Research Square

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Ki-67 pulmonary Immunoreactivity in silver nanoparticles toxicity: size-rate dependent genotoxic impact

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Research Article

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Abstract

Background

Engineered nanoparticles have been recently utilized in numerous domains particularly, silver nanoparticles (AgNPs). Nonetheless, the possible side effects resulting from AgNPs exposure are not fully clarified. The present study aimed to clarify the toxicity of AgNPs toxic effect on lung tissue. This study was extended to investigate the impact of *Glycosmis pentaphylla (G. pentaphylla)* and *Casimiroa edulis (C. edulis)* leaves extracts in addition to mucilage and protein; the purified compounds from *C. edulis* against AgNPs induced pulmonary toxicity.

Methods

Male Swiss albino mice were administered AgNPs orally in two different particle sizes (20 nm and 100 nm) for one month and was further treated via the above mentioned natural product extracts in a dose of 500 mg/ kg for three weeks. Biochemical, molecular, immunohistochemistry, and histopathological investigations were further assessed.

Results

Our results declared an obvious alternation in oxidative stress biomarkers as well as mRNA gene expression of both survivin and matrix metalloproteinase (MMP-9) in addition to exploration of positive nuclei for ki-67. Data declared a significant upregulation of both glutathione s transferase and superoxide dismutase antioxidants up on treatment by *C. edulis* extract. Furthermore, a remarkable downregulation of MMP-9as well as survivin mRNA gene expression in all treated groups. Immunohistopathological examination investigated a significant improvement in the reactivity of ki-67 biomarker upon treatment. Histopathlgical examination confirmed the obtained results.

Conclusion

In conclusion; these functional foods extracts could be considered as a promising candidate as therapeutic regimen against pulmonary toxicity induced via Ag-NPs due to their enrichment with different active constituents.

Introduction

Silver nanoparticles (AgNPs) are commonly utilized in several consumed products. Due to their high antimicrobial property, AgNPs are recommended for attenuating bacterial as well as fungal contamination of food packaging, textiles, implants, cosmetics, and medical devices [1, 2]. Despite the wide range of AgNPs industrial applications, it recorded numerous inflammatory and organs dysfunctions properties [3]. AgNPs can exist as an airborne dust therefore, exposure levels should be put in consideration regarding different sizes of AgNPs that was inhaled [4, 5]. Numerous studies investigated high inflammatory potential associated with AgNPs accumulation in the lung and may lead

to granulomatous lesions. Furthermore, AgNPs from the lung can permeate epithelial barrier via the blood and/or the lymphatic fluid by its bio-kinetic property into remote tissues such as kidney, liver, and spleen causing various toxic effects [6–8]. (2) AgNPs impact was previously assessed in *vitro* [9–11]. Numerous cell lines recorded apoptosis or necrotic cell death within AgNPs acute exposure as a result of reactive oxygen species accumulation, leading to lipid peroxidation, oxidative generated DNA lesions, mutations of genes, swelling for mitochondria, leakage of lysosome, and finally blockage of autophagic flux [11–15] On the other hand, chronic exposure to AgNPs triggers inflammatory and thiol responses, causing damage in mitochondria, phagocytic capacity reduction, and increment in nitric oxide levels through lipopolysaccharide stimulation[16].

Survivin, is an inhibitor member of apoptosis family through inhibition of caspase-mediated cell death by reduction of caspase gene expression in which, it binds to the apoptotic X-linked inhibitor and the pro-apoptotic "Smac/DIABLO" factor that leads to releasing Smac/DIABLO in cytoplasm [17, 18] Furthermore, survivin has an essential role in cell cycle during the G2/M phase [19, 20]. Many human tumors express high level of survivin, and its tissue level is closely related to tumor progression and poor prognosis [21]. However, over expression of survivin in embryonic stage is downregulated in adult tissues, some healthy adult cells declared expressed survivin [22]. Therefore, Survivin over-expression is essential during regeneration of tissue and its suppression may be due to injury [23].

Matrix metalloproteinases (MMPs) are zinc-established enzymes which are responsible for degradation of extracellular matrix proteins, even though their responsibility for activation or inhibition various other effector molecules [24]. Un-regulation of the MMP gene expression is associated with pathogenesis of various lung diseases [25–27].

Immunohistochemical analysis isn't widely applied for the detection of lung toxicity. However, Ki-67 immunostaining assay has been investigated to detect cellular dysfunction [28–30]. The Ki-67 antigen exists in the nucleus but in incomplete form. It was reported to be expressed in all phases of cell cycle except G0 [28]. Epithelial cells that can express Ki-67 are mainly elevated in bronchial cells indicating that it may be an important indicator in lung inflammation, apoptosis and lung cancer[31]. Ki-67 immunohistochemically recorded an elevation in both current and ex-smokers [32]. Furthermore, ki-67 was investigated as an early diagnostic biomarker for lung cancer [33].

Herein, we supposed that the Ki-67 immunohistochemistry index could be overexpressed in pulmonary epithelial cells upon AgNPs intoxication based on different sizes in mice model. In addition to, detection of both survivin and MMP-9 as molecular biomarkers related to lung dysfunction.

Casimiroa edulis (*C. edulis*) tree which is known as "white sapota" is cultivated in Egypt. *C. edulis* leaves and seeds are previously being eaten for their calming activities and helping to sleep [34]. Moreover, their leaves extract was investigated as hypnotic, anticonvulsant, diuretic, antihypertension, antioxidant, anti-inflammatory, anti-Alzheimer and anti-carcinogenic agent [35–38]. Furthermore, different compounds that were extracted from leaves showed adipogenesis activity [39]. Also, essential oil extracted from *C. edulis* declared antimicrobial and anti-coagulant activities [40]. Due to lacking of previous studies on *C.*

edulis leaves extract as a therapeutic regimen for lung injury induced via AgNPs treatment, the current study investigated the promising therapeutic index against AgNPs induced lung toxicity.

Glycosmis pentaphylla (*G. pentaphylla*) is called toothbrush. In alternative medicine, *G. pentaphylla* is used for anemia, cough and rheumatism treatment. Furthermore, it was recorded as an antifungal regimen in addition to its activity in cancer treatment [41, 42]. Methanolic leaf extract also is considered as potential natural antioxidants and antimicrobial agents versus numerous infectious diseases [43]. However, there are no recent researches regarding the therapeutic index of *G. pentaphylla* leaves extract on AgNPs induced lung injury, the current study evaluate the promising therapeutic potential against AgNPs induced lung toxicity.

Materials And Methods

Chemicals and reagents

AgNPs of two particle sizes (20 nm &100nm) were purchased from Sigma-Aldrich CO. (St Louis, Missouri, USA). Biochemical analyses were obtained from Biodiagnostic Kits (Biodiagnostic Co., Egypt). Kits for total RNA extraction, primers and RT-PCR SYBR green kits were obtained from Qiagen (Germany-Helden). All other chemicals are of high analytical grade.

Plant material and extract preparation

Leaves of *C. edulis* and *G. pentaphylla* were collected from botanical garden, Giza, Egypt and Mohammed Ali museum, respectively. Extraction of both *C. edulis* and *G. pentaphylla* were previously established. In addition to, the active constituents, protein and mucilage of *C. edulis* seeds and fruits recpectivelly [38, 44].

Experimental design

Animal groupings and treatments

One hundred-ten male Swiss Albino mice (20–25 g /each) were obtained from National Research Centre (NRC), Cairo, Egypt. Animals were kept in cages for acclimatization and allowed free access for feeding and drinking. All animals were received adequate care and handling according to institutional animal ethics committee of NRC.

After acclimatization (one week), animals were randomly subdivided into eleven groups (10 /each) as the following:

Group 1: Healthy animals.

Group 2: untreated mice [45] that were orally given AgNPs in low size (20 nm) for one month and served untreated as positive control for 20nm AgNPs [45].

Groups 3, 4, 5 & 6: AgNPs 20 nm intoxicated animals which treated with *C. edulis, G. pentaphylla* extracts, protein and mucilage of *C. edulis* seeds and fruits respectively in a dose of 500 mg/kg /each for one month [38].

Group7: untreated mice that were orally given AgNPs in high size 100 nm ([45] for one month and served untreated as positive control for 100 nm AgNPs.

Groups 8, 9, 10 & 11: AgNPs 100 nm intoxicated animals which treated with *C. edulis, G. pentaphylla*, extracts protein and mucilage of *C. edulis* respectively in a dose of 500 mg/kg /each for one month [38].

Determination of some antioxidants

Activity of antioxidant was determined in serum samples for all tested groups. SOD (Superoxide Dismutase) and GST (Glutathione S-transferase) were measured. [46] [47].

RNA extraction and RT-PCR analysis for apoptosis determination

Lung samples of each group were used to extract total RNA individually according to the manufacturer's instructions illustrated in Qiqamp mini kit (Qiagen; USA; Cat No. 74104). Complementary DNA (cDNA) and mRNA gene expression of Survivin (forward and Reverse primer sequence; 5'-GTGAATTTTTGAAACTGGACAG-3' & 5'-CCTTTCCTAAGACATTGCTAAG-3') and MMP-9 (forward and Reverse primer sequence; 5'-GTGCTGGGCTGCTGCTTTGCTG-3' & 5'-GTCGCCCTCAAAGGTTTGGAAT-3') quantitative RT-PCR were assessed using RT-PCR master mix SYBR green kit (Qiagen; USA; Cat No. 204243). Followed by real time PCR reaction amplification was carried out in Stratagene Mx3000 P QPCR System (Agilent Technologies, Santa Clara, CA, USA) in 20 µl reaction volume. Primers sequences were listed in Table 1. Temperature profile was calculated according to optimum annealing temperature for each primer. The relative expression of survivn and MMP genes was obtained by comparative CT ($2^{-\Delta\Delta Ct}$) method against the expression of β-actin (forward and Reverse primer sequence; 5'-CCTGAGGCTCTTTTCCAGCC-3'& 5'-TAGAGGTCTTTACGGATGTCA ACGT-3') as reference gene [48].

Immunohistochemistry Determination of Ki-67

Lung tissue sections (on 4 µm thick) were prepared for Ki-67 antigens immunohistochemistry (IHC) detection. Tissue sections were kept in 0.03% hydrogen peroxide (H_2O_2) at room temperature for 10 min, due to the removing of endogenous peroxidase activity. After that, the reaction is blocked by adding serum (0.5% normal goat serum X0907, Dako Corporation, Carpinteria, CA, USA,, and 0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China in PBS) at room temperature for 30 min. Ki-67 Anti-body (Roche, CONFIRM anti-Ki-67 (30 – 9) Rabbit Monoclonal Primary Antibody: 790–4286) was added (with dilution at 1:200) then incubated kept overnight at 4°C. After that, Sections were subjected for washing three times for 5 min in PBS. Non-specific staining then was blocked using 5% normal serum at room temperature for 30 min. Finally, staining was evaluated with diaminobenzidine as a substrate and sections were counterstained with hematoxylin. PBS replaced the antibody in negative controls [49].

Histopathology

Lung tissues were preserved and fixed in 10% of formalin buffer to form Representative slices. After that, samples were embedded in paraffin 4-µm thick then slides were stained by hematoxylin and eosin (H&E). Finally, slides were examined under light microscope [50].

Statistical analysis

All the obtained data were analyzed using SPSS version 20 software (USA). All values were expressed as the mean ± SE. Significance of differences among all groups were analyzed using one-way analysis of variance (ANOVA) followed by Turkey's multiple comparisons post hoc test where $P < .05$ was considered significant.

Results

Oxidative stress biomarkers estimation

AgNPs (20nm and 100 nm) intoxicated animal investigated a significant reduction in SOD values as well as GST levels as compared to negative healthy animals (Fig. 1) with observation that declared that 20 nm AgNPs induce more oxidative stress than those of 100nm. Treatment with *C. edulis* indicated a significant elevation in SOD of both 20nm as well as 100 nm AgNPs groups. However, treatment of 100 nm silver nanoparticles- intoxicated groups with *G. pentaphylla*, extracts, protein and mucilage of *Casimiroa edulis* declared non-significant improvement of oxidative stress biomarker of SOD and GST values (Fig. 1, Fig. 2).

Modulation of Survivin mRNA gene expression

Silver nanoparticle in 20 nm particle sized intoxicated mice demonstrated a significant elevation in surviving gene expression (3.1 fold change) as compared to negative healthy control (Fig. 3). Meanwhile, AgNPs 100 nm particle size doesn't declare an obvious change and there is no any recorded significance values. Treatment with *C. edulis* indicated a significant downregulation in surviving gene expression with 0.8 fold change. However, treatment of 20 nm AgNPs intoxicated groups with *G. pentaphylla*, declared non-significant improvement of survivin gene expression (2.8 fold change). Furthermore, mucilage declared a significant downregulation of surviving gene expression (1.1 fold change) as compared to negative control. Meanwhile, P extract investigated a significant downregulation of surviving gene expression (1.5 fold change) (Fig. 3).

Regulation of MMP-9 mRNA gene expression

Twenty nm AgNPs (20 nm) particle sized intoxicated group recorded a significant elevation in MMP-9 gene expression (3.7 fold change) as compared to healthy mice group as well as a significant elevation was declared upon 100 nm AgNPs intoxication (1.87 fold change). Treatment with *C. edulis* indicated a significant downregulation in MMP-9 gene expression (1.51 fold change). Furthermore, treatment of 20

nm AgNPs intoxicated groups with *G. pentaphylla*, showed a significant improvement of MMP-9 gene expression recording 1.61 fold changes. However, treatment 20 nm AgNPs by protein recorded non-significant improvement (3.1fold change). On the other hand, a significant improvement was declared upon protein treatment followed by 100 nm AgNPs (1.5 fold change) (Fig. 4).

Ki-67 Immunohistochemistry Detection

Immunohistochemistry determination for ki-67 of lung section from negative control mice declared negatively stained ki-67. However, section for samples from obtained from AgNPs (100 nm) illustrated nearly 10% of pneumocytes and investigated positive nuclei for ki-67. Moreover, AgNPs (20 nm) intoxicated group demonstrated 40% of pneumocytes showed positive nuclei for ki-67 (Fig. 5; part I).

Treatment of AgNPs (20 nm) with *C. edulis* indicated nearly 10% of pneumocytes showing positive nuclei for ki-67. Whereas, lung section of AgNPs (20 nm) treated with *G. pentaphylla* showed about 7% of pneumocytes showing positive nuclei for ki-67.Lung section of AgNPs (20 nm) treated mucilage investigated about 2% of pneumocytes showing positive nuclei for ki-67. Furthermore, lung section from AgNPs (20 nm) treated protein group didn't declare any positive staining of ki-67 (Fig. 5; part II).

Lung section of AgNPs (100 nm) intoxicated group followed by *C. edulis* treatment showing negative staining of ki-67. Lung section from AgNPs (100 nm) treatment with glycosmis, declared about 15% of pneumocytes showed positive nuclei for ki-67. Lung section from AgNPs (100 nm) treated mucilage, showing negative staining of ki-67. Lung section of AgNPs (100 nm) treated protein showed negative staining of ki-67 (Fig. 5; part III).

Histopathological investigation

Histopathological examination of lung section from negative control group demonstrated normal alveoli with thin inter-alveolar septum, type I and type II pneumocytes were also detected, in addition to blood vessel and bronchus as shown in section A, Lung section illustrated positive control AgNPs (20 nm) showing alveoli with mild thick inter-alveolar septum which is infiltrated by mild number of lymphocytes and RBCs. Furthermore, type I and type II pneumocytes were also seen and ruptured alveoli as declared in section B. On the other hand, lung section from intoxicated AgNPs (100 nm) group, showing alveoli with markedly thick inter-alveolar septum, the septum is infiltrated by large number of lymphocytes and RBCs ruptured alveoli as demonstrated in section C (Fig. 6; part I).

Upon *C. edulis* treatment of AgNPs (20 nm), lung section declared alveoli with moderate thick inter-alveolar septum, the septum is infiltrated by moderate number of lymphocytes and RBCs, type I and type II pneumocytes were also seen, ruptured alveoli and Bronchus were declared as illustrated in section A. Meanwhile lung section of AgNPs (20 nm) treated with *G. pentaphylla*, showed almost normal alveoli with thin inter-alveolar septum, type I and type II pneumocytes were also seen in section B: Lung section of AgNPs (20 nm), treated mucilage, showing near normal alveoli with thin inter-alveolar septum, type I and type II pneumocytes were also seen, blood vessel, bronchus as demonstrated in section C. Moreover, lung section from AgNPs (20 nm) treated protein, declared alveoli with mild thick inter-alveolar

septum. Furthermore, the septum is infiltrated by mild number of lymphocytes and RBCs, type I and type II pneumocytes were also seen in addition to ruptured alveoli and bronchus as representative in section D (Fig. 6; part II).

As demonstrated in section A, Lung samples of AgNPs (100 nm) by treated *C. edulis*, observed almost normal alveoli with thin inter-alveolar septum, type I and type II pneumocytes were also seen. Lung section from intoxicated AgNPs (100 nm) followed by treatment with *G. pentaphylla*, showing alveoli with mild-moderate thick inter-alveolar septum, the septum is infiltrated by mild number of lymphocytes and RBCs. Furthermore, type I and type II pneumocytes were also seen and ruptured alveoli (B). On the other hand, Lung section from AgNPs (100 nm) treated mucilage, showing alveoli with mild thick inter-alveolar septum, the septum is infiltrated by mild number of lymphocytes and RBCs, type I and type II pneumocytes were also seen and ruptured alveoli (C). Meanwhile, Lung section of AgNPs (100 nm) treated protein, showing almost normal alveoli with thin inter-alveolar septum, type I and type II pneumocytes were also seen, blood vessel and bronchus as shown in section D (Fig. 6; part III).

Discussion

Prevalence of AgNPs application in medicine as well as industry increased the risk of their accumulation in tissues and organs causing toxic effects on internal organs [51]. Most studies on lung toxicity are conducted by inhalation, but during our oral route experiment, it was found that mice had difficulty in breathing with enlarged lungs size, so the present study evaluated the effect of AgNPs toxicity on lung tissue via monitoring biochemical and histological variations. In general, lung in particular concern is the most vulnerable organ to nanoparticles intoxication post exposure via oral treatment or inhalation.

In the current study, AgNPs intoxication in mice model of both low and high sizes (20 nm and 100 nm) declared lung inflammation as well as genotoxic effects due to accumulation of AgNPs in the lung tissue confirming lung toxicity upon AgNPs intoxication. Furthermore, AgNPs (20 nm) demonstrated more toxic effect than AgNPs (100 nm). Moreover, immunohistochemistry and histopathological alternation was monitored in lung tissue, and oxidative stress besides survivin and MMPs mRNA overexpression.

AgNPs toxic effect was determined according to its physical as well as chemical properties according its size [52]. In previous study, AgNPs ranging from 10–20 nm caused a considerable toxic effect [53]. As Ag + ions are related to the cell death, it is urgent to consider the number of Ag + ions in the inventor suspension of AgNPs [54, 55]. Furthermore, it has been verified that endocytosed AgNPs can be degraded in the lysosomes releasing Ag + ions in the cytosol and further cause cell death.

The oxidative stress has been enhanced by injection of AgNPs (20 nm and 100 nm) in the present study. Where, AgNPs contamination contributed to oxidative stress that resulted in a significant reduction in SOD as well as GST. Previous studies also observed the increment of oxidative stress in the aquatic creatures in the heavy metal contaminated aquatic environment [56]. However, AgNPs supplementation with limited dose (0.5 mg/kg) could improve the obtained oxidative stress; high dose of AgNPs (1mg/kg) increase the oxidative stress validating that AgNPs induce toxicity by the effect of Ag + ions [57].

Herein, the current study deduced the up regulation of survivin mRNA in AgNPs induced lung injury in experimental animals. It was also showed that upon treatment with AgNPs 20 nm treated groups via *C. edulis* and *mucilage* improved mRNA gene expression of survivin overexpression. Detection of survivin indicates that it could be considered as an important mediator of cyto-protection, not only in tumor cells but also in lung injured adult cells. Previously, it was confirmed that survivin gene expression was altered in injured lung epithelial cells in both vitro and vivo via activation of caspase-3 and caspase-7 declaring the anti-apoptotic potential of survivin [58]. However, reports observed that survivin could regulate apoptosis and proliferation of both normal cells and cancer cells through similar pathways such as p21-dependent pathways [59]. Continued investigations regarding mechanisms that regulate the expression of survivin and its function in normal and tumor tissues can develop a novel therapeutic strategy [60].

Dysregulation of MMP-9 has been associated with acute lung injury [61]. In the current study, the expression profiles of MMP-9 gene in AgNPs (20nm) intoxicated group of lung tissues were analyzed and declared overexpression of MMP-9 mRNA levels. On the other hand, AgNPs (100 nm) intoxicated group didn't declare a significant change in MMP-9 expression confirming size dependent genotoxic impact of silver nanoparticle. Our results demonstrated that the expression of MMP-9 was significantly overexpressed in AgNPs (20 nm) intoxicated group as indicated by increased pulmonary inflammation and oxidative stress. This finding suggested that pulmonary overexpression of MMP-9 may be a part of a self-protective response as previously reported by [62, 63].

Ki-67, as a DNA-binding nuclear non-histone protein index is an appealing biomarker of lung cancer where, it was previously reported to be increased in lung cancer patients. Furthermore, it was also previously related to prognosis [64]. However, various investigations recorded an intensive variability in Ki-67 index among diverse individuals that reflect a difference in biological response to other injurious agents [65]. In addition, whereas the increment of Ki-67 index is associated with increasing pre-neoplasia histology, there is an extensive variation in histologic profile, investigating that Ki-67 index could be a promising additional prognostic biomarker in various lung injuries [30]. Different previous studies elucidated that ki-67 could be a prognostic and diagnostic biomarker for lung malfunction [30, 31, 66, 67].

In the present study, sections obtained from AgNPs (100 nm) intoxicated group illustrated nearly 10% of pneumocytes and revealed positive nuclei for ki-67. Whereas, AgNPs (20 nm) intoxicated group demonstrated 40% of pneumocytes showed positive nuclei for ki-67. This results proved the AgNPs (20 nm) has more toxic effect than that induced via AgNPs (100 nm).

Furthermore, the correlation between the expressions of Ki-67 in all studied groups was assessed. The results declared that the expression of Ki-67 is related to the powerful of all used regimen in treatment of injured lung samples. Data recorded indicated an obvious improvement in ki-67 expression in both AgNPs (20 nm) and AgNPs (100 nm) with the superiority of mucilage treated group confirming that ki-67 is an attractive biomarker for lung injury related to AgNPs intoxication.

Treatment of AgNPs intoxicated groups *with C. edulis, G.pentaphylla* extracts. In addition to mucilage and protein bioactive constituents demonstrated a remarkable improvement in all tested biochemical parameters, apoptotic biomarker, and immunohistochemistry for ki-67 in addition to histopathological examination. The noticeable elaboration is due to the antioxidative properties of these compounds as it increases their health benefits [68]. Herein, this beneficial impact may be due to the existence of active phenolic compounds such as phenolic acids, flavonoids, and phenolic triterpenes tannins [44]. In the present study, *G. pentaphylla* extracts of stems and leaves showed an obvious antimicrobial as well as antioxidant activities pentaphylla due to the presence of many valuable compounds as phenolics, saponins, alkaloids and tannins. Furthermore, the antioxidant activity of these extracts is associated with high content of flavonoids [69]. Different previous studies were concerned by studying the hepatoprotective effect of vigour of *C. edulis* and *G. pentaphylla* [44, 70–72]. Here, we have shed light on the prospective role of the bioactive constituents present in the current extracts against lung toxicity induced via AgNPs. Rosmarinic acid and Garllic acid are phenolic compounds which can protect lung tissues against oxidative stress and pathological alternation for its antioxidant property, its functional property as scavenging reactive oxygen species and their power to improve body antioxidant situation [38]. Different investigations identified more bioactive compounds with antioxidant properties in *C. edulis* due to the presence of phenolic contents [73].

Additionally, the active constituent Rutin could influence the inhibition of some cancers due to its antioxidant, anti-angiogenic, antiallergic, anti-inflammatory, and antiviral property [74]. Naringenin, dietary flavonoid, improved the physiological alternations caused by isoniazid certain metabolic alteration that declared anti-inflammatory and anti-oxidative properties [75].

In our study, many histopathological changes in the lung tissue of AgNPs intoxicated animal demonstrated alveoli with markedly thick inter-alveolar septum, the septum is infiltrated by large number of lymphocytes and RBCs ruptured alveoli declaring necrosis, and cellular inflammation. These findings are previously recorded by Hassan and Abdelbaky, who reported damage and necrosis in the lung tissue upon AgNPs intoxication [76]. This damage and inflammation is due to the accumulation of silver ions in the lung tissue Furthermore, treatment with *C. edulis* and *G. pentaphylla* showed an obvious improvement in lung tissue declaring thin inter-alveolar septum, type I and type II pneumocytes, blood vessel and bronchus. This improvement is due to the considerable antioxidative impact due to the presence of phenolic compounds such as flavonoids in addition to, cytotoxic and antimicrobial activities [44].

Conclusions

In summary, the current study proved the adverse action of exposure to AgNPs on lung tissue. Lung toxicity was induced via different pathways including oxidative stress, antioxidant mechanisms, induction of inflammation, and alternation of mRNA gene expression, in addition to over-expression of ki-67. Furthermore, AgNPs intoxication caused alterations in lung histology. Treatment of intoxicated animals with *G. pentaphylla, C. edulis* extracts, mucilage and protein the active constituents investigated

their antioxiositive, cytotoxic and antimicrobial activity due to their enrichment with phenolic compounds such as flavonoids.

In conclusion, these extracts and the two active constituents could be promising candidate as therapeutic regimen against lung toxicity induced by AgNPs. Moreover, these plants as functional foods possess potential positive effects on health beyond main nutrition.

Declarations

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Data availability: Upon request.

Ethics Approval: All experimental procedures were approved by the Animal Care and Ethical Committee of NRC.

Consent to Participate: Not applicable.

Consent for Publication: Not applicable.

Competing Interests: The authors have no competing interests to declare.

References

1. Reidy B, Haase A, Luch A, Dawson KA, Lynch I (2013) Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. Mater (Basel) 6:2295–2350
2. Weldon BA, Oberdörster EMF, Workman G, Griffith T, Kneuer WC, Yu IJ (2016) Occupational exposure limit for silver nanoparticles: considerations on the derivation of a general health-based value. Nanotoxicology 10:945–956
3. Stensberg MC, Wei Q, McLamore ES, Porterfield DM, Wei A, Sepúlveda MS (2011) Toxicological studies on silver nanoparticles: challenges and opportunities in assessment, monitoring and imaging. Nanomed (Lond) 6:879–898
4. Cho WS, Duffin R, Thielbeer F, Bradley M, Megson IL, Macnee W, Poland CA, Tran CL, Donaldson K (2012) Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal oxide nanoparticles. Toxicol sciences: official J Soc Toxicol 126:469–477
5. Seiffert J, Buckley A, Leo B, Martin NG, Zhu J, Dai R, Hussain F, Guo C, Warren J, Hodgson A, Gong J, Ryan MP, Zhang J, Porter A, Tetley TD, Gow A, Smith R, Chung KF (2016) Pulmonary effects of inhalation of spark-generated silver nanoparticles in Brown-Norway and Sprague–Dawley rats. Respiratory Res 17:85

6. Sung JH, Ji JH, Park JD, Yoon JU, Kim DS, Jeon KS, Song MY, Jeong J, Han BS, Han JH, Chung YH, Chang HK, Lee JH, Cho MH, Kelman BJ, Yu IJ (2009) Subchronic inhalation toxicity of silver nanoparticles. Toxicol sciences: official J Soc Toxicol 108:452–461
7. Guo H, Zhang J, Boudreau M, Meng J, Yin JJ, Liu J, Xu H (2016) Intravenous administration of silver nanoparticles causes organ toxicity through intracellular ROS-related loss of inter-endothelial junction. Part fibre Toxicol 13:21
8. Wiemann M, Vennemann A, Blaske F, Sperling M, Karst U (2017) Silver Nanoparticles in the Lung: Toxic Effects and Focal Accumulation of Silver in Remote Organs. Nanomaterials 7:441
9. Dubey P, Matai I, Kumar SU, Sachdev A, Bhushan B, Gopinath P (2015) Perturbation of cellular mechanistic system by silver nanoparticle toxicity: Cytotoxic, genotoxic and epigenetic potentials. Adv colloid interface Sci 221:4–21
10. McShan D, Ray PC, Yu H (2014) Molecular toxicity mechanism of nanosilver. J food drug Anal 22:116–127
11. Wang Z, Xia T, Liu S (2015) Mechanisms of nanosilver-induced toxicological effects: more attention should be paid to its sublethal effects. Nanoscale 7:7470–7481
12. Johnston HJ, Hutchison G, Christensen FM, Peters S, Hankin S, Stone V (2010) A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. Crit reviews Toxicol 40:328–346
13. Huk A, Izak-Nau E, Yamani E, Uggerud N, Vadset H, Zasonska M, Duschl B, Dusinska M (2015) Impact of nanosilver on various DNA lesions and HPRT gene mutations - effects of charge and surface coating. Part fibre Toxicol 12:25
14. Nymark P, Catalán J, Suhonen S, Järventaus H, Birkedal R, Clausen PA, Jensen KA, Vippola M, Savolainen K, Norppa H (2013) Genotoxicity of polyvinylpyrrolidone-coated silver nanoparticles in BEAS 2B cells. Toxicology 313:38–48
15. Wang L, Zhang T, Li P, Huang W, Tang J, Wang P, Liu J, Yuan Q, Bai R, Li B, Zhang K, Zhao Y, Chen C (2015) Use of Synchrotron Radiation-Analytical Techniques To Reveal Chemical Origin of Silver-Nanoparticle Cytotoxicity. ACS nano 9:6532–6547
16. Bobyk L, Tarantini A, Béal D, Veronesi G, Kieffer I, Motellier S, Valsami-Jones E, Lynch I, Jouneau P-H, Pernet-Gallay K, Aude-Garcia C, Sauvaigo S, Douki T, Rabilloud T, Carriere M (2021) Toxicity and chemical transformation of silver nanoparticles in A549 lung cells: dose-rate-dependent genotoxic impact,Environmental Science: Nano. **8**
17. Song Z, Yao X, Wu M (2003) Direct Interaction between Survivin and Smac/DIABLO Is Essential for the Anti-apoptotic Activity of Survivin during Taxol-induced Apoptosis*. J Biol Chem 278:23130–23140
18. Ceballos-Cancino G, Espinosa M, Maldonado V, Melendez-Zajgla J (2007) Regulation of mitochondrial Smac/DIABLO-selective release by survivin. Oncogene 26:7569–7575
19. Li F, Ambrosini G, Chu E, Plescia J, Tognin S, Marchisio P, Altieri D, Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC (1999) Altieri DCControl of apoptosis and mitotic spindle checkpoint by

Survivin. Nature 396: 580–584, *Nature.* **396**, 580-4

20. Dallaglio K, Marconi A, Pincelli C (2012) Survivin: A Dual Player in Healthy and Diseased Skin. J Invest Dermatology 132:18–27
21. O'Driscoll L, Linehan R, Clynes M (2003) Survivin: role in normal cells and in pathological conditions. Curr cancer drug targets 3:131–152
22. Chiou SK, Moon WS, Jones MK, Tarnawski AS (2003) Survivin expression in the stomach: implications for mucosal integrity and protection. Biochem Biophys Res Commun 305:374–379
23. Terasaki Y, Terasaki M, Urushiyama H, Nagasaka S, Takahashi M, Kunugi S, Ishikawa A, Wakamatsu K, Kuwahara N, Miyake K, Fukuda Y (2013) Role of survivin in acute lung injury: epithelial cells of mice and humans. Lab investigation; J Tech methods Pathol 93:1147–1163
24. Greenlee KJ, Werb Z, Kheradmand F (2007) Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiological reviews 87:69–98
25. Chelladurai P, Seeger W, Pullamsetti SS (2012) Matrix metalloproteinases and their inhibitors in pulmonary hypertension. Eur respiratory J 40:766–782
26. Craig VJ, Zhang L, Hagood JS, Owen CA (2015) Matrix metalloproteinases as therapeutic targets for idiopathic pulmonary fibrosis. Am J respiratory cell Mol biology 53:585–600
27. Summer R, Krishna R, Schriner D, Cuevas-Mora K, Sales D, Para R, Roman J, Nieweld C, Gochuico BR, Romero F (2019) Matrix metalloproteinase activity in the lung is increased in Hermansky-Pudlak syndrome. Orphanet J rare Dis 14:162
28. Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182:311–322
29. Aslan DL, Gulbahce HE, Pambuccian SE, Manivel JC, Jessurun J (2005) Ki-67 immunoreactivity in the differential diagnosis of pulmonary neuroendocrine neoplasms in specimens with extensive crush artifact. Am J Clin Pathol 123:874–878
30. Miller YE, Blatchford P, Hyun DS, Keith RL, Kennedy TC, Wolf H, Byers T, Bunn PA Jr, Lewis MT, Franklin WA, Hirsch FR, Kittelson J (2007) Bronchial epithelial Ki-67 index is related to histology, smoking, and gender, but not lung cancer or chronic obstructive pulmonary disease. Cancer Epidemiol biomarkers prevention: publication Am Association Cancer Res cosponsored Am Soc Prev Oncol 16:2425–2431
31. Huang Z, Lyu M, Ai Z, Chen Y, Liang Y, Xiang Z (2021) Pre-operative Prediction of Ki-67 Expression in Various Histological Subtypes of Lung Adenocarcinoma Based on CT Radiomic Features,Frontiers in Surgery.**8**
32. Wen S, Zhou W, Li C-m, Hu J, Hu X-m, Chen P, Shao G-l, Guo W (2015) -h. Ki-67 as a prognostic marker in early-stage non-small cell lung cancer in Asian patients: a meta-analysis of published studies involving 32 studies, *BMC Cancer.* **15**, 520
33. Wei D-m, Chen W-j, Meng R-m, Zhao N, Zhang X-y, Liao D-y, Chen G (2018) Augmented expression of Ki-67 is correlated with clinicopathological characteristics and prognosis for lung cancer patients:

an up-dated systematic review and meta-analysis with 108 studies and 14,732 patients. Respiratory Res 19:150

34. Romero ML, Escobar LI, Lozoya X, Enríquez RG (1983) High-performance liquid chromatographic study of casimiroa edulis: I. Determination of imidazole derivatives and rutin in aqueous and organic extracts. J Chromatogr A 281:245–251
35. Aly H, Elrigal N, Ali S, Rizk M, Ebrahim N (2018) Modulatory effects of Casimiroa edulis on aluminium nanoparticles-associated neurotoxicity in a rat model of induced Alzheimer's disease. J Mater Environ Sci 9:1931–1941
36. Magos GA, Vidrio H, Enríquez R (1995) Pharmacology of Casimiroa edulis; III. Relaxant and contractile effects in rat aortic rings. J Ethnopharmacol 47:1–8
37. Moo-Huchin VM, Estrada-Mota I, Estrada-León R, Cuevas-Glory L, Ortiz-Vázquez E, Betancur-Ancona L, Sauri-Duch E (2014) Determination of some physicochemical characteristics, bioactive compounds and antioxidant activity of tropical fruits from Yucatan, Mexico, *Food chemistry.* **152**, 508 – 15
38. Ali SA, Arafa AF, Aly HF, Ibrahim NA, Kadry MO, Abdel-Megeed RM, Hamed MA, Farghaly AA, Regal E, Fouad NS, Khalil GI, Refaat EA (2020) DNA damage and genetic aberration induced via different sized silver nanoparticles: Therapeutic approaches of Casimiroa edulis and Glycosmis pentaphylla leaves extracts. J Food Biochem 44:e13398
39. Nagai H, Tanaka T, Goto T, Kusudo T, Takahashi N, Kawada T (2014) Phenolic compounds from leaves of Casimiroa edulis showed adipogenesis activity. Bioscience Biotechnol Biochem 78:296–300
40. Awaad AS, Al-Jaber NA, Soliman GA, Al-Outhman MR, Zain ME, Moses JE, El-Meligy RM (2012) New biological activities of Casimiroa edulis leaf extract and isolated compounds. Phytotherapy research: PTR 26:452–457
41. Shaalan EA, Canyon D, Younes MW, Abdel-Wahab H, Mansour AH (2005) A review of botanical phytochemicals with mosquitocidal potential. Environ Int 31:1149–1166
42. Khandokar L, Bari MS, Seidel V, Haque MA (2021) Ethnomedicinal uses, phytochemistry, pharmacological activities and toxicological profile of Glycosmis pentaphylla (Retz.) DC.: A review. J Ethnopharmacol 278:114313
43. Murugan N, Srinivasan R, Murugan A, Kim M, Natarajan D (2020) Glycosmis pentaphylla (Rutaceae): A Natural Candidate for the Isolation of Potential Bioactive Arborine and Skimmianine Compounds for Controlling Multidrug-Resistant Staphylococcus aureus. Front Public Health 8:176–176
44. Ibrahim N, El Hawary S, Mohammed M, Ali S, Kandil Z, Refaat E (2019) Chemical compositions and hypoglycemic activities of the protein and mucilage of Casimiroa edulis (Llave & Lex) seeds and fruits. J Appl Pharm Sci 9:084–091
45. Elkhawass E, Mohallal ME, Soliman MFM (2015) Acute toxicity of different sizes of silver nanoparticles intraperitonally injected in BALB/c mice using two toxicological methods.. 7:94–99

46. Buller AL, Clapper ML, Tew KD (1987) Glutathione S-transferases in nitrogen mustard-resistant and -sensitive cell lines. Mol Pharmacol 31:575
47. Nishikimi M, Appaji Rao N, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 46:849–854
48. Abdel-Megeed RM, El-Alim A, Arafa SH, Matloub AF, Farrag AA, Darwish A, Abdel-Hamid AB, Kadry MO (2020) Crosslink among phosphatidylinositol-3 kinase/Akt, PTEN and STAT-5A signaling pathways post liposomal galactomannan hepatocellular carcinoma therapy. Toxicol Rep 7:1531–1541
49. Aboushousha T, Hammam O, Safwat G, Eesa A, Ahmed S, Esmat ME, Helmy AH (2018) Differential Expression of RAGE, EGFR and Ki-67 in Primary Tumors and Lymph Node Deposits of Breast Carcinoma. Asian Pac J cancer prevention: APJCP 19:2269–2277
50. Hopwood D, Bancroft J, Stevens A (1996) Theory and practice of histological techniques, *Theory and practice of histological techniques*
51. Ali SA, Gooda SM, Naser A, Younis AF, Hamed EA, Ahmed MA, Farghaly YR, Khalil AA, Rizk MZ (2022) Chromosomal aberrations, DNA damage and biochemical disturbances induced by silver nanoparticles in mice: Role of particle size and natural compounds treatment, *Biomarkers*, 1–34
52. De Matteis V, Malvindi MA, Galeone A, Brunetti V, De Luca E, Kote S, Kshirsagar P, Sabella S, Bardi G, Pompa PP (2015) Negligible particle-specific toxicity mechanism of silver nanoparticles: the role of Ag + ion release in the cytosol, *Nanomedicine: nanotechnology, biology, and medicine.* **11**, 731-9
53. Ma W, He S, Ma H, Jiang H, Yan N, Zhu L, Bang JJ, Li PA, Jia S (2020) Silver Nanoparticle Exposure Causes Pulmonary Structural Damage and Mitochondrial Dynamic Imbalance in the Rat: Protective Effects of Sodium Selenite. Int J Nanomed 15:633–645
54. Beer C, Foldbjerg R, Hayashi Y, Sutherland D, Autrup H (2011) Toxicity of silver nanoparticles-Nanoparticle or silver ion? Toxicol Lett 208:286–292
55. Pratsinis A, Hervella P, Leroux JC, Pratsinis SE, Sotiriou GA (2013) Toxicity of silver nanoparticles in macrophages. Small (Weinheim an der Bergstrasse Germany) 9:2576–2584
56. Kumar N, Krishnani KK, Gupta SK, Singh NP (2018) Effects of silver nanoparticles on stress biomarkers of Channa striatus: immuno-protective or toxic? Environ Sci pollution Res Int 25:14813–14826
57. Almofti MR, Ichikawa T, Yamashita K, Terada H, Shinohara Y (2003) Silver ion induces a cyclosporine a-insensitive permeability transition in rat liver mitochondria and release of apoptogenic cytochrome C. J Biochem 134:43–49
58. Fukuda S, Pelus LM (2006) Survivin, a cancer target with an emerging role in normal adult tissues. Mol cancer Ther 5:1087–1098
59. Fukuda S, Mantel CR, Pelus LM (2004) Survivin regulates hematopoietic progenitor cell proliferation through p21WAF1/Cip1-dependent and -independent pathways. Blood 103:120–127

60. Terasaki Y, Terasaki M, Urushiyama H, Nagasaka S, Takahashi M, Kunugi S, Ishikawa A, Wakamatsu K, Kuwahara N, Miyake K, Fukuda Y (2013) Role of survivin in acute lung injury: epithelial cells of mice and humans. Lab Invest 93:1147–1163
61. Wang C, Li D, Qian Y, Wang J, Jing H (2012) Increased matrix metalloproteinase-9 activity and mRNA expression in lung injury following cardiopulmonary bypass. Lab investigation; J Tech methods Pathol 92:910–916
62. Mahor D, Kumari V, Vashisht K, Galgalekar R, Samarth RM, Mishra PK, Banerjee N, Dixit R, Saluja R, De S, Pandey KC (2020) Elevated serum matrix metalloprotease (MMP-2) as a candidate biomarker for stable COPD. BMC pulmonary medicine 20:302
63. Zhang H, Mao YF, Zhao Y, Xu DF, Wang Y, Xu CF, Dong WW, Zhu XY, Ding N, Jiang L, Liu YJ (2021) Upregulation of Matrix Metalloproteinase-9 Protects against Sepsis-Induced Acute Lung Injury via Promoting the Release of Soluble Receptor for Advanced Glycation End Products, *Oxidative medicine and cellular longevity.* **2021**, 8889313
64. Naheed S, Holden C, Tanno L, Pattini L, Pearce NW, Green B, Jaynes E, Cave J, Ottensmeier CH, Pelosi G (2022) Utility of KI-67 as a prognostic biomarker in pulmonary neuroendocrine neoplasms: a systematic review and meta-analysis. BMJ open 12:e041961
65. Shaikh SB, Prabhu A, Bhandary YP (2020) Curcumin Suppresses Epithelial Growth Factor Receptor (EGFR) and Proliferative Protein (Ki 67) in Acute Lung Injury and Lung Fibrosis In vitro and In vivo. Endocr metabolic immune disorders drug targets 20:558–563
66. Chirieac LR (2016) Ki-67 expression in pulmonary tumors. Translational lung cancer research 5:547–551
67. Wang D, Ye W, Shi Q (2021) Prognostic Value of Ki-67 Expression in Advanced Lung Squamous Cell Carcinoma Patients Treated with Chemotherapy. Cancer Manage Res 13:6429–6436
68. Awaad AS, El-Sayed N, Maitland D, Mabry T (2006) Phenolic Antioxidants from Casimiroa edulis. Leaves, *Pharmaceutical biology.* **44**, 258–262
69. Murugan N, Srinivasan R, Murugan A, Kim M, Natarajan D (2020) Glycosmis pentaphylla (Rutaceae): A Natural Candidate for the Isolation of Potential Bioactive Arborine and Skimmianine Compounds for Controlling Multidrug-Resistant Staphylococcus aureus,Front Public Health.**8**
70. Adefegha SA (2018) Functional foods and nutraceuticals as dietary intervention in chronic diseases; novel perspectives for health promotion and disease prevention. J Diet Supplements 15:977–1009
71. Park SY, Kim Y-S, Ahn C-B, Je J-Y (2016) Partial purification and identification of three antioxidant peptides with hepatoprotective effects from blue mussel (Mytilus edulis) hydrolysate by peptic hydrolysis. J Funct Foods 20:88–95
72. Kumar R, Kumar R, Prakash O, Srivastava R, Pant A (2019) GC-MS analysis of the hexane extract of Limnophila indica (L.) Druce, its total phenolics, in-vitro antioxidant, anti-inflammatory and antifeeding activity against Spilosoma obliqua. J Entomol Zool Stud 7:970–975
73. El-Kady T, El-Rahman A, Toliba AO, Abo El-maty S (2017) Evaluation of Mango Seed kernel Extract as natural occurring phenolic rich antioxidant compound. Bull Natl Nutr Inst Arab Repub Egypt 48:1–30

74. Sherif MH, Hussein MM, Eid RS, Abas A-SM (2019) Biochemical Study On the Effect of Morin and Rutin in amelioration of Titanium dioxide Nanoparticle Toxicity in rat liver. Biochem Lett 15:167–185
75. Abdel-Ghaffar O, Ali AA-M, Soliman SA-M (2018) Protective effect of naringenin against isoniazid-induced adverse reactions in rats. Int J Pharmacol 14:667–680
76. Hasan E, Abdelbaky F (2018) Dermal Application of Silver Nano-particles on Adult Mice: A Histopathological and immunohistochemistry study of kidney and lung. Ain Shams Journal of Forensic Medicine and Clinical Toxicology 30:52–59

Table 1

Table 1 is not available with this version

Figures

Fig 1:

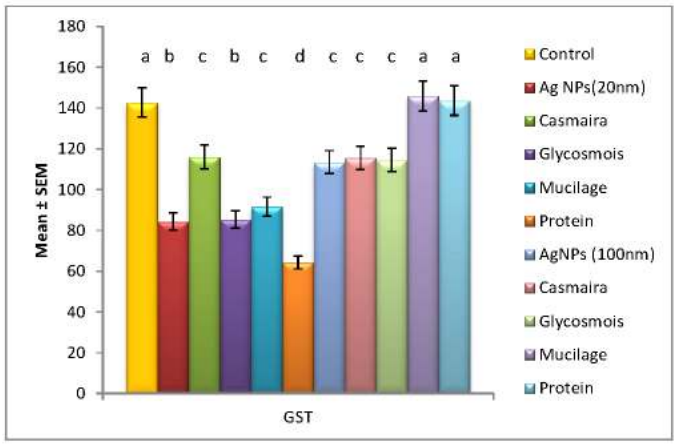

Figure 1

Effect of *C. edulis, G. pentaphylla,* mucilage and protein extracts on superoxide Dismutase antioxidant post AgNPs (20 & 100 nm) intoxication. Data were expressed as means ± SEM (n=10). P <0.05 was considered significant. Groups had the same letter are not significantly different from each other, while those had different letters are significantly different from each other.

Fig 2:

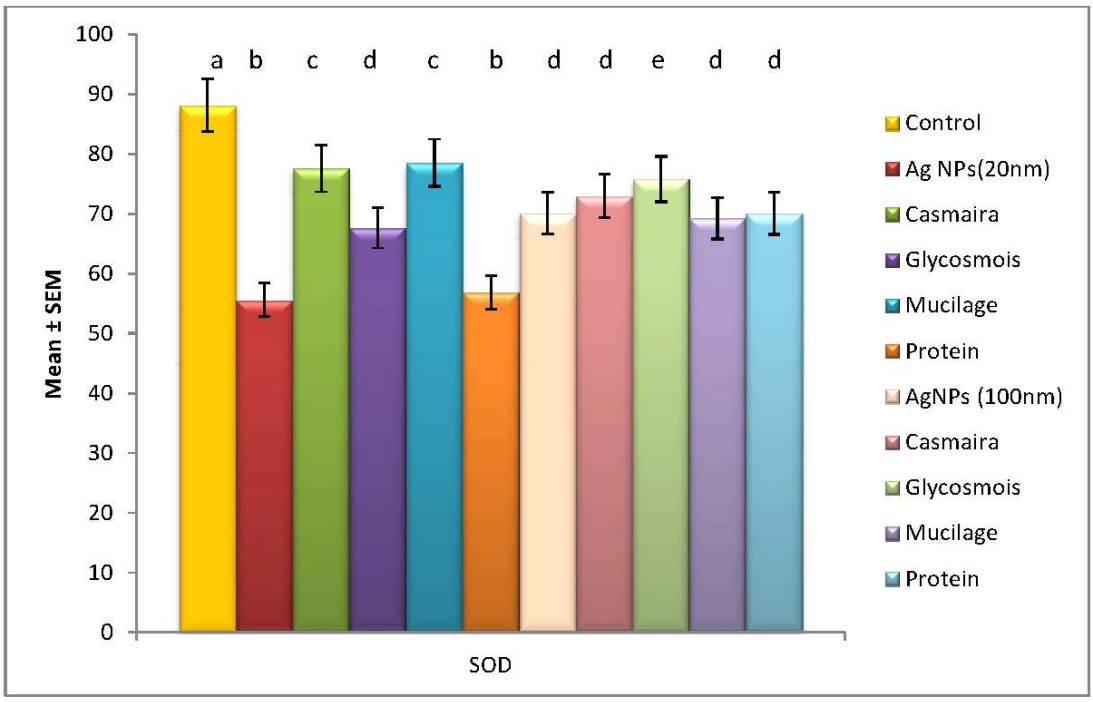

Figure 2

Effect of *C. edulis, G. pentaphylla,* mucilage and protein extracts on superoxide Dismutase antioxidant post AgNPs (20 & 100 nm) intoxication. Data were expressed as means ± SEM (n=10). P <0.05 was considered significant. Groups had the same letter are not significantly different from each other, while those had different letters are significantly different from each other.

Fig. 3

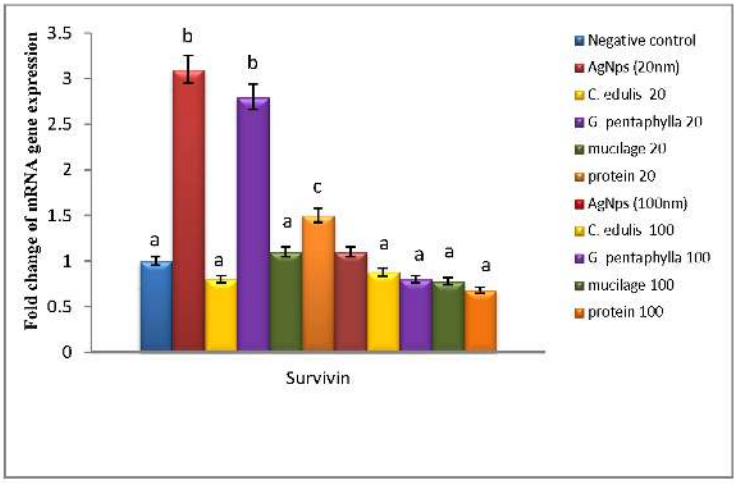

Figure 3

Effect of *C. edulis, G. pentaphylla*, mucilage and protein extracts on mRNA gene expression of Survivin post AgNPs (20 & 100 nm) intoxication. P< 0.05 was considered significant. Groups had the same letter are not significantly different from each other, while those had different letters are significantly different from each other.

Fig. 4:

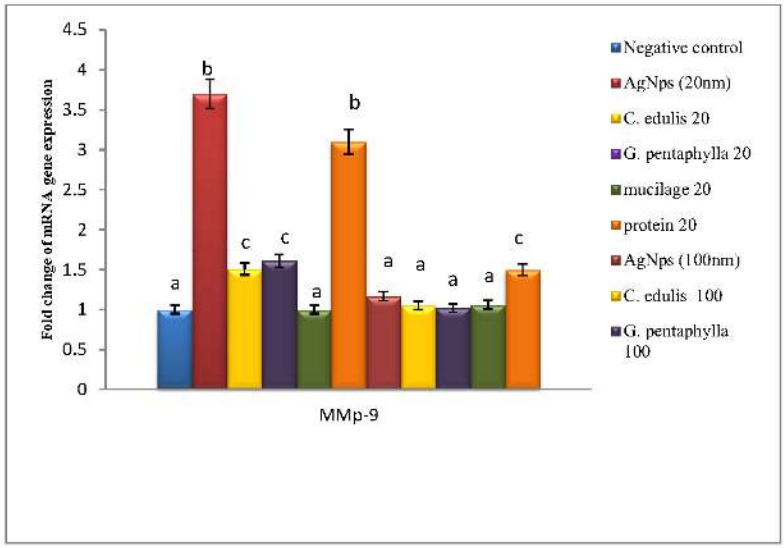

Figure 4

Effect of *C. edulis, G. pentaphylla*, mucilage and protein extracts on mRNA gene expression of matrix metalo-proteinase (MMP) post AgNPs (20 & 100 nm) intoxication. P< 0.05 was considered significant. Groups had the same letter are not significantly different from each other, while those had different letters are significantly different from each other.

Fig. 5:

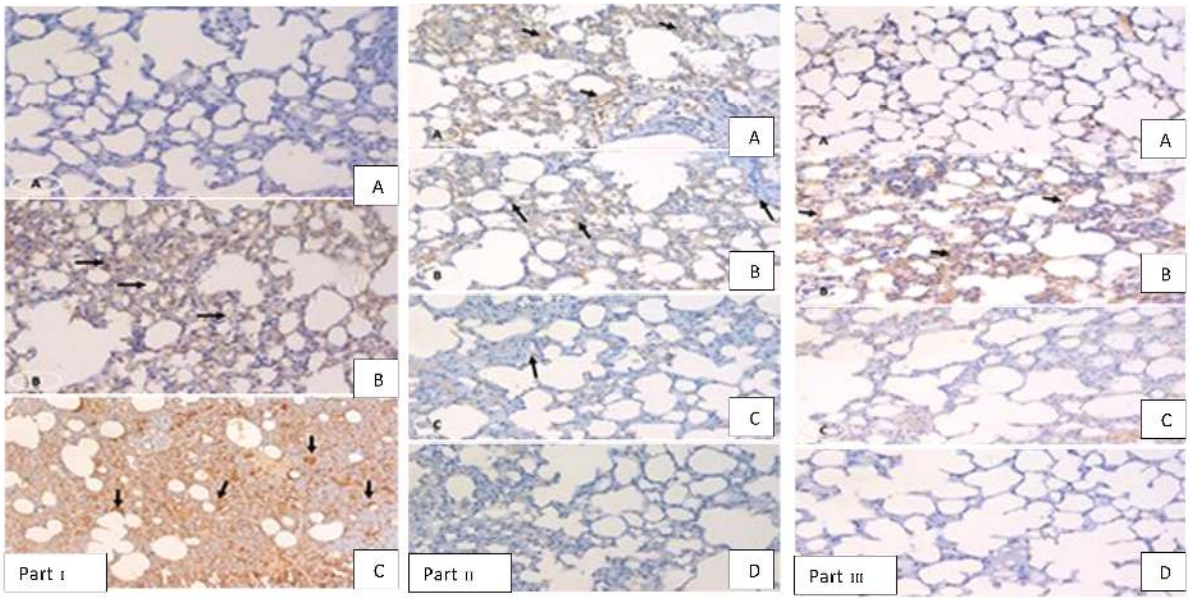

Figure 5

Lung section from healthy mice showed negative staining of ki-67. However, section for samples from obtained from Ag-NPs (100nm) illustrated nearly 40% of pneumocytes and investigated positive nuclei for ki-67. Moreover, Ag-NPs (20 nm) intoxicated group demonstrated 10% of pneumocytes showed positive nuclei for ki-67.

Lung section from 20 nm (AgNPs) treated with *C. edulis***,** showing about 10% of pneumocytes showing positive nuclei for ki67(as brownish color)(black arrows), (IHC, ki67 ,x400).**(B)**:Lung section of 20 nm (AgNPs) treated with *G. pentaphylla***,** showing about 7% of pneumocytes showing positive nuclei for ki67(as brownish color)(black arrows), (IHC, ki67 ,x400).**(C)**:Lung section of 20nm(AgNPs) treated mucilage**,** showing about 2% of pneumocytes showing positive nuclei for ki67(as brownish color)(black arrows), (IHC, ki67 ,x400).**(D):** Lung section from 20 nm (AgNPs) treated protein**,** showing negative staining of ki67 (IHC, ki67,x400).

Lung section of 100nm(AgNPs) treated *C. edulis*, showing negative staining of ki67 (IHC, ki67,x400).**(B):** Lung section from 100nm (AgNPs) treated with *G. pentaphylla*,showing about 15% of pneumocytes showing positive nuclei for ki67(as brownish color)(black arrows), (IHC, ki67 ,x400).**(C)**: Lung section from 100nm (AgNPs) treated mucilage,showing negative staining of ki67 (IHC, ki67,x400).**(D)**:Lung section of 100 nm (AgNPs) treated protein, showing negative staining of ki67 (IHC,ki67,x400).

Fig. 6:

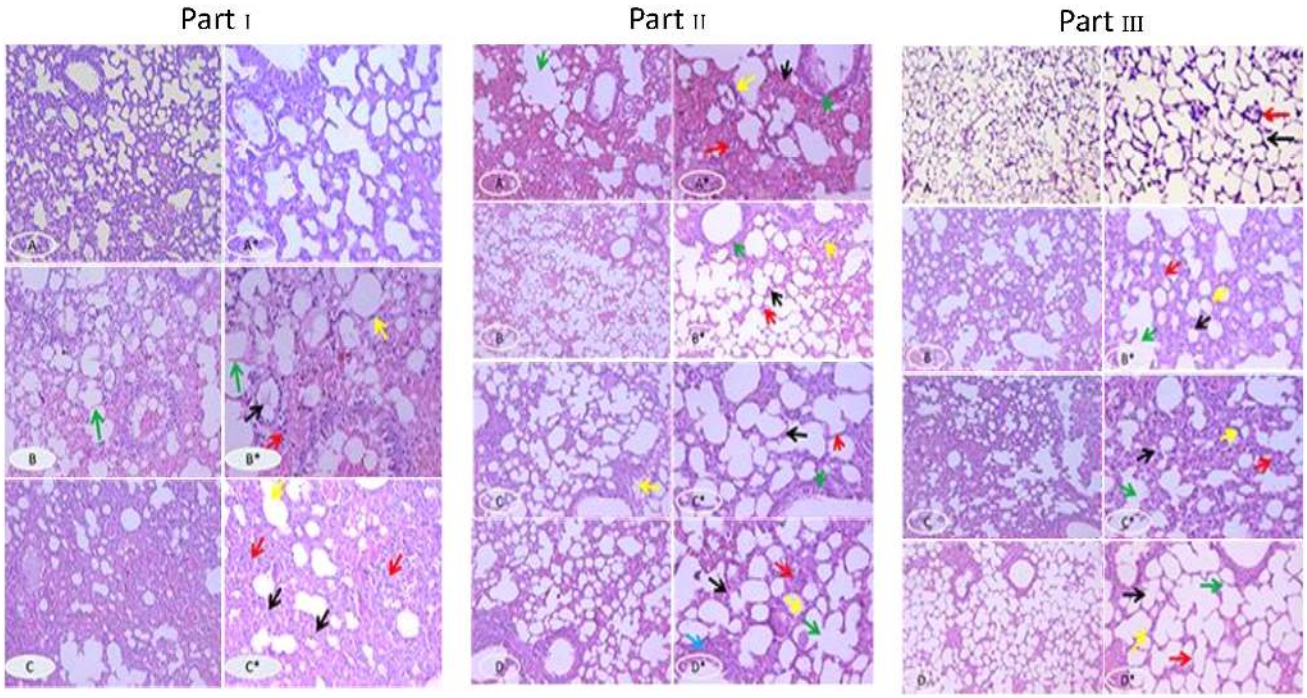

Figure 6

part I: Lung section from healthy mice group showing normal alveoli with thin inter-alveolar septum (black arrow), type I and type II pneumocytes were also seen (red arrow), blood vessel (yellow arrow), bronchus (green arrow) (H&E, x200 ,x400). **(B)**: Lung section from 20nm (AgNPs) showing alveoli with mild thick inter-alveolar septum (black arrow),the septum is infiltrated by mild number of lymphocytes and RBCs (red arrow), type I and type II pneumocytes were also seen (yellow arrow), ruptured alveoli (green arrow) (H&E, x200 ,x400). **(C)** Lung section from 100nm (AgNPs), showing alveoli with markedly

thick inter-alveolar septum (black arrow), the septum is infiltrated by large number of lymphocytes and RBCs (red arrow), ruptured alveoli (yellow arrow) (H&E, x200,x400)

part II: Lung section from 20nm (AgNPs) treated with *C. edulis***,** showing alveoli with moderate thick inter-alveolar septum (black arrow),the septum is infiltrated by moderate number of lymphocytes and RBCs (red arrow), type I and type II pneumocytes were also seen (yellow arrow), ruptured alveoli (green arrow), Bronchus (green arrow) (H&E, x200 ,x400). **(B)**:Lung section of 20nm (AgNPs) treated with *G. pentaphylla***,** showing almost normal alveoli with thin inter-alveolar septum (black arrow), type I and type II pneumocytes were also seen (red arrow), blood vessel (yellow arrow), bronchus (green arrow) (H&E, x200 ,x400) **(C)**:Lung section of 20nm (AgNPs)treated mucilage**,** showing near normal alveoli with thin inter-alveolar septum (black arrow), type I and type II pneumocytes were also seen (red arrow), blood vessel (yellow arrow), bronchus (green arrow) (H&E, x200 ,x400). **(D):** Lung section from 20nm (AgNPs) treated protein**,** showing alveoli with mild thick inter-alveolar septum (black arrow),the septum is infiltrated by mild number of lymphocytes and RBCs (red arrow), type I and type II pneumocytes were also seen (yellow arrow), ruptured alveoli (green arrow), Bronchus (blue arrow) (H&E, x200 ,x400).

part III: Lung section of 100nm (AgNPs) treated *C. edulis***,** showing almost normal alveoli with thin inter-alveolar septum (black arrow), type I and type II pneumocytes were also seen (red arrow (H&E, x200 ,x400).**(B):** Lung section from 100nm (AgNPs) treated with *G. pentaphylla* group**,** showing alveoli with mild-moderate thick inter-alveolar septum (black arrow),the septum is infiltrated by mild number of lymphocytes and RBCs (red arrow), type I and type II pneumocytes were also seen (yellow arrow), ruptured alveoli (green arrow) (H&E, x200 ,x400).**(C)**: Lung section from 100nm (AgNPs) treated mucilage**,** showing alveoli with mild thick inter-alveolar septum (black arrow),the septum is infiltrated by mild number of lymphocytes and RBCs (red arrow), type I and type II pneumocytes were also seen (yellow arrow), ruptured alveoli (green arrow) (H&E, x200 ,x400). **(D)**:Lung section of 100nm (AgNPs) treated protein**,** showing almost normal alveoli with thin inter-alveolar septum (black arrow), type I and type II pneumocytes were also seen (red arrow), blood vessel (yellow arrow), bronchus (green arrow) (H&E, x200 ,x400).