Original Article

Histological and Immuno-histochemical Responses of Zinc oxide Nanoparticles and Ethanolic Extract of *Senecio glaucus* L. With the Potential Anti-apoptotic Effect of Gallic Acid in Lungs' Rats

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ABSTRACT:

Background: Zinc oxide nanoparticles (ZnO NPs) are used in different fields. Mureer or *Seneico glaucus* L. plant (SP) is the most important plants spreading in the deserts that animals feed on it. ZnO NPs and SP can be used separately or combined as supplements of alternative insecticides in the agriculture program for crop protection. Gallic acid (GA) is an important antioxidant that found in many types of fruits. **Objectives**: The purpose of this study was to evaluate the effects of either single or dual treatments of ZnO NPs and SP on lung tissue through histological and immunohistochemical investigations, and to estimate the prospective role of GA in rats for 30 days. **Material & methods:** Rats were allocated into eight groups with orally administrated: Control, GA (100 mg/kg), ZnO NPs (150 mg/kg), SP (400 mg/kg), GA+ZnO NPs (100,150 mg/kg), GA+SP (100,400 mg/kg), ZnO NPs+SP (150,400 mg/kg), and GA+ZnO NPs+SP (100,150,400 mg/kg). **Results:** Results indicated that either single or dual treatments of ZnO NPs and SP induced dense thickening in the alveolar wall, interstitial inflammation, and induced an overexpression of caspase-3 immune response. Further, the effect of dual treatment was more obvious than the influence of alone one. Inversely, GA ameliorates these undesirable changes and down-expressed of caspase-3 immune response. **Conclusion:** Our findings revealed that ZnO NPs and SP are pulmonary-toxic agents and GA might be cyto-protective and anti-apoptotic agent against the induced pulmonic-toxicity.

Keywords: Zinc oxide nanoparticle, *Senecio glaucus* L. Gallic acid, Lung, Caspase-3 protein.
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I-Introduction

Zinc oxide nanoparticles (ZnO NPs) are used in many applications all over the world such as feeding substances, crystals, lotions, drug delivery, and bio-imaging due to their unique properties (Zhou et al., 2023). They induce reactive oxygen species (ROS) production, leading to a number of the toxicological manners, such as inflammation, necrosis, and apoptosis (APOP) mechanisms (Moustafa et al.,2023). There is a strong communication between distinctive physiochemical features of the tiny size range (1-100 nm) and fatal result of ZnO NPs in the living cells. They can accumulate in the lung organ that induces inflammatory reaction and APOP procedure. Then, they persuade zinc hemostasis imbalance and disrupt the level of zinc ions in intracellular tissues (Dong and Ma,2019). From prior criticism, APOP convinces an intrinsic APOP alleyway, comprising from B-cell lymphoma-2 (Bcl-2) family and caspases proteins. Once motivated, initiator caspases are unrepressed into the cytosol, permitting cleavage and initiation of the effector caspases, such as caspase-3,8 (Micheau et al.,2014).

Mureer or *Senecio glaucus* subsp. coronopifolius (Maire) C. Alexander L. plant (SP) reputes to (*Senecio* species, Asteraceae family) found in the grimy and hot places all over the world. They already have been used as insecticidal (He et al.,2022) and anti-inflammatory (Ren et al.,2016). They contain plentiful natural constituents that are responsible for the toxic effects in the living organisms (Yu et al.,2018).

Commonly, the lung is the major organ responsible for respiration in the mammalian body. The homeostatic capacity of the alveolar epithelium is allied to controlling of the inflammatory response (Guillot et al.,2013). Each beneficial antioxidants supporting the protection against various toxins, which can inhibit systemic toxicity (EL-Shafey,2023).

Gallic acid,(3,4,5-trihydroxy benzoic acid)(GA), is a secondary metabolite found in mango, gallnut, grapes, pomegranate, and green tea. Rapidly absorbed from the gastrointestinal tract into the bloodstream. It acts as reno-protective (El-Shafey et al., 2023), anti-inflammatory (Jin et al., 2018), and cyto-

protective activities (Mehraban et al.,2020). The connection between the structure of GA and its pulmonary-protective consequence, it has an aromatic structure that can capture the free radicals (Sroka and Cisowski,2003)

The aim of this study: The intention of this study was planned to appraise the toxic effects convinced by either alone or dual treatments of ZnO NPs and SP via oral gavage through estimating histopathological and immunological analyses in lung tissue. Likewise, our study was proposed to evaluate the promising ameliorative effect of GA against lung toxicity provoked by ZnO NPs and SP in adult male rats.

II-Materials and Methods

II.1-Chemicals and reagents:

Zinc oxide nanoparticles (ZnO NPs) (<50nm) (BET), sodium carboxy-methyl cellulose (Na-CMC) salt, and gallic acid (GA) were credited from (Sigma Aldrich, St. Louis, Missouri, USA). 70% of ethanol solvent was ascended from (EL-Naser Syndicate, Egypt). In totaling, lactate dehydrogenase enzyme (LDH) and alkaline phosphatase (ALP) kits were come from (Egyptian Enterprise for Biotechnology (S.A.E), Egypt). As a final point, the primary antibody of caspase-3 was acquired from (Santa Cruz Establishment, USA) and the secondary antibody was subscribed from (Sigma Firm, St.Louis, Missouri, USA). Other used amalgams were first-class makings.

II.2-Plant extraction:

Mureer plant was collected from Egypt desert, permitting it to Uniprot database with Taxon identifier (183639) (Boulos,2002) that was identified by Pro.Dr.Abdel-Halim Abdel-Mogaly, Botanist, Herbarium of Horticultural Research Institute, Agricultural Research Center. 1.5 kg of the all parts of the plant (leaves, stem, root, and flowers) were dried-up in the laboratory of (Plant Protection Research Institute, Agricultural Research Center) and excavated in 70% ethanol for 3 days. At the culmination of drenching time, the solution was sieved with a Whatman paper and concentrated at 60˚C in a rotary evaporator (IKA-WERK, RV10, China). The extract was kept at -20˚C.

II.3-Estimation of doses of (ZnO NPs, SP, and GA):

ZnO NPs, SP, and GA suspensions were executed using 0.5% Na-CMC as a suspending agent, which was sonicated for 20 min in a bath sonicator at the tome (5 ml/kg**)** (Mahmoud et al.,2014).

II.4-Experimental design:

Our study was used forty albino male rats (*Rattus norvegicus*), weighed 180-220 g (b.wt). This study was endorsed to use the Animal Ethics Commission of Zagazig University as an approval integer (ZU-IACUC/1/F/42/2019) in the faculty of Medicine. The rats were kept at temperature (23±1˚C), humidity (55±5%), and a 12 h dark/light cycle with *ad libitum* access to food and water. Afterwards a week of the acclimation, animals were well adjusted and estranged into eight equivalent groups:

- **Group 1 (Control group)** (Mahmoud et al.,2017): rats were received 0.5% Na-CMC (Dhiyaaldeen et al.,2014)

- **Group 2 (GA-treated group):** rats were received 100 mg/kg (b.wt) of GA (Mansouri et al.,2013), which was suspended in 0.5% Na-CMC (Sen et al., 2013).

- **Group 3 (ZnO NPs-treated group):** rats were received 150 mg/kg (b.wt) of ZnO NPs, which was suspended in 0.5% Na-CMC (Srivastav et al.,2016).

-**Group 4 (SP-treated group):** rats were received 400 mg/kg of SP (EL Sheikh et al.,2021).

- **Group 5 (ZnO NPs+GA-treated group):** rats were received both ZnO NPs and GA at doses (150 and 100 mg/kg).

- **Group 6 (GA+SP-treated group):** rats were received both GA and SP at doses (100 and 400 mg/kg).

-**Group 7 (ZnO NPs+SP-treated group):** rats were received both ZnO NPs and SP at doses (150 and 400 mg/kg).

- **Group 8 (ZnO NPs+SP+GA-treated group):** rats were received ZnO NPs, SP, and GA at doses (150, 400, and 100 mg/kg).

- The pretreatment of GA was used beforehand other agents. The management of all agents was applied every day by day for 30 days by oral administration. At the culmination of the trial passé, rats were sacrificed by cervical dislocation. Then, lung tissues were fixed in 10% formalin for histopathological and immuno-histochemical investigations.

II.5-Histopathological investigation:

Lung samples were fixed using 10% neutral buffered formaldehyde. After applicable obsession, the samples were parched in mounting situations of ethyl alcohol and fixed in paraffin wax. 5-µm thick segments were censored using the rotatory microtome and blemished with hematoxylin and eosin (H&E.,) staining for learning the broad-spectrum histological erection of the lung and nearsighted beneath a light microscope (Bancroft and Layton,2012).

II.6-Immunohistochemistry investigation:

The paraffin-embedded lung was attuned into a 4 µm piece and fixed on a positively charged slide for the expression of a caspase-3 protein. The immuno-histochemical scrutiny was equipped using peroxidase/anti-peroxidase (PAP). The nonspecific peroxidase rejoinder was upset with methanol encompassing 0.1% H_2O_2. The wedge was hatched with customary goat serum to move nonspecific response once the sample was nurtured with the rigorous antibodies against caspase-3 (dilution,1:2000). The tissue segment was eroded with phosphate buffer and nurtured with secondary antibodies (dilution,1:2000). Beforehand, they have eroded in phosphate buffer again and nurtured with the PAP complex (dilution,1:200). The peroxidase reaction was attained using a solution of 3,3'-diaminobenzidine tetra-hydrochloride encompassing from 0.01 % H_2O_2 in Tris-HCl buffer (0.05 M, pH=7.6). After immunostaining, the lung section was counterstained with (H&E.,) staining and comprehended beneath a light microscope (Ramos-Vara et al.,2008).

III. Results

III.1-Histopathological investigation:

(Figure 1 a-h) showed that normal tissue structure with normal sized alveolar spaces, interalveolar septa, and bronchioles with columnar ciliated epithelial lining (Figure 1a). Additionally, healthy pulmonary parenchyma cells were observed in GA-treated group (Figure 1b). On a hand, thickening in the alveolar walls, and the inter-alveolar septa, and infiltration in the alveolar interstitial with dissemination of the inflammatory cell appeared in ZnO NPs-treated group (Figure 1c). Dense thickening alveolar walls and more dispersal interstitial inflammatory cells in

SP-treated group (Figure 1d). On the other hand, there are inflammatory cells and misfortune of typical thickness of the alveoli befallen in GA+ZnO NPs-treated group (Figure 1e). A similar improvement was showed in GA+SP-treated group (Figure 1f). Thickening in the alveolar walls, increased interstitial inflammatory hemorrhage, and hypertrophy of the alveolar cells emerged in ZnO NPs+SP-treated group (Figure 1g). Thickening in the alveolar walls and medium interstitial inflammatory hemorrhage induced in GA+ZnO NPs+SP-treated group (Figure 1h).

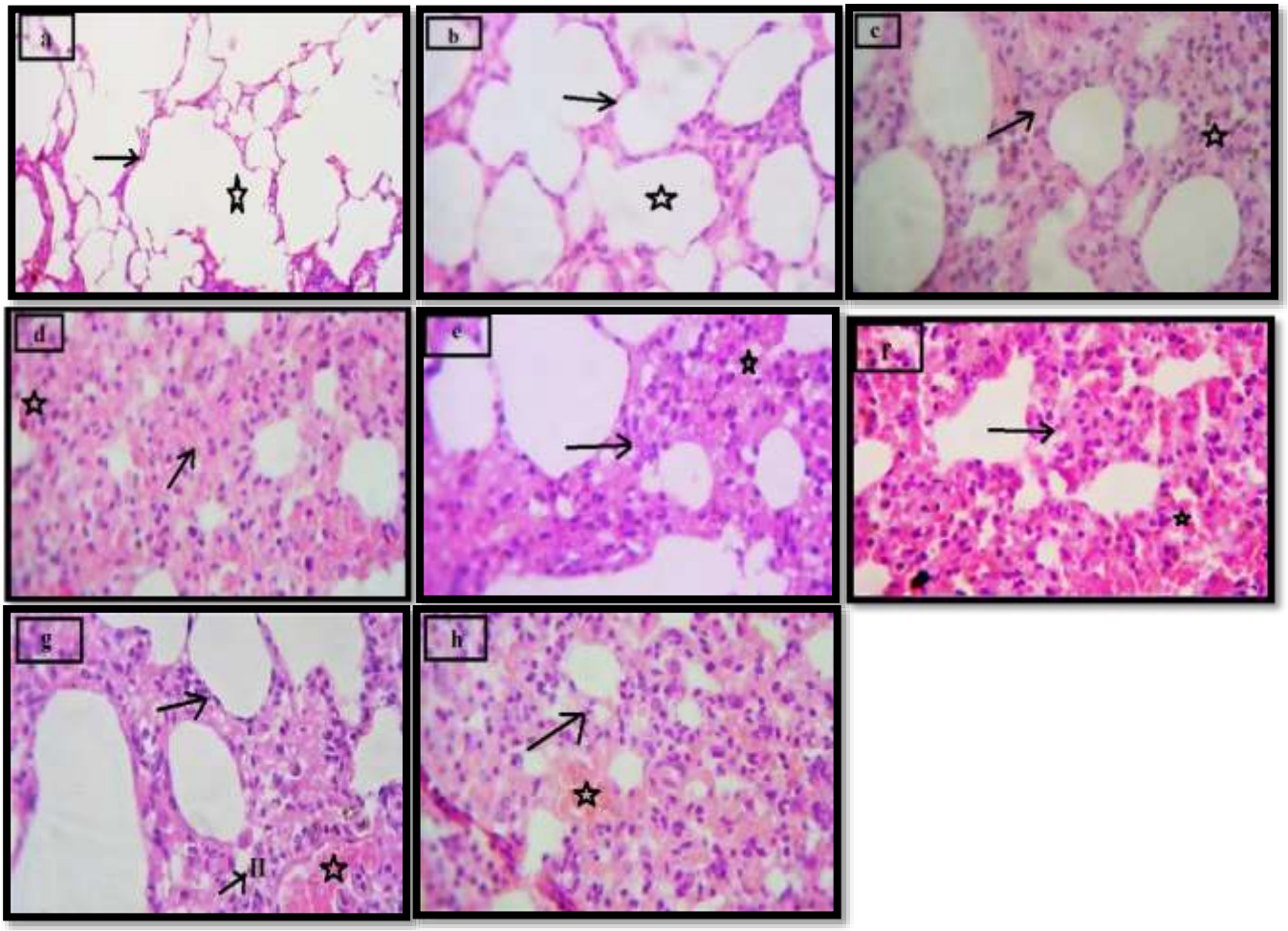

Figure (1 a-h): Photograph of the histological studies of effect of Zinc oxide nanoparticles (ZnO NPs), mureer or *Senecio glaucus* L. plant (SP), and gallic acid (GA) on a section of lung tissue in different groups. 1a) Control group showing normal spongy pulmonary parenchyma (air bronchioles, simple columnar ciliated epithelial lining, smooth muscle numerous alveoli, and alveolar sacs) (star) and normal alveolar septa (arrow)(H&E.,x200).1b) GA-treated group showing normal healthy pulmonary parenchyma (star) with normal alveolar septa (arrow)(H&E.,x400). 1c) ZnO NPs-treated group showing thickening of an alveolar wall (arrow) and destruction of inter-alveolar septa with alveolar inflammatory cell areas (star) (H&E.,x200). 1d) SP-treated group showing extensive thickening of an alveolar wall (arrow) and more spreading interstitial inflammatory areas (star). 1e) GA+ZnO NPs-treated group showing a small area of inflammation with reversal of normal thickness of alveoli (arrow) and mild inflammatory cells (star). 1f) GA+SP-treated group showing mild thickening of alveolar walls (arrow) and slightly marked interstitial inflammatory hemorrhage (star)(H&E.,x400). 1g) ZnO NPs+SP- treated group showing severe thickening of an alveolar wall (arrow), a large area of marked interstitial inflammatory hemorrhage (star), and hypertrophy of alveolar cells (H) (H&E.,x400). 1h) GA+ZnO NPs+SP- treated group showing mild thickening of alveolar walls (arrow) and a small area interstitial inflammatory interstitial hemorrhage (star) (H&E.,x400).

III.2-Immuno-histochemical results

Our outcomes displayed that either single or dual treatments of ZnO NPs and SP persuaded positive caspase-3 immune response (Figure 2 c,d,g). Yet, the adding

of GA to them convinced a weak positive immune response of caspase-3 (Figure 2 e,f,h). Similarly, the addition of GA to ZnO NPs and SP separately or combined groups enhanced the lung cells from apoptotic status and declined in the expression of caspase-3 immune response that inhibited of APOP process in the lung tissue.

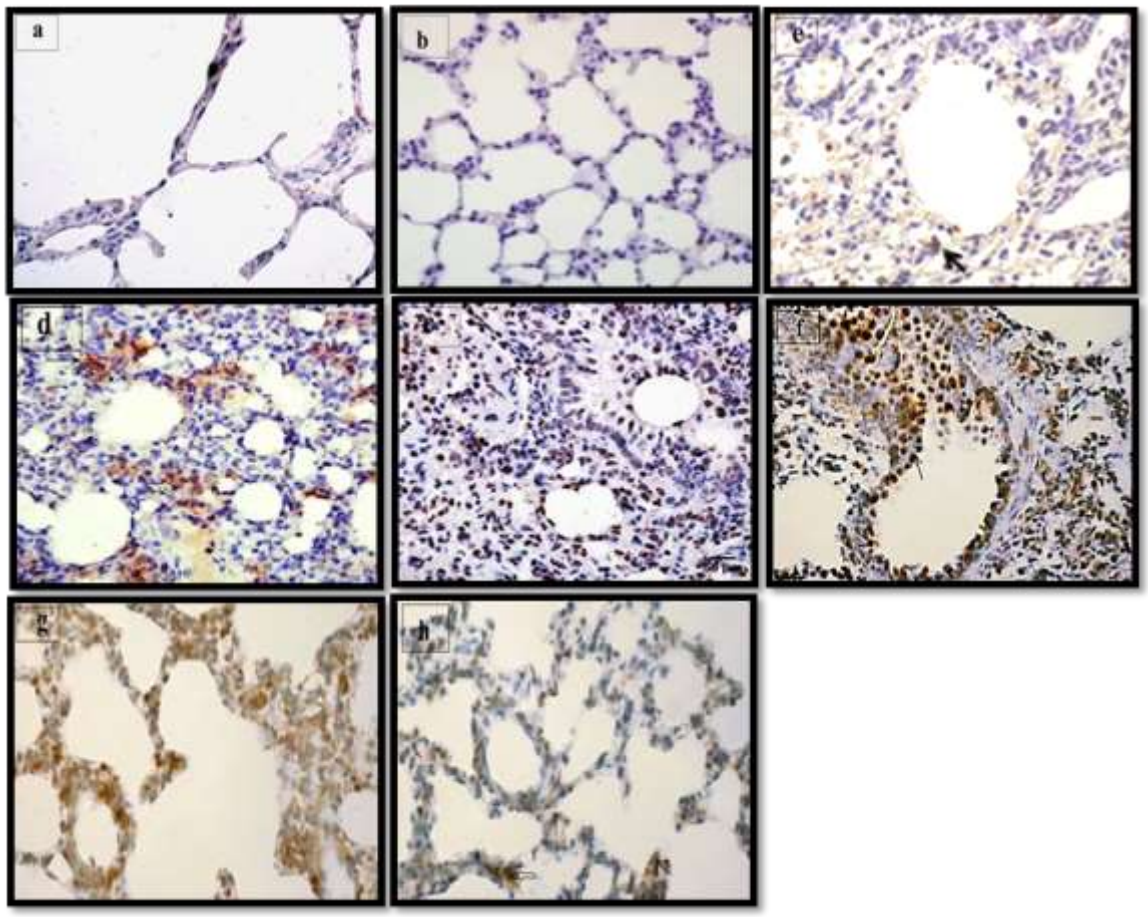

Figure (2 a-h): Photograph of the immunohistochemical studies of effect of zinc oxide nanoparticles (ZnO NPs), mureer or *Senecio glaucus* L. plant (SP), and gallic acid (GA) on immunohistochemical staining of caspase-3 protein in the lung section of all treated groups. Control group (2 a), GA-treated group (2b) showing negative caspase-3 immune response (H&E.,x400). 2d): ZnO NPs-treated group showing strong positive caspase-3 immune response (H&E.,x200). 2d) SP-treated group showing strong positive caspase-3 immune response (arrow) (H&E.,x200). 2e) GA+ZnO NPs-treated group showing weak positive caspase-3 immune response (arrow)(H&E.,x400). 2f) GA+SP-treated group showing a weak positive immune response of caspase-3 (arrow) (H&E.,x400). 2g) ZnO NPs+SP-treated group showing very strong positive caspase-3 immune response (H&E.,x400). 2h) GA+ZnO NPs+SP-treated group showing moderate positive caspase-3 immune response (arrow) (H&E.,x400).

IV. Discussion

Our study aimed to estimate the detrimental effects of single or dual treatments of ZnO NPs and SP on lung organ in rats, and the prophylactic consequence of GA against them. Our data exposed that either single or dual treatments of ZnO NPs and SP showed hemorrhage, inflammation, and stiffening in the alveolar walls. Furthermore, on the level of immuno-histochemical appraisals, they elicited up-regulation in the expression of caspase-3 in the lung tissue. The histopathological and immuno-histochemical studies were confirmed that ZnO NPs and SP parade as pulmonary-toxic and pro-apoptotic mediators.

In order to recognize the crucial injurious pathway, NPs may arouse oxidative stress to intracellular tissues, leading to mitochondrial hemostasis disturbance (Cameron et al.,2022). They induced necrosis and inflammation, and DNA mutation and convinced APOP mechanism (Zhang et al., 2023). These upshots were in the equivalent stripe with Cifuentes etal.,(2016); Paredes et al.,(2016) signposted that *Senecio* plants convinced abnormality in the standard bloodstream and ion hemostasis imbalance that initiated vasodilation in the blood vessels.

Caspase-dependent appliance or cysteine-aspartic acid protease caspase family induces DNA and protein annihilations after the cell impairment. Entirely, caspase-3 is an ordinarily activated death protease and is catalyzed explicit cleavage of the abundant important cellular proteins. It is also obligatory for some features of APOP events and it is indispensable for apoptotic chromatin compression and DNA obliteration in all cell varieties (Espinosa-Oliva et al.,2019).

Our results were in the equivalent with, González-Vega et al.,(2022); Ma et al.,(2019) who described that NPs impelled changes in the lung cell lines because of the inspiration of modification in the level of transcriptional and translational genes. Likewise, these data were in the matching inclination with Li et al.,(2017) who pronounced that NPs caused lung inflammation and congestion in the blood vessels. The activation of NF-κB appliance is indispensable for

expression of the pro-inflammatory cytokines supported in an infiltration of the inflammatory cells via a Fas/FasL alleyway (Snyder et al.,2019).

SP contains a large amount of saponins, which are responsible for its lung-toxic assets that accumulated in the alveolar cells and prompted vacuoles (Xin et al.,2016). Thus, they are the chief explanations, which are responsible for the modulation in pulmonary utility and edifice, such as outsized alveolar edema, alveolar septal fibrosis, infiltration in the polymorphic nuclear leukocytes, and bleeding foci in the lung tissue. Our fallouts also were in coherence with Burns,(1972) who revealed that feeding on *Senecio* plants instigated damage in the pulmonary arteries. Still, Hooper, (1974); Rasool et al.,(2022) informed that these plants convinced pulmonary inflammation and lung infiltration due to accretion of its phytochemical components in the blood vessels. The infiltration in the pulmonary cells is accredited to irritating in the secretion of myeloperoxidase enzyme (lysosomal protein), which was unconstrained from the neutrophil and stimulated abnormal immune response. The inflammatory cell clusters led to thickened septa (María et al.,2018).

In dissimilarity, our fallouts disclosed that the pretreatment of GA to either single or dual treatments of ZnO NPs and SP repaired pulmonary structure and down-regulated ofcaspase-3 protein immune response in treated groups. The defensive effect of GA was confirmed by histopathological scrutiny, which mitigated lung damage via reestablishing of the inflammation and hemorrhage and immunohistochemical analysis. Generally, GA can impede an induction of the inflammatory immune response and APOP mechanism in the injured lung cells (Pham et al.,2018). Reactive oxygen species (ROS) are the foremost basis to overturn various living cells through inducing the redox equilibrium between oxidants and antioxidants and enhancing tissue inflammation. Then, it restores antioxidant ability (Winiarska-Mieczan et al.,2020). Our results were in a comparable link with Alazragi,(2020); Singla et al.,(2020) revealed that GA mitigated pulmonary inflammation and fibrosis in the cured animals.

V. Conclusion

Lastly, this study showed that ZnO NPs and SP induced many histologic amendments in the lung tissue and a strong expression in caspase-3 protein due to stimulus of APOP appliance. Hence, they exhibit as pulmonic-toxic and pro-apoptotic agents. Inversely, our study revealed that GA recovered some of these histological and immuno-histochemical analyses and inhibited caspase-3 protein expression. Hence, this study assumed that GA deeds as a respiratory-protective and anti-apoptotic agent.

VI. Declaration of interest

The authors declare that there are no competing conflicts of interest.

VII. Funding sources

This research was not funded by any funding agency.

VIII. References

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الملخـــــص العربـــي

التأثيرات التشريحية و التشريحية المناعية المستحدثة بواسطة جسيمات أكسيد الزنك النانوية ونبات الشيخة الرمادية مع الدور المأمول المضاد لموت الخلايا المبرمج لحمض الجاليك على رئة الجرذان

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تم تصميم هذه الدراسة لتقييم التأثيرات النسيجية التشريحية و تشريحية مناعية للمعاملات الفردية و المزدوجة لجسيمات أكسيد الزنك النانوية ونبات الشيخة الرمادية في النسيج الرئوى ، والتنبؤ بالتأثير الوقائى الدور المأمول المضاد لموت الخلايا المبرمج لحمض الجاليك لمدة 30 يومًا في الجرذان و قد تم تقسيم أربعين من الجرذان البيضاء إلى ثماني مجموعات مع تناولها للمعاملات عن طريق الفم: **1**) المجموعة الضابطة. **2**) مجموعة حمض الجاليك: (100 مجم/ كجم). **3**) مجموعة جسيمات أكسيد الزنك النانوية: (150مجم/كجم) . **4**) مجموعة نبات الشيخة الرمادية: (400مجم/ كجم). **5**) مجموعة جسيمات أكسيد الزنك النانوية + حمض الجاليك: (150،100مجم / كجم). **6**) مجموعة نبات الشيخة الرمادية + حمض الجاليك: (400 ، 100 مجم/ كجم). **7**) مجموعة جسيمات أكسيد الزنك النانوية + نبات الشيخة الرمادية: (150 ،400 مجم/ كجم). **8**) مجموعة حمض الجاليك+جسيمات أكسيد الزنك النانوية + نبات الشيخة الرمادية: (150،100 ،400 مجم/كجم). وقد أظهرت نتائجنا أن معاملات جسيمات أكسيد الزنك النانوية و نبات الشيخة الرمادية تسببوا فى حدوث التهاب ،تضخم ،تنكس رئوى ، وإلتهابات وفجوات. وأيضا عملوا على زيادة التعبير البروتينى لبرتين Caspase-3 وقد أوضحت حقائقنا أيضًا أن التأثيرالسام للمجموعة المزدوجة من جسيمات أكسيد الزنك النانوية ونبات الشيخة الرمادية كان أقوى من تأثيرالمجموعة المتعاملة بواحدة من هاتين المادتين. وعلى العكس من ذلك ، نتائجنا وجدت أن حمض الجاليك قلل من الإصابة بالتدمير الرئوى والعمل على تحسين الإختلال الرئوى النسيجى الناتج منهم وعالج تلفها وخفض التعبيرالبروتينى لبرتين Caspase-3 . و باختصار،قد عرضت هذه الدراسة نموذجا باعتبار جسيمات أكسيد الزنك النانوية و نبات الشيخة الرمادية كعوامل لها أثر سلبى على الرئة ؛ بينما قد يعمل حمض الجاليك كعامل وقائى ضد سمية الرئة ،وقد أشادت هذه الدراسة لمفهوم جديد للمبيدات الحشرية البديلة من خلال استخدام جسيمات أكسيد الزنك النانوية و نبات الشيخة الرمادية فى الإستخدامات الزراعية المستخدمة في برنامج مكافحة الآفات ،وينصح ايضا بتناول حمض الجاليك كمصدر وقائى ضد المسببات السامة للرئة.