of interstitial pulmonary fibrosis. The immunohistochemical staining of α–smooth muscle actin (α-SMA) showed marked increase in the expression of α-SMA positive myofibroblasts, filament-laden parenchymal cells typical of lung fibrosis in pulmonary tissue positive cells in CCl_4 injected rats. α-SMA is a differentiation marker of smooth muscle cells and is also present in a special type of fibroblast called myofibroblast [14]. Previous reports demonstrated that cells containing α-SMA are found in maturing alveolar interstitium [36]. Myofibroblasts expressing α-SMA are instrumental in wound contraction during normal wound healing [37]. α-SMA within myofibroblasts becomes organized in filamentous bundles, called stress fibers, that allow the retractile movement producing wound contraction [38]. Following the same line, Vyalov et al., [14] showed that α-SMA appeared in desmin-positive alveolar fibroblasts as early as 24 hours after intratracheal bleomycin instillation; the modulation of α-SMA in these cells was preceded by a lymphomonocytic infiltration of alveolar septa. Twenty-four hours to 3 days after bleomycin administration, a proliferation of alveolar myofibroblasts occurred. Fibrosis with laying down of collagen fibers took place after the above mentioned cellular modifications. Our results support the view that septal fibroblastic cells can modulate into typical α-SMA -containing myofibroblasts during experimental bleomycin-induced pulmonary fibrosis.

In such a modulation a possible role of cytokines, particularly of transforming growth factor-beta, is considered. In previous studies, claimed that the lymphomonocytic infiltrate is responsible for the local production of fibrogenic cytokines-8 that later on might mediate the collagen production [39]. The present study revealed **t**hat injection of CCl_4 caused decrease in GST expression in the bronchiole and thickened alveoli of the pulmonary tissue as a result of its oxidative stress**.** The importance of GSTs in the protection against oxidative stress in testis is underscored by recent studies showing that when GST activity is inhibited, products of lipid peroxidation accumulate, resulting in germ cell apoptosis [40]. Treatment with CCl_4 caused decreased levels of the reduced glutathione (GSH) as reported by Rajesh and Latha, [41]. In the lung homogenate GST activity of CCl_4 treated group was lower compared to that in the normal group [24]. Inflammatory lung diseases are characterized by chronic inflammation and oxidant/antioxidant imbalance, a major cause of cell damage. The development of an oxidant/antioxidant imbalance in lung inflammation may activate redox-sensitive transcription factors such as nuclear factor-kB, and activator protein-1 (AP-1), which regulate the genes for pro-inflammatory mediators and protective antioxidant genes. GSH is a vital intra- and extracellular protective antioxidant against oxidative/nitrosative stresses, which plays a key role in the control of pro-inflammatory processes in the lungs. Alterations in alveolar and lung GSH metabolism are widely recognized as a central feature of many inflammatory lung diseases such as idiopathic pulmonary fibrosis, acute respiratory distress syndrome, cystic fibrosis and asthma [42].

The stimulation of GST due to SAC administration in lung indicates that SAC can confer protection against the toxic effect of xenobiotics. GST a group of enzymes that play a major role in the detoxification pathway and help in the conversion of reactive chemicals to non reactive polar compounds which can be excreted from the body [43]. The GST activity was found to be increased in the lung tissue of rats treated with herbal aqueous extract of *Podophyllum hexandrum* for 15 days prior to CCl_4 treatment, which was close to the normal level in lung tissue [24]. Another Study also showed that Glycyrrhiza glabra herbal extracts could protect organs against CCl_4 induced oxidative stress by altering the levels of increased lipid peroxidation and enhancing the decreased activities of antioxidant enzymes, like GST as well as enhanced the decreased level of the reduced GSH as delineated by Rajesh and Latha, [41]. N-acetyl cysteine (NAC), a cysteine-donating compound, acts as a cellular precursor of GSH and on deacetylation becomes cysteine. It reduces disulphide bonds, but also has the potential to interact directly with oxidants. NAC, is also used as a mucolytic agent (to reduce mucus viscosity and improve mucociliary clearance). NAC has been used in an attempt to enhance lung GSH levels and reduce inflammation in patients with COPD and IPF with variable success [44]. The present study recorded increased PCNA-LI (28.9 % positive cells) in lung tissues following the injection of CCl_4. This come in accordance with Zhuo et al. [45] who found that the incidences of PCNA and p53 antigen positive hepatocytes, and the numbers of nuclear apoptotic bodies were markedly higher in the CCl_4-treated animals than in those protected with taurine.

The resulting cell death may be related to the inhibition of DNA synthesis or damage as a result of the free radicals. Apoptosis is a critical mechanism controlling cellularity in various tissues [46]. Again, Aoshiba et al. [47] showed that the chronic exposure to xenobiotics to rats caused increase in apoptosis and increased levels of Fas ligand expression, which may be one of the pathogenic mechanisms in rats. It is reported that inhaled toxic material such as silica and particle matters induce apoptosis of affected tissues, beside, the increased oxidative stress, mitochondrial dysfunction and mitochondrial cytochrome C release. On the other hand, PCNA-LI in rats injected with CCl_4 and treated with SAC recorded 11.82 %. This may be related to its immnunomodulatory effect by enhancing natural killer (NK) cell function in lung tissue, which considered as the first line of defense [26].

Detection of the cytokine INF- γ, IL-6 and IL-2, revealed that CCl_4 exposure elevated their concentrations relative to control group. SAC administration counteracted CCl_4 effect on cytokine production. IL-6 acts as a growth factor for mature B cells and induces their final maturation into antibody-producing plasma cells. It is involved in T cell activation and differentiation, and participates in the induction of IL-2 and IL-2 receptors expression. Some of the regulatory effects of IL-6 involve inhibition of TNF production, providing negative feedback for limiting the acute inflammatory response. Upregulation of IL-6 production has been observed in a variety of chronic inflammatory and autoimmune disorders such as type I diabetes [48], mesangial proliferative glomerulonephritis, and neoplasma such as renal cell carcinoma, multiple myeloma, lymphoma and leukemia [48]. IL-2 originally known as T cell growth factor (TCGF). It is secreted mainly by activated T helper cells. It acts as a growth factor/activator for T cells, NK cells, and B cells [49].

It therefore plays a critical role in regulating both cellular and humoral chronic inflammatory responses. Binding of IL-2 to the IL-2 receptor on T lymphocytes leads to cell proliferation, increased lymphokine secretion (INF-γ, lymphotoxin, IL-4, IL-3, IL-5). The interferons are a group of cytokines originally identified by their antiviral activity. Type I interferons include INF-α, a product of leukocytes, and INF-β, a product of fibroblasts. They exhibit antiviral as well as anti-proliferative properties. Type II interferon, is an immune interferon or INF-γ, produced by activated T cells and NK cells cytotoxicity [50]. INF-γ is known to stimulate many of the effector functions of mononuclear phagocytes. While INF-α and –β bind to a common receptor, INF-γ recognize a distinct and specific cell surface receptor. INF-γ has been implicated in the pathogenesis of a variety of autoimmune and chronic inflammatory conditions [51]. Inflammation is commonly associated with fibrogenesis [52]. CCl_4 is metabolized in the liver by cytochrome P450 into the free radical CCl_3. The free radical attacks cells and causes necrosis, which promotes inflammatory responses in the liver. Pro-inflammatory IFN-γ, and IL-6 are major players in hepatic inflammation. Concentrations of serum TNF-α and IL-6 are remarkably increased in patients with alcoholic cirrhosis [53]. IFN-γ plays a crucial role in modulating immune responses. The level of IFN-γ was positively correlated fibrogenesis [54]**.** The innate cellular response was preceded by an increase of pro-inflammatory cytokines IL-1α, IL-1β, IL-6, CINC-1, and GM-CSF in BALF 1-2 days post-exposure. Consistent with our observations of the subsequent recruitment of inflammatory cells to the airways, the expression of these cytokines in concert provides strong signals for neutrophil chemoattraction [55], activation of the Th17 pathway [56]. Previous studies in other rat strains have shown dose-dependent transient increases in granulocytes and monocytes in the lungs, as well as epithelial and fibro-proliferative changes upon challenge with titanium dioxide ($TiO_{2)}$ [57,58].

Certain triggers (key toxins) can trigger the switching of cytokine production from TH-2 anti-inflammatory cytokines to the pro-inflammatory cytokines. These TH-1 cytokines in turn initiate the production of IL-2 and interferon γ. These cytokines prompt phagocytes to produce oxygen rich species (ROS) and nitrogen rich species (NOS). These reactive oxygen species initiate the production of a further cytokine, tumour necrosis factor α (TNF-α) which further changes the cell function by interfering with mitochondrial oxidative phosphorylation by upsetting the enzyme NADPH oxidase, the rate-controlling enzyme of the hexose monophosphate shunt, and stimulates nitric oxide synthase, producing further nitric oxide [59]. The present study revealed the immunomodulatory effect of SAC which appeared by its inhibitory effect on pro-inflammatory cytokines production investigated in this work. These results indicated that SAC suppressed inflammation caused by CCl_4, which might lead to the protection of the lung from injury. Following the same line, Fu et al. [54] reported that curcumin might protect the liver against CCl_4-induced injury by suppressing inflammation by reducing the levels of the pro-inflammatory cytokines IFN-γ, and IL-6.

CONCLUSION

Collectively, these findings indicate that the S-allyl sycteine **(SAC)** has an anti-proliferative activity, which is possible mediated by induction of apoptosis and alterations of the cell cycle, beside its inhibitory effect on pro-inflammatory cytokines production, which thereby modulates collagen synthesis and so attenuated the pulmonary fibrosis as shown by reversed histopathological changes.

There is a variety of pharmacological agents that have been proposed as therapies for pulmonary fibrosis; however, there are currently no effective treatments [60]. Corticosteroids and cyclophosphamide are widely used as standard protocols of current therapy in idiopathic pulmonary fibrosis, but the effects are not optimal for universal use [60]. In addition, corticosteroids block a broad array of pathways and thereby cause several side effects, including an increased risk of infection. In the present study, our findings indicate that **SAC** may become a novel potential treatment for pulmonary fibrosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

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