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PROTECTIVE EFFECT OF *CAMELLIA OLEIFERA* ABEL. ON SILICA-INDUCED PULMONARY FIBROSIS IN RATS

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Abstract

Background: *Camellia oleifera* Abel. belongs to the family Theaceae and genus Camellia. It is commonly cultivated in southern China. The seeds of *C. oleifera* have been reported to exhibit a diversity of pharmacological activities which include, but are not limited to, antioxidant, anti-cancer and antimicrobial. Pulmonary fibrosis is one of lethal causes of mortality across the globe and accretion of considerable amount of reactive oxygen species (ROS) in lungs have been implicated in the onset of this disease. Given the known antioxidant activity of *C. oleifera* seed extract (CSE), the present study was designed to evaluate the influence of CSE on the silica-induced pulmonary fibrosis rat models.

Materials and Methods: Protective effect of CSE was determined in silica-induced pulmonary fibrosis rat models. Malondialdehyde (MDA), hydroxyproline (HP) and superoxide dismutase 2 (SOD-2) activity were determined by standard biochemical assays. Histopathological analysis was carried out by H and E staining. Phyto-constituents of CSE were identified by LC/MS analysis.

Results: The results of this study indicated that CSE lowered the MDA and hydroxyproline content in silica-treated rats. Additionally, CSE also caused a significant increase in the expression of SOD-2 leading to scavenging of ROS. Hematoxylin and eosin (H and E) staining of lung tissue sections revealed that CSE maintained the integrity of parenchymatous cells of lungs and prevented the development of pulmonary fibrosis. To gain insights about the phytochemical constituents of CSE, LC/MS analysis was carried out and several antioxidant phenolics and flavonoids were tentatively identified.

Conclusion: Taken together, we conclude that CSE prevents development of pulmonary fibrosis and the protective effect of CSE may be due to its ability to induce SOD-2 expression and due to the presence antioxidant phyto-constituents.

Keywords: Pulmonary fibrosis, Silica, *Camellia oleifera*, Reactive oxygen species, Superoxide dismutase.

Introduction

Plants have been used as a source of medicine for the treatment of several diseases and disorders in different systems of medicine (Wei et al., 2012). *Camellia oleifera* Abel. belongs to family Theaceae and genus Camellia. It is commonly cultivated in southern China. The annual production of *C. oleifera* seed is around one million tons (Wei et al., 2012). These seeds are rich source of several secondery metabolites and have been reported to exhibit tremendous pharmaceutical potential. The *C. oleifera* saponins have been shown to have antioxidant activity, antihyperlipidemic,

antifungal, anticancer and several other activities (Yoshikawa et al., 2005; Kuo et al., 2010; Mu et al., 2013). Given the antioxidant activity *C. oleifera*, the present study was designed to evaluate the effect of *C. oleifera seed* extract on silica-induced pulmonary fibrosis. Pulmonary fibrosis has been reported to have caused considerable mortality either alone or in combination with tuberculosis (Bingham, 2011). Although several studies have been carried out on silica-induced lung injury but the explicit molecular mechanism is still largely unknown. However, it has been reported that silica particles can incite the accretion of reactive oxygen species (ROS) and the discharge of hydrolytic enzymes which cause severe injuries to lungs (Murray and Nadel, 2000). Therefore, it is believed that drugs that exhibit antioxidant activities can prove useful for the treatment of silica-induced lung injury. Consistent with this, *C. oleifera* seeds have been reported to contain a number of antioxidant molecules and around 70 different saponins, several terpenoids, phenolics and flavonoids have been identified from it (Zhang et al., 2014). Therefore, in the current study the influence of *C. oleifera* seed extract (CSE) was for the first time evaluated in a rat model of pulmonary fibrosis developed by endotracheal silica. The changes in oxidative stress state was examined by estimating the plasma levels of malondialdehyde (MDA), and hydroxyproline (HP) as an index of collagen synthesis. Moreover, histopathological analysis was also carried to evaluate the influence of CSE on the anatomy of lung tissue. The results indicated that CSE extract exerts protective effect and improves upon the silica-induced lung injury and this protective effect was mainly found to be due to the capacity of CSE to prevent the accumulation of ROS. To identify, the active constuients, we carried out the LC/MS analysis of CSE and several constuients such as flavonoids and phenolics were identified.

Materials and Methods

Preparation of extract

Dried seeds of *C. oleifera* were kindly provided by Xiaoliu Liu who obtained them from Medical School, Wuhan University of Science and Technology, Wuhan, Hubei, China. The authentic identification of the *C. oleifera* seeds was carried out by Xiaoliu Liu and submitted to herbarium in the department under accession number HTMC112-2016. The seeds were ground to a fine powder. Afterwards, 250 g of the powder was extracted with 500 ml of 70% ethanol and incubated at room temperature for 4 days. The extract (CSE) was then filtered and the solvent (ethanol/water) was removed using rotary evaporator under vacuum at 60 ^{o}C. The dried extract (CSE) was stored at 4 ^{o}C for further experimentation.

Animals

Male Wistar albino rats (180–200 g) were purchased from were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Animals were kept in well ventilated rooms with controlled setting of light/dark cycle, temperature of 24 ± 2 and humidity of 40-60%. The study was approved by Medical ethics committee of Tongji Medical College of Huazhong University of Science and Technology under approval number METU-C9112A/17.

Intratracheal instillation of silica

Ketamine hydrochloride (i.p.; 50 mg/kg) was used to anesthetize the rats and instilled intratracheally (IT) with silica suspension (50 mg in 0.1ml saline/rat). The normal animals were adminstrated with an equal volume of sterilized saline only. After 90 days of silica injection, the rats were sacrificed and examined for pulmonary fibrosis.

Animal grouping

Animals were randomly divided into 5 groups of 10 mice per group: group I consisted of normal control mice that were administered with normal saline only, group II received a single IT instillation of silica, group III consisted of animals that received 100 mg/kg CSE only. Group IV and V received a single IT instillation of silica plus 50 mg/kg and 100 mg/kg CSE respectively. The day of IT injection of silica or saline was taken as day 0.

Biochemical assays

The MDA levels of the plasma were estimated by the methods as described previously (Buege and Aust, 1978; Yagi, 1984). The obtained values were compared to a series of 1, 1, 3, 3-tetraethoxypropane standard solutions. To obtain the plasma samples, 1ml of blood was collected from left ventricle, and centrifuged for 5min at 1000g, and then diluted 20 times with phosphate buffer solution, pH of 7.4. The results were expressed in the units of mmol/L.

To determine the HP, left lobe of lung was used and the HP content was determined by a colorimetric protocol of Edwards and O'Brien (1980). The absorbance of the samples was taken at 500 nm and the HP content was expressed as mg/g tissue.

Histopathological analysis

For histopathological analysis, lungs were washed twice with PBS and fixed in 4% paraformaldehyde at 4 °C for 24 h. Afterwards, lung tissues were embedded in paraffin and then sectioned. Subsequently, sectioned tissues were stained with hematoxylin and eosin (HE staining). Pathological changes, such as fibrosis, severity of lesion inflammatory cell infiltration and edema, were observed under Ti–S bright field microscope (Nikon, Melville, NY, USA) (Zhang et al., 2014)

Western blot analysis

Cells from the lung tissues of all the groups were harvested and lysis was done with lysis buffer. The proteins levels in the cell extracts were determined by BCA assay by specific antibodies. Equal concentrations of protein from each sample were loaded and run on denaturing SDS gel (12%). Finally, the proteins were then electroblotted on polyvinylidene difluoride membranes. The β-actin was used as internal control.

LC/MS analysis

The chemical constituents of CSE were tentatively identified by LC–ESI-MS using LC–MS QqQ-6410B (Agilent Technologies) comprising of a chromatographic system connected to a spectrometer having an ESI source. The MS conditions were as; MS range 100–1000 Da, The mass spectra were attained in both positive and negative modes. The gas temperature was 325 ◦C, nebulizer gas 45 Psi and capillary voltage was kept at 4000 V. HPLC analysis was done using chromolith RP-18e column (4.6 mm ID, 50 mm length). The temperature of the column was kept at 30 ◦C. The mobile phase was water and 0.1% FA and acetonitrile. The gradient elution programe was; 0–10 min, 10–50% B; 10–15 min, 50% B; 15–17 min, 50–100% B and 17–19 min, 100%. The flow rate was kept at 0.5 ml/min and the injection volume was 2.5 µl.

Statistical analysis

All experiments were carried out in triplicates and expressed as mean ± SD. Statistical analysis was carried out by one-way ANOVA using GraphPad 7. Significant values were tested with Tukey's test. $*p<0.01$ and $**p<0.001$ were taken as an indication of a statistically significant difference.

Results

CSE prevents lipid peroxidation

MDA is a marker of oxidative stress and is the product of lipid peroxidation. We determined the MDA levels in lung tissues of all the groups on the 90th day and the results are summarized in Figure 1. The results indicated that the MDA levels were significantly ($^{\#}p<0.01$) higher in silica-treated group (Group III) than the normal rats (Group I). No significant difference was observed between the normal rats (Group 1) and the rats adminstrated with 100 mg/kg of CSE (Group II) Moreover, it was observed that administration of CSE to the silica-treated rats groups (i.e. Group IV and Group V), showed significantly ($*p<0.01$ and $**p<0.001$) lower MDA levels as compared to the silica-treated group (Group III). Taken together these results indicate SCE scavenges ROS in lung silica-treated rats preventing lipid peroxidation and hence lower MDA content.

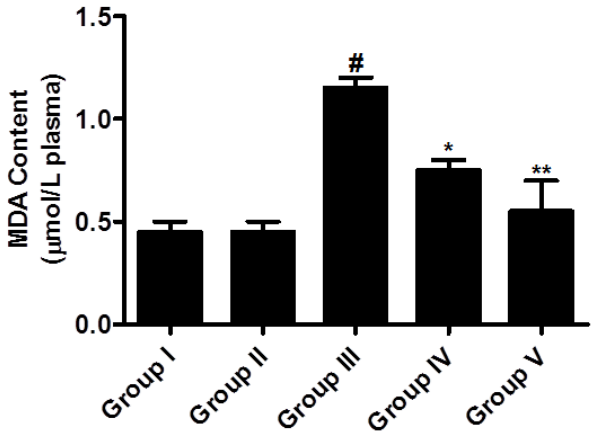

Figure 1: Effect of CSE on the MDA concentrations in Group I (normal rats), Group II (Rats receiving CSE 100 mg/kg), Group III (rats receiving single IT silica), Group IV (rats receiving single IT silica plus 50 mg/kg of CSE) and Group V (rats receiving single IT silica plus 100 mg/kg of CSE). All experiments were carried out at least in triplicates. The differences between the Group I and Group III were significant at $^{\#}p<0.01$ and the differences between the Group III and Groups IV or V were significant at $^{*}p<0.01$ and $^{**}p<0.001$ respectively.

CSE lessens the Hydroxyproline (HP) levels

We also determined the collagen formation in the pulmonary tissue by estimating the concentration of the HP of the lungs on 90th day of treatment. The HP concentration of the lungs of each group is depicted in Figure 2. The results indicated a significant increase ($^{\#}p<0.01$) in HP concentrations of the lung in silica-treated group (Group III) in comparison to the control group (Group I). However, it was observed that 50 mg/kg (Group IV) and 100 mg/kg (Group V) of CSE administration lead to a slight but significant ($^{*}p<0.01$) reduction of HP concentrations.

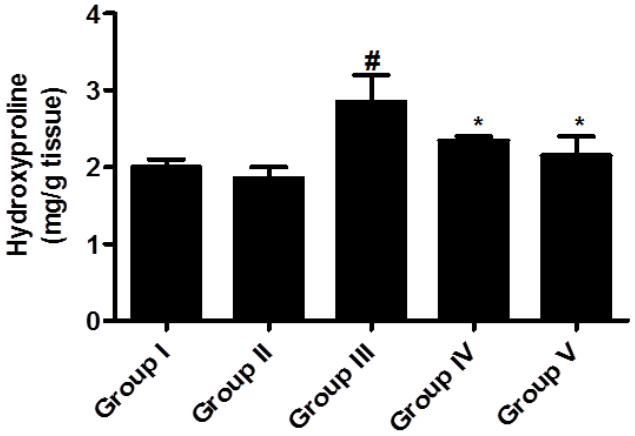

Figure 2: Effect of CSE on the hydroxyproline (HP) concentrations in all rat groups. All experiments were carried out at least in triplicates. The differences between the Group I and Group III were significant at $^{\#}p<0.01$ and the differences between the Group III and Groups IV/V were significant at $^{*}p<0.01$.

CSE enhances expression of SOD-2

Since CSE prevented lipid peroxidation, we speculated that it might induce the expression of antioxidant enzymes (Figure 3). Therefore we determined the expression of superoxide dismutase 2 (SOD-2) in all the treatment groups by western blotting. It was observed that CSE significantly ($p<0.01$) enhanced the protien expression of SOD-2 in lungs of both CSE treated normal (Group II) as well as the silica-treated plus CSE (Group IV and V) rats.

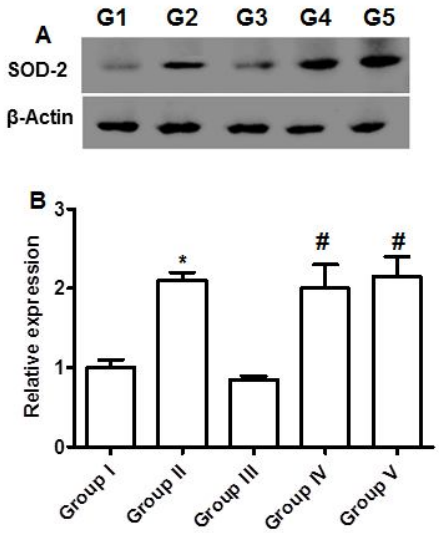

Figure 3: Effect of CSE on the protien expression of SOD-2 (A) Western blots showing expression of SOD-2 (B) quantification of SOD-2 expression by densitometry. All experiments were carried out at least in triplicates. The differences between the Group I and Group II were significant at $^{\#}p<0.01$ and the differences between the Group III and Groups IV/V were significant at $^{*}p<0.01$.

Histopathological analysis

To examine the influence of CSE on the silica-induced lung injuries, we carried out the histopathological analysis by light microscopy 90 days after treatment (Figure 4). The results indicated that lungs of rats in control group (Group I), which received IT saline only and normal rats receiving 100 mg/kg of CSE (Group II) exhibited normal lung structure and no lesions were observed. However, after 90 days of instillation of silica (Group III) caused perivascular and peribronchilal fibrosis together with silicotic nodules and dispersed hyalinized collagen (Figure 4). However, in groups which received single IT silica plus 50 mg/kg (Group IV) or 100 mg/Kg (Group V) of CSE- such events were less pronounced and the numbers of nodules were less (Figure 4). Indicating that administration of CSE confers protective effect to silica-treated rats

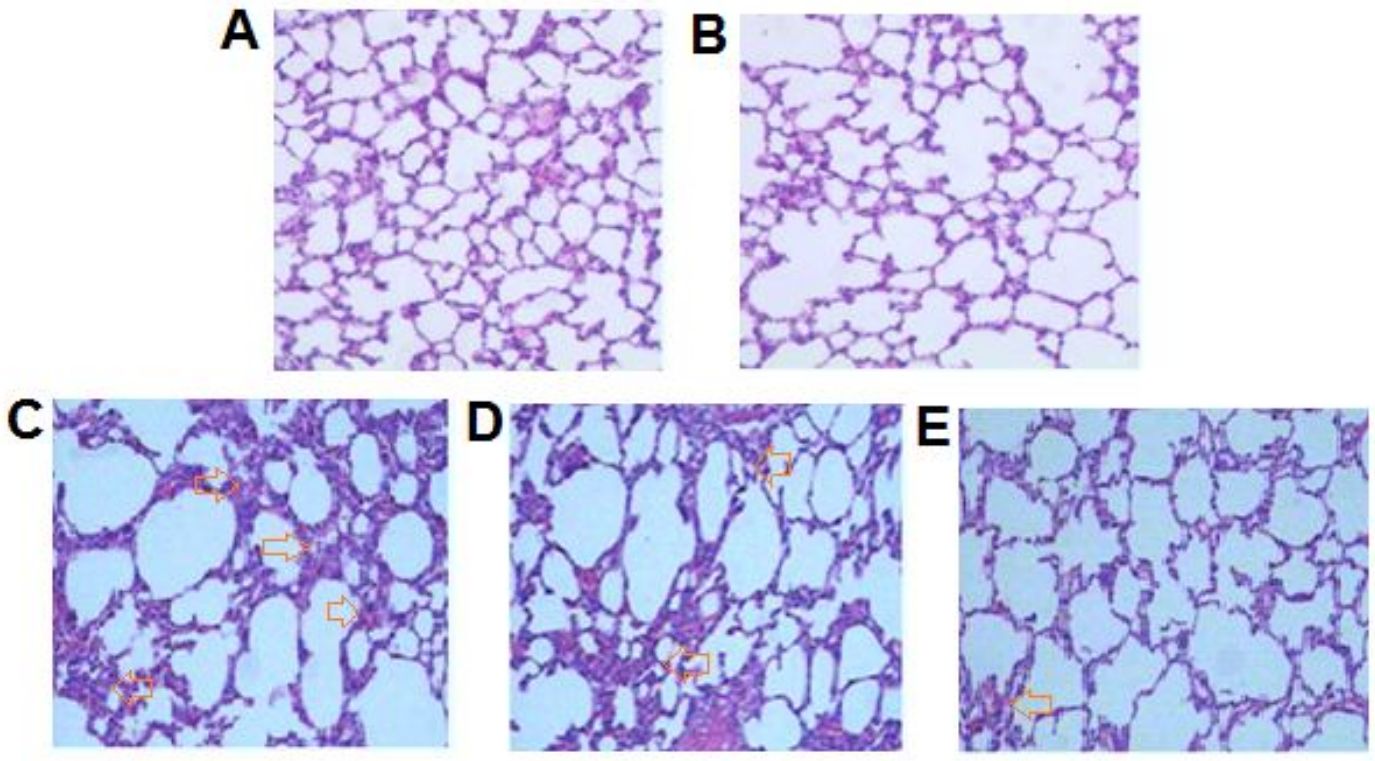

Figure 4: Photomicrographs of lung tissues of (A) Group I (normal rats) (B) Group II (Rats receiving CSE 100 mg/kg), (C) Group III (rats receiving single IT silica), (C) Group IV (rats receiving single IT silica plus 50 mg/kg of CSE) and (D) Group V (rats receiving single IT silica plus 100 mg/kg of CSE). The photomicrographs are representatives of several replicates.

Phytochemical analysis of CSE

To gain insights about the phytochemical constituents of CSE, we carried out LC/MS analysis (Figure 5). The LC/MS analyses lead to the tentative identification of several phenolics and flavonoids. These include theogallin, gallic acid, Gallocatechin, epigallocatechin, caffeine, Quercetin 3-glucoside and Catechin gallate. These compounds were identified based on the mass and in the light of literature (Yao et al., 2004).

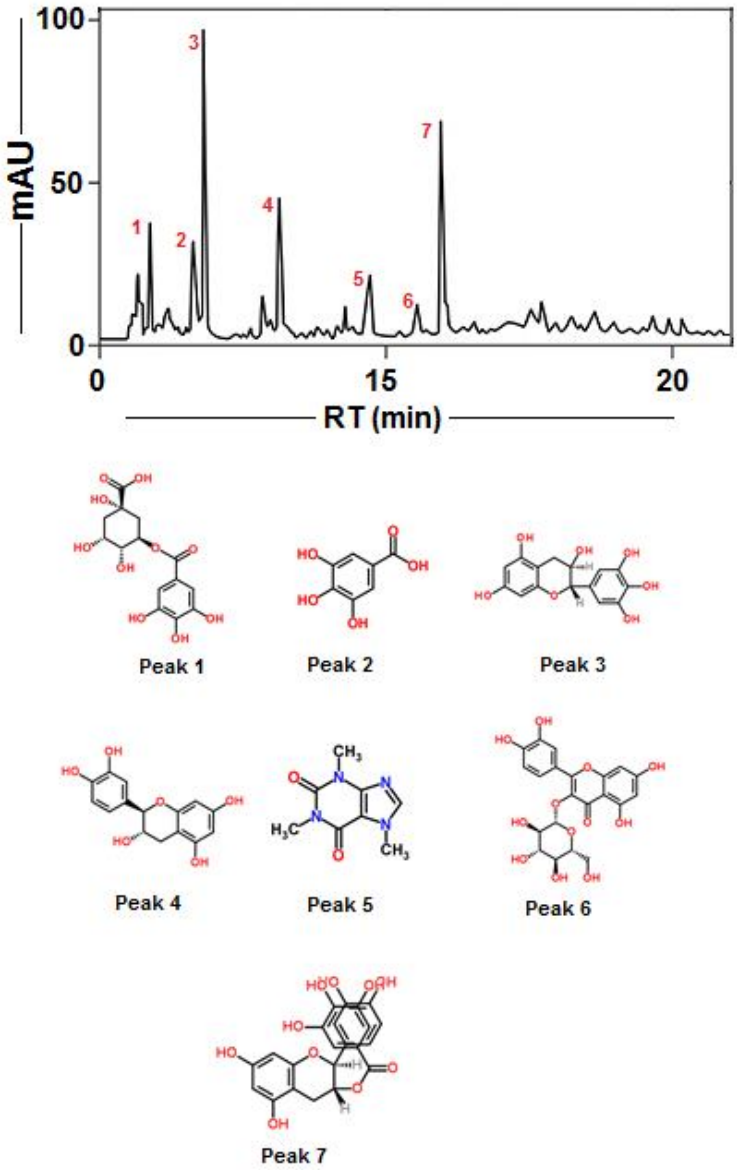

Figure 5: Phytochemical analysis of CSE by LC/MS. The peaks 1-7 represent theogallin, gallic acid, Gallocatechin, epigallocatechin, Caffeine, Quercetin 3-glucoside and Catechin gallate respectively.

Discussion

Pulmonary fibrosis is a recurrent reaction to a wide array of injuries lungs. Though several mechanisms that initiate pulmonary fibrosis have been proposed, oxidative stress involves rapid proliferation and significant buildup of connective tissue ultimately affecting normal parenchyma. The pathogenesis of pulmonary fibrosis comprises of injuries to endothelial and epithelial cells, in flow of inflammatory cells which generate chemicals that mediate the proliferation and stimulation of fibroblasts (Gorinstein et al., 1994; Uchida et al., 1995; Buege and Aust, 1978). Silica-induced pulmonary fibrosis is somewhat different from other types of pulmonary fibrosis, e.g. by bleomycin. Silica particles which are insoluble are accumulated in lung parenchyma is successively gulped by macrophages and additional injuries are results of internal reactions in these cells (Ahn et al., 2002). It has been reported that certain exogenous agents can initiate the development of pulmonary fibrosis through accretion of considerable amounts of ROS in animal models (Franklin et al., 1997). Moreover, there are concrete evidences that indicate accretion of ROS by dusts essentially or through the inflammatory response is one of the important routed for initiation of pulmonary fibrosis (Marshall et al., 1997; Kinnula et al., 2005; Caillet et al., 2006). Subsequently, the accumulation of significant

amounts of ROS cause lipid peroxidation of cellular membranes and inactivate the enzymes (Kelley, 1990). Consistent with this, antioxidants from natural products are considered important targets for prevention of pulmonary fibrosis. A number of studies have reported that plant extracts such as grape seed extract, have proved beneficial in the prevention of pulmonary fibrosis (Ahn et al., 2002; Donnelly et al., 2004; Lorenz et al., 2003). The present study was, therefore, carried out to evaluate the influence of *Camellia oleifera* seed extract on silica-induced lung injury. The results of microscopic and biochemical analysis revealed that treatment after silica instillation reduced and conferred protective effects against silica toxicity in rats. The CSE lead to significant reduction in the MDA concentrations of CSE-treated groups indicating that CSE prevented lipoid peroxidation. However, it should also be noted that CSE caused reduction in the hydroxyproline (HP) levels in silica-treated rats. To investigate the mechanism by which CSE confers protective effect against silica-induced lung injury, we determined the expression of superoxide dismutase 2 (SOD-2) in the lung tissues of all the rat groups. The results indicated that CSE enhanced the expression of SOD-2 in lungs of silica-treated rats. However, our study does not exclude the possibility of involvement of other antioxidant enzymes in CSE-conferred protective effects in silica-induced lung fibrosis. To gain insights about the constituents of CSE potentially responsible for the protective effect, we carried out the LC/MS analysis of CSE and several compounds including flavonoids and phenolics were identified. These include theogallin, gallic acid, Gallocatechin, epigallocatechin, caffeine, Quercetin 3-glucoside and Catechin gallate.

Taken together, we conclude that CSE has a protective effect and may prevent silica-induced lung injury and fibrosis. The protective effects are mainly due to the activation of antioxidant systems such as SOD-2 and the presence of several antioxidant constuients.

Declaration: Authors declare that this research presents no conflict of interests.

References

1. Ahn, H. S., Jeon, T. I., Lee, J. Y., Hwang, S. G., Lim, Y., Park, D. K. (2002). Antioxidative activity of persimmon and grape seed extract: in vitro and in vivo. Nutrition Research, **22**: 1265-1273.
2. Buege, J. A., and Aust, S. D. (1978). Microsomal lipid peroxidation. Methods in enzymology **52**, 302-310.
3. Caillet, S., Salmiéri, S., Lacroix, M. (2006). Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric method. Food Chemistry, **95**; 1-8.
4. Donnelly, L. E., Newton, R., Kennedy, G. E., Fenwick, P. S., Leung, R. H., Ito K., Russell, R.E., Barnes, P. J. (2004). Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. American Journal of Physiology-Lung Cellular and Molecular Physiology, **287**: L774-L783.
5. Edwards, C. A., and O'Brien, W. D. (1980). Modified assay for determination of hydroxyproline in a tissue hydrolyzate. Clinica chimica acta, **104**: 161-167.
6. Franklin, T. J. (1997). Therapeutic approaches to organ fibrosis. The international journal of biochemistry & cell biology **29**: 79-89.
7. Gorinstein, S., Zemser, M., Weisz, M., Halevy, S., Deutsch, J., Tilis, K., Feintuch, D., Guerra, N., Fishman, M., Bartnikowska, E. (1994). Fluorometric analysis of phenolics in persimmons. Bioscience, biotechnology, and biochemistry, **58**: 1087-1092.
8. Kelley, J. (1990). Cytokines of the lung. Am Rev Respir Dis, **141**, 765-788.
9. Kinnula, V. L., Fattman, C. L., Tan, R. J., Oury, T. D. (2005). Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. American journal of respiratory and critical care medicine, **172**: 417-422.
10. Kuo, P. C., Lin, T. C., Yang, C. W., Lin, C. L., Chen, G. F., Huang, J. W. (2010). Bioactive saponin from tea seed pomace with inhibitory effects against Rhizoctonia solani. Journal of agricultural and food chemistry **58**, 8618-8622.
11. Lorenz, P., Roychowdhury, S., Engelmann, M., Wolf, G., Horn, T. F. (2003). Oxyresveratrol and resveratrol are potent antioxidants and free radical scavengers: effect on nitrosative and oxidative stress derived from microglial cells. Nitric Oxide, **9**: 64-76.
12. Marshall, R. P., McAnulty, R. J., Laurent, G. J. (1997). The pathogenesis of pulmonary fibrosis: is there a fibrosis gene? The international journal of biochemistry & cell biology, **29**: 107-120.
13. Mu, L. H., Huang, C. L., Zhou, W. B., Guo, D. H., Liu, P. (2013). Methanolysis of triterpenoid saponin from Ardisia gigantifolia stapf. and structure–activity relationship study against cancer cells. Bioorganic & medicinal chemistry letters, **23**: 6073-6078.
14. Murray J, Nadel J. Textbook of respiratory medicine (2000). 3rd ed. Philadelphia, PA: WB Saunders, 143–186.

15. Uchida, S., Ozaki, M., Akashi, T., Yamashita, K., Niwa, M., Taniyama, K. (1995). Effects of epigallocatechin-3-ogallate (green tea tannin) on the life span of strokeprone spontaneously hypertensive rats. Clinical and Experimental Pharmacology and Physiology, **22(**s1).
16. Wei, Y. Y., Cai, H. Y., Wang, L. S., Xia, T., Du, X. F. (2012). Production of multi-enzyme enriched bio-feed from camellia oleifera seed cake in solid state fermentation by *Aspergillus niger*. Journal of Anhui Agricultural University, **39**: 41-46.
17. Yagi, K. (1984). Assay for blood plasma or serum. Methods in enzymology 1**05**, 328-331.
18. Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., Duan, J., Raymont, K., Lisle, A., Xu, Y. (2004). HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. Food Chemistry **84**: 253-263.
19. Yoshikawa, M., Morikawa, T., Yamamoto, K., Kato, Y., Nagatomo, A., Matsuda, H. (2005). Floratheasaponins A− C, Acylated Oleanane-Type Triterpene Oligoglycosides with Anti-hyperlipidemic Activities from Flowers of the Tea Plant (Camellia sinensis) 1. Journal of natural products **68**: 1360-1365.
20. Zhang, X. F., Yang, S. L., Han, Y. Y., Zhao, L., Lu, G. L., Xia, T., Gao, L. P. (2014). Qualitative and quantitative analysis of triterpene saponins from tea seed pomace (*Camellia oleifera* abel) and their activities against bacteria and fungi. Molecules **19**: 7568-7580.