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EFFECT OF COMBINATION OF METHANOLIC EXTRACT OF *GLYCYRRHIZA GLABRA* ROOTS AND *CITRUS LIMON* LEAVES AGAINST SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

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Keywords:

Silicosis, Respirable Crystalline Silica (RCS), Pirfenidone, Pulmonary oedema, Pulmonary fibrosis, Oxidative stress

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ABSTRACT: Aim & Background: Silicosis is a prevalent occupational lung disease caused by inhalation of Respirable Crystalline Silica (RCS), leading to irreversible pulmonary fibrosis and restrictive lung disease. This study investigates the therapeutic potential of a combination of methanolic extracts from *Glycyrrhiza glabra* (licorice) root and *Citrus limon* (lemon) leaf (CMEGC) against silica-induced silicosis in *Wistar* rats, leveraging their known antioxidant and anti-inflammatory properties. **Methods:** The experimental protocol involved dividing rats into six groups, including a control group, a silicosis model group, a Pirfenidone-treated group, and groups treated with the combined extracts at two different dosages (200 mg/kg and 400 mg/kg). Silicosis was induced through aerosol inhalation of SiO_2. Various parameters, including body weight, hematological markers, pulmonary edema, TNF-α levels, oxidative stress markers, and lung collagen content, were assessed to evaluate the efficacy of the treatments. **Results & Conclusion:** Results indicated that the combined extracts significantly improved body weight and reduced pulmonary edema, TNF-α levels and oxidative stress markers compared to the silicosis model group. Histopathological analysis confirmed reduced lung inflammation and fibrosis in treated groups. The study concludes that the combination of *Glycyrrhiza glabra* and *Citrus limon* extracts holds promise as a potential therapeutic strategy for silicosis, warranting further investigation and development.

INTRODUCTION: Silicosis is the most common occupational lung disease in the world, caused by inhalation of Respirable Crystalline Silica (RCS) and is characterized by gradual, irreversible pulmonary fibrosis that eventually results in restrictive lung disease [1, 2].

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Silicosis poses a major risk to those employed in the mining and construction sectors [3, 4]. X-rays are not a viable way to diagnose silicosis in its early stages, despite the fact that the disease is diagnosed based on radiologic findings and occupational history [5].

Inhaling small silica dust particles causes them to deeply enter the lung's tiny alveolar sacs and ducts. Reactive oxygen species (ROS), which are produced in huge quantities when alveolar macrophages activate in reaction to foreign material, directly harm the lung parenchyma

around the alveolar macrophages [6-8]. Pro-inflammatory cytokines including IL-1β, IL-4, and TNF-α are released, and cell signaling pathways are enhanced, as a result of cellular damage and increased production of inducible Nitric Oxide Synthase (iNOS) in the bronchoalveolar. When these elements come together, lung tissue exhibits abnormal fibroblast growth and collagen deposition [4,8]. In silicotic models, pirfenidone and nintedanib have demonstrated a good track record of treating idiopathic pulmonary fibrosis, and the FDA has granted licenses for their use [9]. Tetrandrine improves lung function by lowering inflammation and lung fibrosis [10, 11]. Furthermore, it has also been demonstrated that silica-induced pulmonary inflammation and fibrosis in silicosis models can be inhibited by other potential drugs, including anti-cytokines and antioxidants, agents that affect the autophagic-lysosome system and the agents that increase cAMP levels, and microRNAs [12].

Licorice is one of the best herbal remedies for lowering toxicity and enhancing the potency of other herbal remedies when taken in combination, as per the traditional Chinese medical concept that "nine out of ten formulae contain licorice" [13]. And according to pathogenesis of silicosis, oxidative stress and inflammatory response are the two main factors for lung injury, so, the *Glycyrrhiza glabra* was combined with *Citrus limon*, having a good antioxidant and anti-inflammatory property. Thus, the current study demonstrated the effect of combination of methanolic extract of *Glycyrrhiza glabra* root and *Citrus limon* leaf against SiO_2 induced silicosis in *Wistar* rats.

MATERIAL AND METHODS:

Chemicals: Silicon dioxide (SiO_2) powder was purchased from Akshar Exim Co. Pvt. Ltd. Pirfenidone was obtained as a commercial tablet (Pirfenex, Mfd. by CIPLA LTD, Sikkim, India). All chemicals used in this study were of the highest grade.

Plant Material: The roots of *Glycyrrhiza glabra* and the leaves of *Citrus limon* were collected from local sources, Vadapalani, Chennai district, Tamil Nadu during December. The plant material was taxonomically identified and authenticated by Siddha Central Research Institute, Arumbakkam, Chennai-600106.

Experimental Protocol:

Sample Preparation and Extraction:

***Glycyrrhiza glabra*:** The roots were cleaned, air-dried at room temperature, and then ground into coarse powder using a mechanical grinder. This powder was extracted with methanol using a Soxhlet apparatus for 12 hours. The methanol extract was concentrated to yield a solid mass, which was then collected, dried, and stored at 4°C for future use. The percentage yield of the extract was found to be 11.92% w/w.

***Citrus limon*:** The roots were cleaned with water, air-dried at room temperature, and ground into powder using a mechanical grinder. This powder was mixed with methanol (1g in 10ml) and stirred for 24 hours. The mixture was then filtered through Whatman No. 1 filter paper. The filtrate evaporated at 35°C using a rotary evaporator under reduced pressure. The resulting dry extract was stored at 4°C for future analysis. The percentage yield of the extract was found to be 11.76 % w/w.

Preliminary Phytochemical Analysis: The methanolic extract of *Glycyrrhiza glabra* roots and *Citrus limon* leaves was subjected in a different test tube to preliminary phytochemical screening for the presence or absence of phytoconstituents in accordance with procedure given by Egbuna C *et al*., Phytochemical test methods published in 2018 [14].

Acute Toxicity Study: Acute oral toxicity studies for combination of methanolic extract of *Glycyrrhiza glabra* and *Citrus limon* were conducted as per OECD guidelines 423 (Acute toxic class method).

Animal Grouping and Treatment: Animals were housed in C. L. Baid Metha College of Pharmacy, Thoraipakkam, Chennai-97. All experiments were carried out according to the guidelines for care and use of experimental animals approved by CPCSEA guidelines. The study was approved by Institutional Animal Ethics Committee (IAEC). IAEC Reference no: 04/321/PO/Re/S/01/CPCSEA/dated 24/11/23 valid up to 25/11/24.

Animals were divided into six groups at random (n=6 per group): (i) Control group; (ii) Silicosis was induced by aerosolic inhalation of SiO_2 - 50mg/kg for 4 weeks; (iii) Silicosis was treated by

Pirfenidone (100mg/kg orally) for 35 days from day 11-45; (iv) Silicosis was treated by combination of methanolic extract of *Glycyrrhiza glabra* root and *Citrus limon* leaf extract (200/kg) for 35 days from day 11-45; (v) Silicosis was treated by combination of methanolic extract of *Glycyrrhiza glabra* root and *Citrus limon* leaf extract (400mg/kg) for 35 days from day 11-45.

The silica suspension (1mg/ml) was prepared and was induced in rats of all the groups by aerosolic inhalation method. And on day 45, all the animals were weighed and then the blood was collected through tail vein for hematological parameters. Afterwards, animals were sacrificed using chloroform and their lungs were rapidly excised for further analysis.

Parameters Assessed:

Body Weight Determination: The body weight of each animal in each group was noted on day 0, 7, 14, 21, 28, 35, 45.

Hematological Analysis: Blood samples in each group of an animal was collected on day 45 and hematological parameters were determined by routine hematological method with a hemocytometer.

Estimation of Total Protein in Serum: Blood samples in each group of an animal was collected on day 45, in a clot activating tube and allowed to clot by leaving undisturbed at room temperature for 15 min. And then the blood was centrifuged at 2,000 x g for 10 minutes in a centrifuge. The resultant supernatant was designated as serum. Then the total protein in serum was determined.

Evaluation of Pulmonary Oedema: To evaluate pulmonary oedema, the weight of the lung in each group of animals was taken.

Determination of TNF-α in Tissue Homogenates: TNF-α ELISA Kit was used to determine the levels of TNF-α in rat lung tissue of all the 5 groups following instructions of the manufacturer.

Evaluation of Oxidative Stress: Rat lung tissue was homogenized, centrifuged at 4^{o}C at 12,000 x g for 15 min and the supernatant was extracted. The levels of Malondialdehyde (MDA) and the activities of Superoxide dismutase (SOD) in the homogenate were determined using commercial kits (MDA assay kit & T-SOD assay kit) as per the manufacturer's instruction.

Measurement of Hydroxyproline and Collagen Contents in Lungs: To assess collagen deposition in the lungs, hydroxyproline content was measured. A lung sample was soaked in 5% KOH overnight at 37°C, then hydrolyzed with 10N NaOH and incubated with Chloramine-T for 3 hours at 25°C. Ehrlich's reagent was added to the mixture, which was then incubated for 20 minutes at 60°C. The resulting color's absorbance was measured spectrophotometrically at 550 nm.

Nitric Oxide Content in Tissue Homogenates: Nitric oxide content was measured in tissue homogenates. Lung homogenates were treated with NaOH and incubated for 5 minutes at 25°C. ZnSO4 was added for deproteination, and the mixture was centrifuged. A portion of the resulting supernatant was mixed with VCl3 and Griess reagent. After a 45-minute incubation at 37°C, the samples were analyzed spectrophotometrically at 540 nm.

Histopathological Analysis: To assess pulmonary inflammation and alveolar structure in rats and evaluate the success of the silicosis model, the lung weight was measured to calculate the lung organ coefficients.

A uniform sample from the right upper lung was fixed in 4% paraformaldehyde. After dehydration, the tissue was embedded in paraffin wax and sliced into 4 µm thick sections for Hematoxylin & Eosin (H&E) staining.

Statistical Analysis: Appropriate descriptive and analytical statistics were used. The analysis was done using Graph pad Prism 10 software. The values were expressed as mean ± SEM from all 6 animals in each group. Further to find the difference between groups statistical analysis using one-way ANOVA followed by Dunnet's "t" test was used. $P<0.05$ was considered as significant.

RESULTS:

Comparison:

1. Group I vs Group II, Group III, Group VI and Group V [considered as a].

2. Group II vs Group III, Group VI and Group V [considered as b]

3. Group III vs Group IV and Group V [considered as c]

The Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's 't' test, ns – non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

TABLE 1: EFFECT OF CMEGC ON BODYWEIGHT IN SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

Groups Treated	Body Weight (gm)			
	Day 0	Day 14	Day 28	Day 45
Group-I	231.0±1.00	236.0±1.00	243.0±1.00	248.0±2.00
Group-II	202.0±2.00 a***	181.5±1.50 a****	162.0±2.00 a****	137.5±2.50 a****
Group-III	211.0±1.00 a** b^{ns}	221.5±1.50 a** b****	227.0±1.00 a** b****	253.0±3.00 a^{ns} b****
Group-IV	232.5±2.50 a^{ns} b*** c**	237.0±1.00 a^{ns} b**** c**	241.5±1.50 a^{ns} b**** c**	263.5±0.50 a* b**** c^{ns}
Group-V	212.0±2.00 A** b^{ns} c^{ns}	242.5±2.50 a^{ns} b**** c**	246.5±0.50 a^{ns} b**** c***	271.5±1.50 a** b**** c**

TABLE 2: EFFECT OF CMEGC ON TOTAL WBC COUNT AND TOTAL PROTEIN IN SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

Groups Treated	Total WBC Count (cells/mm^3)	Eosinophil (%)
Group-I	8538 ±37.50	3.75±0.05
Group-II	15290±100.0 a****	7.70±0.1 a****
Group-III	10220±60.00 a* b***	5.00±0.1 a** b****
Group-IV	13500±500.0 a*** b* c***	5.90±0.1 a**** b*** c**
Group-V	11585±45.00 a** b*** c*	5.20±0.1 a*** b**** c^{ns}

TABLE 3: EFFECT OF CMEGC ON TOTAL PROTEIN IN SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

Groups Treated	Total Protein (gms/dl)
Group-I	6.100±0.1000
Group-II	9.050±0.1500 a**
Group-III	7.700±0.2000 a** b*
Group-IV	7.200±0.1000 a* b** c^{ns}
Group-V	6.550±0.2500 a^{ns} b***c*

TABLE 4: EFFECT OF CMEGC ON PULMONARY OEDEMA IN SIO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

Groups Treated	Pulmonary Oedema (Weight of the lungs in g)
Group-I	2.470±0.02
Group-II	4.625±0.15 a****
Group-III	2.625±0.15 a** b****
Group-IV	2.945±0.15 a**** b**** c***
Group-V	2.880±0.01 a**** b****c***

TABLE 5: EFFECT OF CMEGC ON TNF-A IN SIO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

Groups Treated	TNF-α (pg/mg)
Group-I	281.0±2.00
Group-II	576.5±2.50 a****
Group-III	319.0±1.00 a*** b****
Group-IV	348.0±2.00 a**** b**** c***
GROUP-V	294.5±1.50 a* b**** c**

TABLE 6: EFFECT OF CMEGC ON MDA, SOD AND NITRIC OXIDEIN SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

Groups Treated	MDA (nmol/mg)	SOD (pg/mg)	Nitric oxide (µmol/g)
Group-I	13.00±1.00	299.0±2.00	0.125±0.015
Group-II	69.50±1.50 a****	259.0±3.00 a***	0.640±0.030 a****
Group-III	30.50±1.50 a*** b****	513.5±3.50 a**** b****	0.305±0.015 a** b***
Group-IV	36.00±1.00 a*** b**** c^{ns}	610.0±2.0 a**** b**** c****	0.430±0.020 a*** b** c*
Group-V	22.00±1.00 a* b**** c*	641.0±1.0 a**** b**** c****	0.215±0.005 a^{ns} b**** c^{ns}

TABLE 7: EFFECT OF CMEGC ON HYDROXYPROLINEIN SIO_2 INDUCED SILICOSIS IN MALE *WISTAR* RATS

Groups Treated	Hydroxyproline (ng/ml)
Group-I	61.00±1.00
Group-II	98.00±1.00 a****
Group-III	70.00±1.00 a* b****
Group-IV	83.50±1.50 a*** b** c**
Group-V	77.50±1.50 a** b*** c*

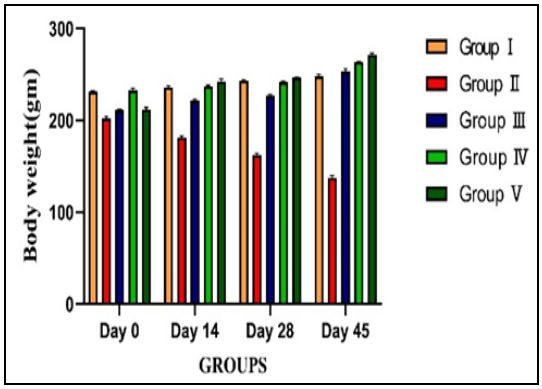

FIG. 1: EFFECT OF CMEGC ON BODY WEIGHT IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

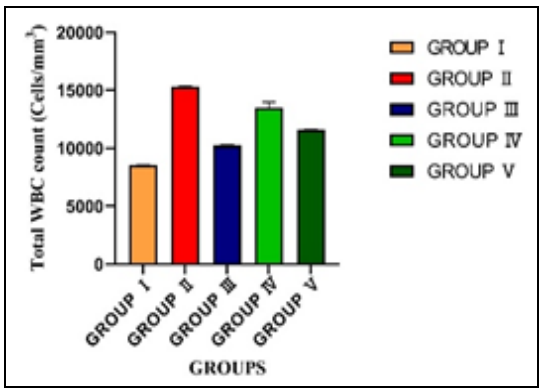

FIG. 2: EFFECT OF CMEGC ON BODYTOTAL WBC COUNT IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

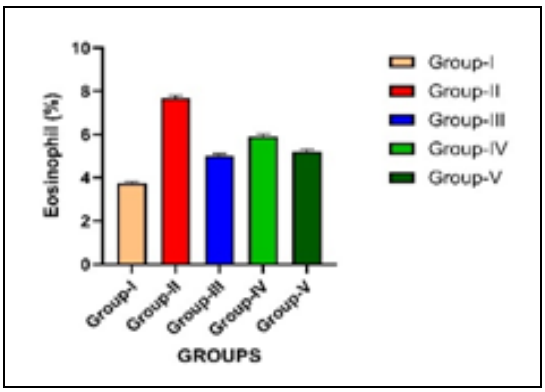

FIG. 3: EFFECT OF CMEGC ON EOSINOPHILS IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

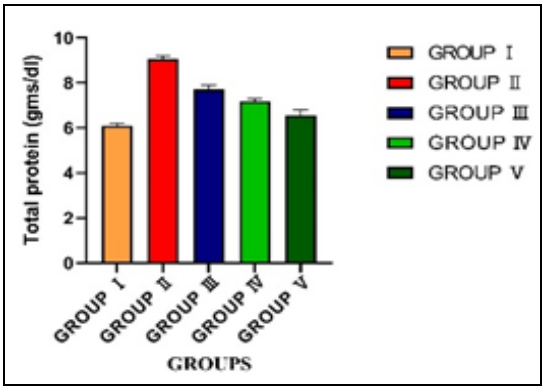

FIG. 4: EFFECT OF CMEGC ON TOTAL PROTEIN IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

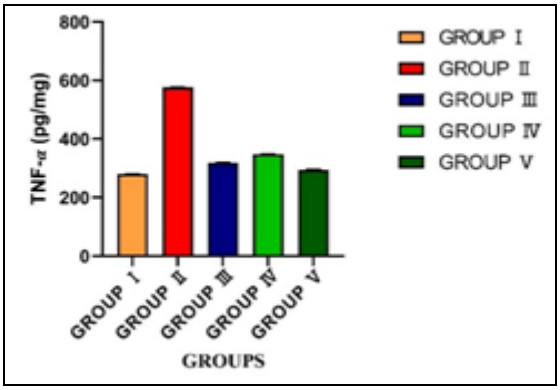

FIG. 5: EFFECT OF CMEGC ON TNF-α IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

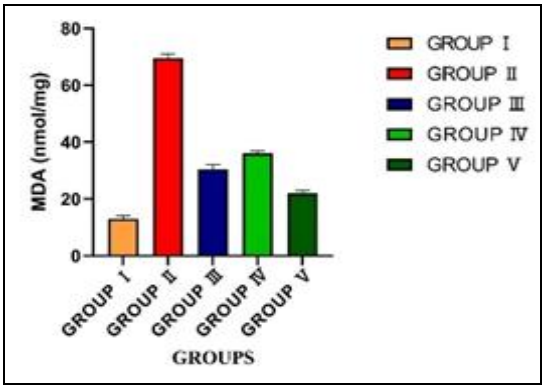

FIG. 6: EFFECT OF CMEGC ON MDA IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

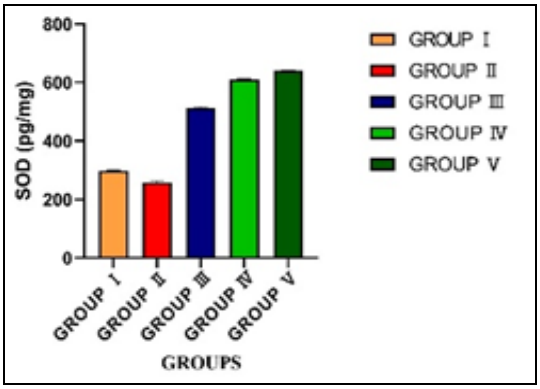

FIG. 7: EFFECT OF CMEGC ON SDO IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

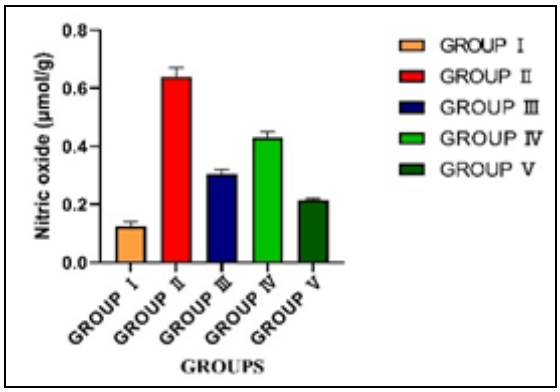

FIG. 8: EFFECT OF CMEGC ON NITRIC OXXIDE IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

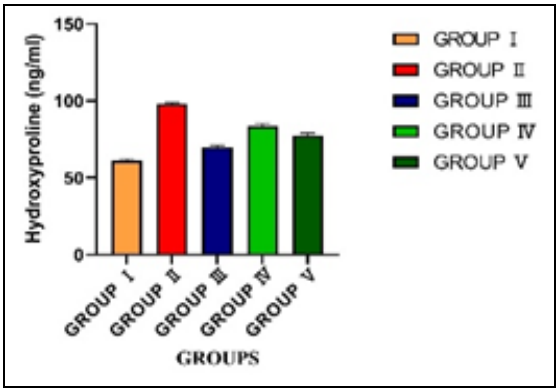

FIG. 9: EFFECT OF CMEGC ON HYDROOXIPROLINE IN SiO_2 INDUCED SILICOSIS IN MALE WISTAR RATS

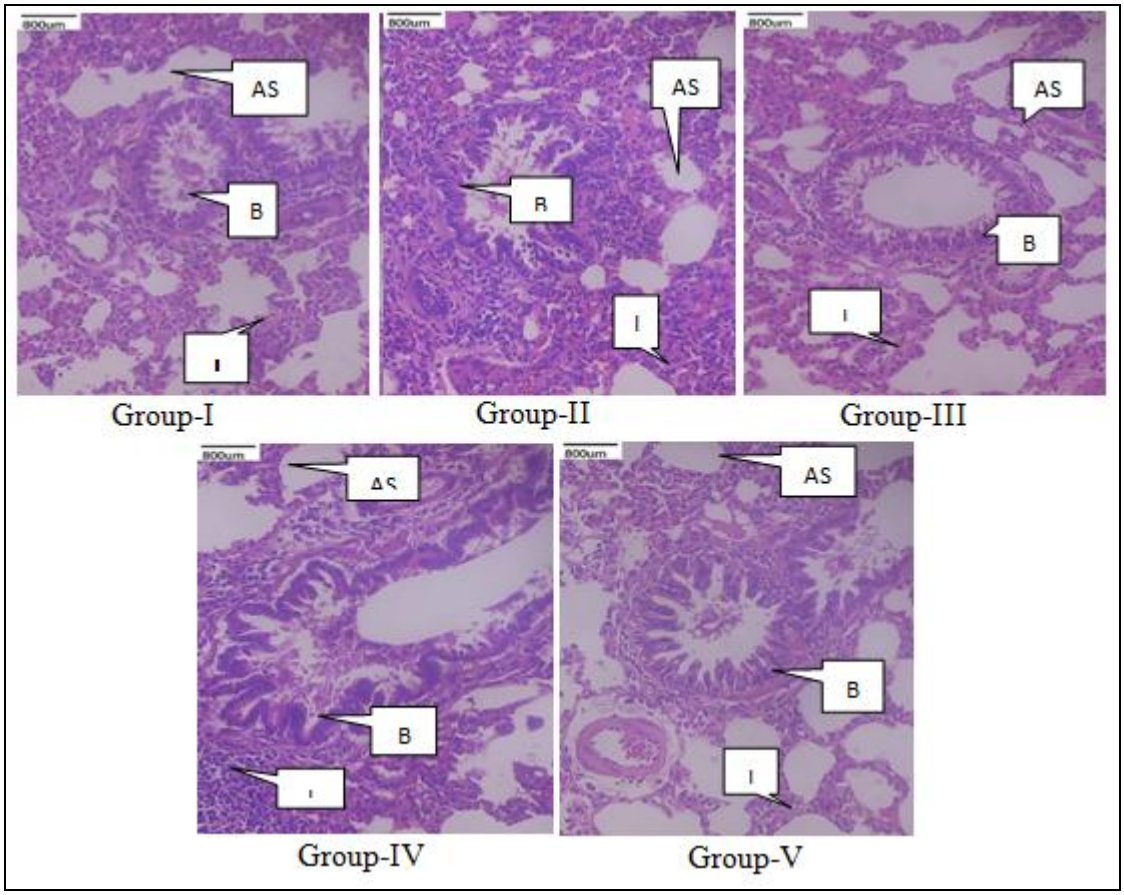

FIG. 10: HISTOPATHOLOGICAL EXAMINATION OF CMEGC IN SiO_2 INDUCED SILICOSIS IN WISTAR RATS

DISCUSSION: Silicosis, a chronic interstitial lung disease, leads to lung structure degradation, inflammation, and fibrosis. It causes progressive breathlessness, pulmonary hypertension, and respiratory failure-related death. Despite clear evidence of its cause, millions of workers worldwide are still exposed to hazardous levels of respirable crystalline silica. There is currently no effective treatment for silicosis, and patients typically receive only symptomatic therapy. Pirfenidone (PFD), one of the two approved treatments for idiopathic pulmonary fibrosis (IPF), has gained attention for its anti-fibrotic properties. However, its high cost and side effects limit widespread use. Therefore, new, more effective medications are needed to treat silicosis [1, 2, 9]. The pathophysiology of silicosis indicates that enhancing antioxidant and anti-inflammatory functions could help prevent silica-induced lung damage and fibrosis. Natural products offer a promising approach for treating various inflammatory and fibrotic conditions.

Glycyrrhiza glabra (Licorice) from the Leguminosae family is considered a vital remedy in Ayurvedic medicine. Licorice roots have been used to treat numerous conditions and are known for their anti-inflammatory, antioxidant, antiviral, immunomodulatory, antimicrobial, anticancer, anti-ulcer, hepatoprotective, anti-nephritic, anti-diabetic, and memory-enhancing properties. Licorice is also effective in reducing toxicity and boosting the effectiveness of other herbal remedies [13, 15]. So, in this study, *G. glabra* was combined with *Citrus limon* from the Rutaceae family, known for its strong antioxidant and anti-inflammatory properties.

In preliminary phytochemical analysis, *G. glabra* showed the presence of Alkaloids, Carbohydrates, Steroids, Protein, Tannins, Phenols, Flavonoids, Glycosides, Saponins and Terpenes Whereas*c. Limon*showed the Presence of Alkaloids, Steroids, Tannins, Phenols, Flavonoids, Glycosides, Saponins and Terpenes.

Acute oral toxicity studies for combination of methanolic extract of *Glycyrrhiza glabra* and *Citrus limon* were conducted as per OECD guidelines 423 (Acute toxic class method). There were no considerable changes before and after treatment of the experiment and no signs of toxicity were observed at the dose of 2000mg/kg.

Silicosis itself can have significant impacts on a body weight due to factors such as respiratory impairment, decreased physical activity, and systemic inflammation [16]. Following exposure to silica, compared with the positive control group, the weight of the rats in the silica model group was considerably reduced. Whereas the groups which received 200mg/kg and 400mg/kg of CMEGC, in comparison with silica model group (negative control group) showed a significant increase in body weight. So, the significant increase in body weight in 400mg/kg suggests that the extract may have potential therapeutic benefits for conditions associated with weight loss. Numerous studies have explored the link between WBC counts and the severity of silicosis. When silica dust is inhaled, it initiates an acute inflammatory response in the lungs, marked by the recruitment of immune cells such as neutrophils and macrophages. This inflammatory process results in tissue damage and the release of pro-inflammatory cytokines [17]. So, an elevated level of WBC counts was observed in response to silica exposure in negative control group in comparison with positive control group. The extract administered to the treated group (200mg/kg and 400mg/kg of CMEGC) may possess anti-inflammatory properties and may modulate the immune response in a way that limits the activation of immune cells, such as neutrophils and monocytes, which contribute to inflammation and tissue damage in silicosis. This modulation may lead to a dampened increase in WBC count, compared to negative control group.

In the early phase of inflammation after silica dust exposure, total protein content may rise due to the acute-phase response. This response involves the release of inflammatory cytokines, which can prompt the liver to produce acute-phase proteins, thereby increasing total protein levels.[18]So, a significant increase in total protein content was observed in negative control group in comparison with positive control group. Whereas 400mg/kg of CMEGC treatment group showed a non-significant decrease in total protein content in comparison with negative control group.

An increase in lung weight in negative control group when compared with positive control group indicates pulmonary congestion and oedema, whereas 200mg/kg and 400mg/g of CMEGC group showed a significant decrease in lung weight and indicates a significant decrease in pulmonary oedema.

Silica particles are engulfed by alveolar macrophages, which then release TNF-α and other pro-inflammatory cytokines. TNF-α attracts more immune cells to the lungs and activates inflammatory pathways. It drives the fibrotic response in silicosis by stimulating the production of extracellular matrix proteins and fibrogenic mediators like TGF-beta, leading to collagen and fibrotic component deposition in lung tissue. TNF-α also causes cellular injury and apoptosis in lung epithelial cells, contributing to tissue damage and dysfunction, and affects the balance between pro-survival and pro-apoptotic signals, determining the fate of lung cells [19]. So, an elevated levels of TNF-α in negative control group in comparison with positive control group whereas 400mg/kg of

CMEGC group showed a significant decrease in TNF-α. In silicosis, assessing oxidative stress involves measuring levels of malondialdehyde (MDA) and superoxide dismutase (SOD) [20]. MDA indicates lipid peroxidation due to reactive oxygen species (ROS) attacking cell membranes, reflecting oxidative stress. Higher MDA levels in the negative control compared to the positive control show increased oxidative stress in the lungs. Treatment with 400mg/kg of CMEGC significantly lowers MDA levels, indicating reduced oxidative stress. Whereas SOD is an antioxidant enzyme that converts harmful superoxide radicals into less damaging molecules like hydrogen peroxide. Reduced SOD levels in the negative control suggest heightened oxidative stress post-silica exposure. In contrast, increased SOD expression in the 200mg/kg and 400mg/kg CMEGC groups suggests enhanced antioxidant defense in the lungs.

Hydroxyproline, found predominantly in collagen, serves as a marker for assessing fibrosis severity in silicosis [3]. Increased hydroxyproline levels in the negative control group indicate higher collagen deposition and fibrotic tissue accumulation in the lungs compared to the positive control group.

Treatment with 400mg/kg of CMEGC significantly reduces hydroxyproline levels, suggesting a decrease in lung fibrosis. Nitric oxide (NO) is a vital signaling molecule involved in immune regulation and inflammation. In silicosis, NO production is increased by the inflammatory response and oxidative stress caused by silica dust exposure [21]. Elevated NO levels observed in the lungs of the negative control group, compared to the positive control group, indicate activation of inducible nitric oxide synthase (iNOS) in response to inflammation. Treatment with 400mg/kg of CMEGC notably reduces NO levels, indicating a decrease in lung inflammation and oxidative stress.

The histopathological result showed severe distortion of alveolar spaces (AS) with interstitium (I) showed severe inflammation and bronchus showed a severe destruction of lining epithelium (B) in negative control group whereas 400mg/kg of CMEGC showed a Very less distortion of alveolar spaces (AS) with interstitium (I) showed very mild inflammation and bronchus showed a very less severe destruction of lining epithelium (B).

CONCLUSION: The present study reported that 200mg/kg and 400mg/kg of Combination of methanolic extract of *Glycyrrhiza glabra* root and *Citrus limon* leaf (CMEGC) showed a significant effect on the silicotic lung. The effect of CMEGC was evaluated by various parameters and based on the result obtained, it is suggested that the Combination of methanolic extract of *Glycyrrhiza glabra* root and *Citrus limon* leaf (CMEGC) could be beneficial for the treatment of silicosis, since it possesses a good anti-inflammatory, antioxidant and anti-fibrotic activity needed for the reduction of disease progression on the basis of understanding the pathophysiology. And these effects are maybe due to the presence of flavonoids, saponins and terpenes like glycyrrhizin and limolene. And, further research needs to be carried out in the future to isolate, evaluate and formulate the various chemical constituents of *Glycyrrhiza glabra* and *Citrus limon* on a molecular level.

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