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Immunological, histological and immunohistochemical alternations induced by zinc oxide nanoparticles and mureer plant in spleen albino rats with the prospective anti-inflammatory action of gallic acid

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Abstract: The current study was proposed to evaluate the mortal impacts of either alone or mixed treatments of zinc oxide nanoparticles (ZnO NPs) and mureer or *Senecio glaucus* L. plant (SP) on spleen tissue via immunological and histological studies and to estimate the likely immunomodulatory effect of gallic acid (GA) for 30 days in rats. Rats were classified into eight groups with orally treated: Control, GA (100mg/kg), ZnO NPs (150mg/kg), SP (400mg/kg), GA+ZnO NPs (100,150mg/kg), GA+SP (100,400mg/kg), ZnONPs+SP (150,400mg/kg) and GA+ZnONPs+SP (100,150,400mg/kg). Interleukin-6 (IL-6) level was measured using an enzyme-linked immunoassay (ELISA). Also, the pro-apoptotic protein (caspase-3) expression was estimated using an immunohistochemistry assay. Our data revealed that ZnO NPs and SP triggered a significant increase in the levels of IL-6 and total lipids (TL) and the activity of lactate dehydrogenase (LDH), ($p<0.001$). Furthermore, they overexpressed caspase-3 and caused lymphoid depletion. They revealed that the immunotoxic outcome of mixed treatment was more than the outcome of the alone treatment. However, GA restored the spleen damage from these adverse results. Finally, this study indicated that ZnO NPs and SP might be immunotoxic and splenotoxic agents; however, GA may be displayed as an anti-inflammatory and splenic-protective agent.

Keywords: Zinc oxide nanoparticles, mureerplant, gallic acid, spleen, pro-inflammatory cytokine (il-6), pro-apoptotic protein (caspase-3).

INTRODUCTION

Today, nanotechnology is an industrialized science that can create invented nanoparticles. Curiously, nanoparticles (NPs) have already taken part in the manufacturing of a diversity of profitable uses in plentiful energetic arenas, including medicine, industry and farming. Moreover, they have a very small size with a range (1-100 nm), giving them the ability to permit through any biological membranes. Based on the literature, NPs have used in a wide range of marketable imposts in the bazaar; however, they convinced oxidative stress and inflammatory reactions (George *et al*., 2018; Durazzo *et al*., 2020). Amongst the factory-made dominant nanoparticles, zinc oxide nanoparticles (ZnO NPs) are the best-known NPs used in various dynamic solicitations, including sunscreen, batteries, ceramics, cosmetics and sensors. ZnO NPs have dissolved in body fluids and passed easily inside the cell. Then, they may persuade many disparities in the mitochondrial electron potential path, which may drive the creation of pro-inflammatory cytokines (Singh, 2019; Holmes *et al*., 2020). Still, they are mainly stored in the liver organ, which could be the target organ followed by the spleen organ (Yuan *et al*., 2019).

Expending of natural plants is broadly used as a substitute custom of insecticidal yields. Amid them, mureer or *Senecio glaucus* subsp. coronopifolius (Maire) C. Alexander L. (SP) plant belongs to the largest family of Asteraceae, which is distributed in dry and steamy regions. It has antimicrobial, antioxidant and reducing power actions (Mohamed *et al*., 2022). Likewise, *Senecio* plants are used as therapeutic resources in a traditional medication, such as treatment of cancer, cough suppressant, eczema inflammation and muscular pain (Faraone *et al*., 2018). Unfortunately, they may cause numerous clinicopathological changes in consumed animals (García *et al*., 2020). No previous studies have examined the poisonous impressions of SP on the spleen in rats, so this study focused on it. In particular, the whole parts of *Senecio* plants have many chemical elements, such as flavonoids, lipids, proteins, phenolic compounds and saponins that encourage cell death in biological cells (Jameel *et al*., 2021).

Immunologically, both the liver and spleen play energetic roles in immune homeostasis. Thus, the notion of a liver-spleen axis acts as junctions for immunity and metabolism of different compounds (Bozward *et al*., 2021). Despite the fact that the spleen is an important organ of the immune system, which is responsible for defending the body against any inspired harm. It is a secondary lymphoid organ, which includes parenchyma cells of white pulp (WP) and red pulp (RP). The WP contains highly organized accumulations of B and T lymphocytes around arterioles (Hermida *et al*., 2018).

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Therefore, they can secret pro-inflammatory cytokines that are decisive molecules, releasing from lymphocytes to induce an immune response against any grievance, such as interferon-1 (INF-1), tumor necrosis factor (TNF-α), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) during nuclear factor kappa B (NF-κB) mechanism initiation (Ciebiera *et al*., 2018). In particular, zinc homeostasis interruption disturbs the innate and adaptive immune responses, which are responsible for the induction of prolonged inflammatory disorders. The extrinsic apoptosis pathway or death receptor pathway is responsible for the creation of pro-inflammatory cytokines, persuading caspases or cysteine-aspartic acid protease family activation, such as caspase-3 protein (Micheau, 2018). To preclude the inimical effects of different toxins, IL-6 is studied that promotes specific differentiation of naïve $CD_{4+}T$ cells, performing an important function in the linking between both immune responses (Velazquez-Salinas *et al*., 2019).

Looking for impeccable protection to counter inflammatory cytokines, mediated injury stays a huge scientific challenge. Gallic acid (GA), (3,4,5-trihydroxy benzoic acid), is a phenolic compound dispersed in diverse families of the plant kingdom, such as blueberry, blackberry, strawberry, plums and so on. It displays prevailing cytoprotective and anti-inflammatory activities in living cells (Liu *et al*., 2020; Kulkarni and Swamy, 2020). With regard to distinguishing the link between the structure of GA and its immune-protective effect, GA has an aromatic structure in its conformation that scavenges free radicals. Besides, it can connect with metals that cause tissue destruction. Similarly, the anti-inflammatory properties of GA appear through the inhibition of the pro-inflammatory mediator expression (Bai *et al*., 2021).

Therefore, the main purpose of this study was to investigate the underlying mechanism of immunotoxicity persuaded by ZnO NPs and SP via examining of the biochemical, immunological and histological studies in the spleen tissue and to explore the imaginable splenic-protective role of GA against splenic-toxicity in model rats.

MATERIALS AND METHODS

Chemicals and reagents

Zinc oxide nanoparticles (ZnONPs) (<50nm) (BET), sodium carboxymethyl cellulose (Na-CMC) salt and gallic acid (GA) were commercially gotten from Sigma Aldrich (St.Louis, Missouri, USA). 70% of ethanol solvent was purchased from (EL-Naser Company, Egypt). An interleukin-6 (Il-6) kit was procured from (NOVA Company, Beijing, China). Correspondingly, total lipids (TL) kit was bought from (Biodiagnostic Company, Egypt). In addition, the lactate dehydrogenase enzyme (LDH) kit was bought from (Egyptian Company for Biotechnology (S.A.E), Egypt). Other substances and components were used for high-grade types.

Analysis of suspended ZnO NPs in Na-CMC

The description of suspended ZnO NPs in Na-CMC was performed to investigate the hydrodynamic diameter of surface charge, using Particles Sizing System (PPS) (INC Santa Barbara, Calif, USA-Model: ZPW338-V2-14/ZPW388.tbl).

Plant extraction and investigation of phytochemical constituents

The plant was collected from Cairo-Ismailia Highway, Egypt, according to the Uniprot database with Taxon identifier (183639) (Ghazanfar *et al*., 2019).1500 g of total parts of the plant was shrunk in the laboratory and extracted in 70% of ethanol for 3 days in glass jars. Then, the solution was filtered with a Whatman paper, using a rotary evaporator (IKA-WERK,RV10, China) at 60˚C to obtain the final extract and arid at 45˚C to acquire the greenish extract. The harvest of extraction was retained at -20˚C for the trial.

Dose preparation

ZnO-NPs, SP and GA suspensions were suspended in 0.5% of Na-CMC for 15 min into a sonicator.

Experimental protocol

Forty albino male rats (*Rattus norvegicus*), weighted 180-220g b.wt, were used in this study. This study was allowed by the Animal Ethics Committee of Zagazig University as an approval number (ZU-IACUC/1/F/42/ 2019) in Medicine Faculty. The rats were retained at an organized temperature (23±1˚C), humidity (55±5%) and a 12h dark/light cycle with *ad libitum* access to food and water. Afterward a week of acclimation, the rats were separated into eight groups and each group has 5 rats. 1) Control group: Rats were treated with 0.5% Na-CMC as a vehicle (5ml/kg of 0.5% Na-CMC/rat) (Dhiyaaldeen *et al*., 2014). 2) GA-treated group: Rats were treated with (100 mg/kg of GA) (Mansouri *et al*., 2013), suspending in 0.5% Na-CMC (Sen *et al*., 2013). 3) ZnO NPs-treated group: Rats were treated with (150 mg/kg of ZnO NPs), suspending in 0.5% Na-CMC (Srivastav*et al*., 2016). 4) SP-treated group: Rats were treated with (400 mg/kg of SP) (El Sheikh *et al*., 2021). 5) GA+ZnO NPs-treated group: Rats were treated with (GA and ZnO NPs) at (100 and 150 mg/kg). 6) GA+SP-treated group: Rats were treated with (GA and SP) at (100 and 400 mg/kg). 7) ZnONPs+SP-treated group: Rats were treated with (ZnO NPs and SP) at (150 and 400 mg/kg). 8) GA+ZnONPs+ SP-treated group: Rats were treated with (GA, ZnO NPs and SP) at (100, 150 and 400 mg/kg). The management of GA was achieved before the handling of other elements about 10 min. The supervision of all these agents was taken via gavage, suspending in 0.5% Na-CMC (w/v).

At the finale of 30 days, rats were sacrificed by cervical dislocation and collected: Serum and spleen tissues. They were excised and divided into two portions: The first portion of spleen tissue was immediately uninvolved, crushed, weighed and homogenized with 0.9% NaCl in a homogenizer. The homogenates were centrifuged at 3.000×g rpm and the later supernatants were kept on -80 ˚C and the second portion was fixed in 10% formalin buffered saline for histopathological and immunohistochemical studies.

Biochemical biomarker evaluation

Splenic lactate dehydrogenase activity (LDH) assessment

The lactate dehydrogenase (LDH) activity was assessed according to the technique of kit: 20µl of splenic homogenate was added to 1ml of working solution, diversified and read at a preliminary absorbance after 30 sec. Then, it was read again after 1, 2 and 3 min and measured the modification absorbance per min (Zimmerman and Hennery, 1979).

Serum total lipids level estimation

Total lipids test (TL) was measured according to Zollner and Kirsch, (1962) method and detected at the absorbance of 545 nm.

Immunological biomarker estimation

Splenic pro-inflammatory cytokine interleukin-6 (IL-6) level evaluation

Splenic IL-6 was determined using ELISA (Enzyme-Linked Immunosorbent) bioassay according to the manufacturer's instructions on NOVA kit. The standard and samples were pipetted into wells with immobilized antibodies specific for rat IL-6 and incubated for 30 min at 37˚C. After incubation and washing, a horseradish peroxidase-conjugated streptavidin was pipetted into the wells and incubated for 30 min at 37˚C. A and B chromogens were added to the wells, incubated for 15 min at 37˚C to appear a specific color and measured at 450 nm. The results were expressed at (pg/mg protein).

Histopathological investigation

Spleen specimens were fixed using 10% neutral buffered formaldehyde. After proper fixation, the specimens were dehydrated and embedded in paraffin wax. 5-µm thick sections were cut using a rotatory microtome, stained with hematoxylin and eosins (H&E.,) staining for studying the general histological structure of the spleen according to Bancroft and Layton method (2012) to observe under a light microscope.

Immunohistochemistry investigation

The paraffin-embedded spleens were amended into 4-µm pieces and fixed on positively charged slides for the expression of a caspase-3 protein. The immunohistochemical reaction was prepared using the peroxidase/anti-peroxidase (PAP) according to the method of Ramos-Vara *et al*, (2008). The nonspecific peroxidase reaction was upset with methanol, comprising from 0.1% H_2O_2. The slides were incubated with normal goat serum to dodge nonspecific reaction once the samples have gestated with the exact antibodies against caspase-3 (dilution, 1:2000). The tissue sections were washed with phosphate buffer and incubated with secondary antibodies (dilution, 1:2000). The peroxidase reaction was carried out using a solution of 3,3'-diaminobenzidine tetrahydrochlorideen (0.01% H_2O_2 in Tris-HCl buffer). After immunostaining, the spleen sections were counterstained with (H&E.,) staining, recognizing underneath a light microscope.

STATISTICAL ANALYSIS

Data were described as a mean±standard deviation (mean±SD) using statistical software package SPSS for Windows 20.0 to do a comparison between the biological analyses using a one-way ANOVA test. It was followed by Tukey's post hoc test for the comparison between several groups. The level of significance was considered at $P<0.05$ (IBM Corp. SPSS, 2011).

RESULTS

The assessment of the hydrodynamic diameter of surface charge of the suspended ZnO NPs

Our results showed that the hydrodynamic diameter of surface charge was found to be (-50.54 mV), using PPS to suspended ZnO NPs (fig. 1).

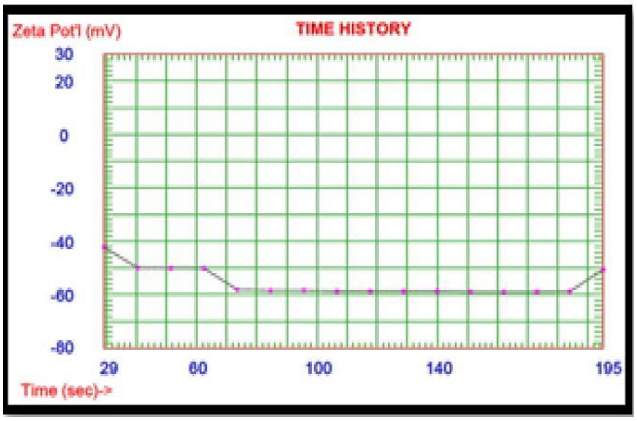

Fig. 1: Photograph of hydrodynamic diameter of surface charge of suspended ZnO NPs using particles sizing system (PPS).

Evaluation of Biochemical and immunological biomarkers

To evaluate the immunotoxic impact of ZnO NPs and SP, our study estimated some biochemical and immunological biomarkers in spleen lysate and serum, such as (interleukin-6 level (IL-6) (pg/mg protein), lactate dehydrogenase activity (LDH) (U/mg protein) and total

lipids level (TL) (mg/dl) (table 1).Our results noticed that either alone or mixed treatments of ZnO NPs and SP produced a significant elevation in splenic il-6 level, splenic LDH activity and serum TL level compared to the control group, ($p<0.001$). In addition, the toxic effects of the combined treatment of ZnO NPs and SP were stronger than the effect of the alone treatment of them. Moreover, the adverse impact of ZnO NPs-treated group was more than the impact of SP-treated group on these tested biomarkers. Similarly, our results illustrated that there was no significant relationship in all tried parameters between GA-treated group and the control group.

In Contrast, our outcomes showed that the pretreatment of GA caused a significant decrease in IL-6 level, LDH activity and TL level relative to the alone or combined treated groups as follows: (GA+ZnO NPs relative to ZnO NPs, GA+SP relative to SP and GA+ZnONPs+SP relative to ZnONPs+SP), ($p<0.001$). Thus, our data determined that GA ameliorated against the production of pro-inflammatory cytokine and LDH enzyme due to its ability to inhibition of inflammatory mechanism. Furthermore, our records revealed that GA alleviated against fatty accumulation and exhibited as an anti-lipidemic agent.

Histopathological observation

Our histological investigation showed that the splenic-toxic effects of ZnO NPs, SP and the immuno-protective effect of GA against them (fig. 2a-h). Microscopic examination of the spleen tissue section in the control group showed a normal histological structure of the lymphoid follicle called the white pulp; the reddish area, consisting of spleen parenchyma called the red pulp and capsule body of spleen (fig. 2a). GA-treated group appeared a healthy spleen structure of white pulp and red pulp (fig. 2b). On a hand, ZnO NPs-treated group prompted a large area of degeneration in the white pulp and congestion in the red pulp (fig. 2c). Moreover, there was an abnormal appearance of spleen tissue in SP-treated group, including hyperplasia in the white pulp, splenic congestion in the red pulp and thick trabecular (fig. 2d). On the other hand, GA+ZnO NPs-treated group transpired a mild lymphoid depletion in the splenic lymphoid follicle of white pulp (fig. 2e). Similarly, there was an enhancement in the structure of spleen tissue and become closer to normal white pulp and red pulp forms, observing in GA+SP-treated group (fig. 2f). Inaptly, ZnONPs+SP-treated group induced a deposition in the hemosiderin pigment of the splenic parenchyma, congestion in the central artery of the white pulp and mostly reticular cell hyperplasia with congestion in the red pulp (fig. 2g). Finally, GA+ZnONPs+SP-treated group provoked a mild lymphoid depletion in the follicle of white pulp (fig. 2h). Thus, the poisonous impact of SP-treated group was more than the impact of ZnO NPs on the histological structure of spleen tissue. In addition, the noxious impact of both treatments was stronger than the alone treatment of the spleen organ.

Immunohistochemical investigation

Our data indicated that ZnO NPs and SP induced overexpression in the pro-apoptotic protein (caspase-3) immune reaction in spleen tissue and GA persuaded low expression in the pro-apoptotic protein (caspase-3) immune reaction through stimulation the apoptosis mechanism (fig. 3a-h). There was a negative immune reaction of caspase-3 expression in the control and GA-treated groups (fig. 3a,b). On a hand, there was a strong positive immune reaction of caspase-3 expression with the appearance of deep brown staining in ZnONPs-treated group (fig. 3c). Furthermore, a very strong positive immune reaction of caspase-3 expression was observed in SP-treated group with the appearance of deep brown staining (fig. 3d). On the other hand, our data proposed that the co-treatment of GA was able to control the pro-apoptotic signal. A mild positive immune reaction of caspase-3 expression was seen in GA+ZnO NPs- and GA+SP-treated groups (fig. 3e,f). In Contrast, both treatments prompted a very strong positive immune reaction of caspase-3 expression in ZnONPs+SP-treated group (fig. 3g). Furthermore, the co-treatment of GA to both treatments persuaded a moderate positive immune reaction of caspase-3 expression (fig. 3h). Thus, these results confirmed that the pro-apoptotic influence of mixed treatment of ZnO NPs and SP was more than the influence of the alone treatment of them. Furthermore, the pro-apoptotic impact of SP-treated group was stronger than the impact of ZnO NPs-treated group.

DISCUSSION

To the preeminent of our information, this study pointed to investigate the immunotoxic effects of alone or mixed treatments of ZnO NPs and SP and to estimate the anti-inflammatory influence of GA, using histopathological, biochemical and immunohistochemical bioassays in rats. Our data found the individual or combined treatments of ZnO NPs and SP significantly increased splenic IL-6 production, serum TL level and splenic LDH activity compared to the control group, ($p<0.001$). Furthermore, our records observed that mixed treatments instigated many histopathological repetitions in the spleen organ, convincing a diminution in the lymphoid cells, congestion in the immune cells and splenic degeneration.

Especially, SP-treated group provoked hyperplasia. Likewise, mixed treatment persuaded a resilient positive immune reaction of caspase-3 expression; however, the alone treatment of them triggered a mediocre positive immune reaction of caspase-3 expression. From gotten findings, the alone or mixed treatments of ZnO NPs and SP may be acted as pro-inflammatory agents. Based on earlier reviews, numerous researches have revealed that nanomaterials instigated many immunological effects through producing many pro-inflammatory cytokines. They modify the normal immune response, mediating unspecific and specific reactions (Schubauer-Berigan *et al.*, 2020).

Table 1: Influence of Zinc oxide nanoparticles (ZnO NPs), mureer or *Senecio glaucus* L. plant (SP) and Gallic acid (GA) on biochemical and immunological biomarkers: [splenic IL-6 (pg/mg protein) activity, splenic LDH (U/mg protein) activity and serum TL level (mg/dl)].

Groups	IL-6 (pg/mg protein)	LDH (U/mg protein)	TL (mg/dl)
Control	52.65±0.25	1561.89±1.46	400.16 ± 2.45
GA	52.49±0.20$^{n.s.g}$	1564.38±1.81$^{n.s.g}$	393.79± 3.59 $^{n.s.g}$
ZnO NPs	49.64±0.34$^{***\ a}$	1832.36±2.43 $^{***\ a}$	1164.43± 3.71$^{***\ a}$
SP	53.73±0.25***	21.63.39±2.87 ***	792.64±1.89 ***
GA+ZnO NPs	45.51±0.30$^{***\ d}$	1532.27±2.78 $^{***\ d}$	845.03 ± 2.89 $^{***\ d}$
GA+SP	35.64±0.32$^{***\ e}$	1666.52±2.26 $^{***\ e}$	552.58±2.18 $^{***\ e}$
ZnONPs+SP	58.59±0.28$^{***\ b,c}$	1864.03±2.43 $^{***\ b,\ c}$	1341.81±7.23 $^{***b,\ c}$
GA+ZnONPs+SP	133.61±0.22^{***f}	1702.61± 1.93^{***f}	772.04±2.29^{***f}

Data were assumed as mean±SD, (n=5 rats per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons between groups. Compared to the control group, highly significant: $^{***}(P < 0.001)$ and $^{n.s.}$(P is non-significant). a,b,c,d,e,f,g letters represent the relations between treated groups at $P < 0.05$: [^{a}ZnO NPs relative to SP, ^{b}ZnONPs+SP relative to ZnO NPs, ^{c}ZnONPs+SP relative to SP, ^{d}GA+ZnO NPs relative to ZnO NPs, ^{e}GA+SP relative to SP, ^{f}GA+ZnONPs+SP relative to ZnONPs+SP, ^{g}GA relative to control].

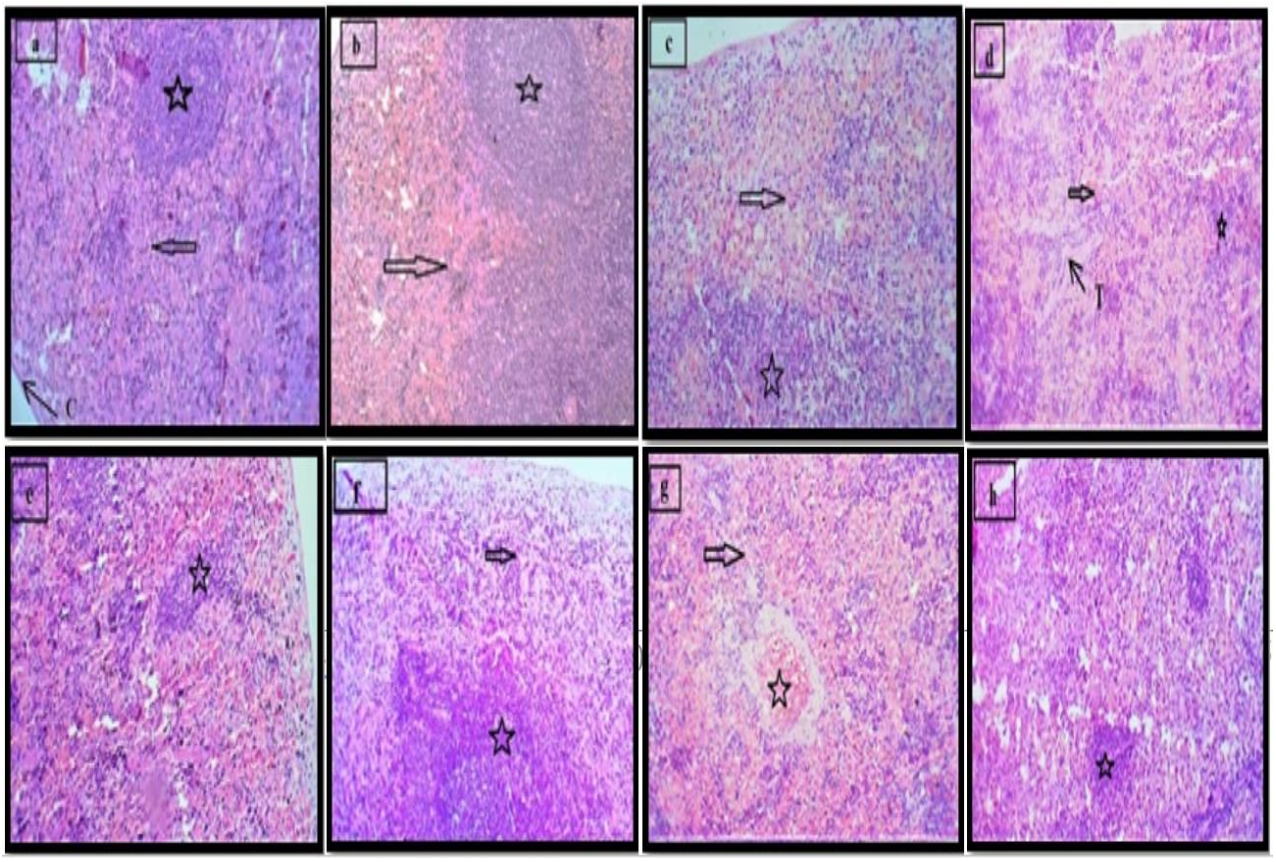

Fig. 2: (a-h). Photograph of the histopathological structure of spleen tissue in different groups: a) Control group showing normal spleen consists of lymphoid follicles (star) called white pulp and reddish areas of spleen parenchyma cells called red pulp (arrow) and capsule body of spleen (C) (H&E.,x100). b) GA-treated group showing a healthy normal spleen structure of white pulp (star) and red pulp (arrow). c) ZnO NPs-treated group showing: Mostly degeneration of white pulps (star) and congestion of the red pulp (arrow). d) SP-treated group showing hyperplasia of white pulps (star), congestion of the red pulp (arrow) and thick trabeculae (T). e) GA+ZnO NPs-treated group showing mild lymphoid depletion in the lymphoid follicles of white pulps (star). f) GA+ SP-treated group showing normal white pulp (arrow) and red pulp (star). g) ZnONPs+SP-treated group showing deposition of hemosiderin pigment in the splenic parenchyma (arrow), reticular cell hyperplasia with congestion in the red pulp (star). h) GA+ZnONPs+SP-treated group showing mild

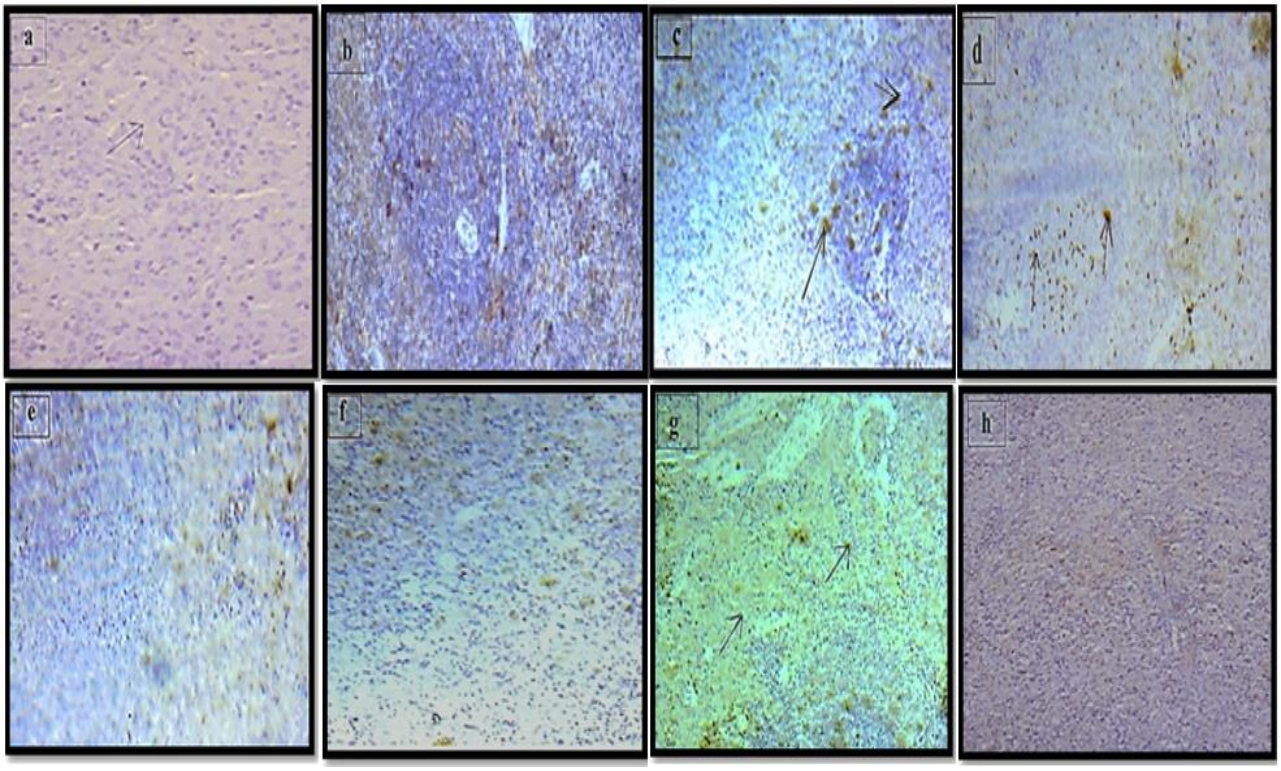

Fig. 3: (a-h). Photograph of the immunohistochemical structure of spleen tissue in different groups: a) Control group showing a negative immune reaction of caspase-3 expression (H&E., x400). b) GA-treated group showing a negative immune reaction of caspase-3 expression. c) ZnO NPs-treated group showing a strong positive immune reaction of caspase-3 expression with the appearance of brown staining. d) SP-treated group showing a strong positive immune reaction of caspase-3 expression with the appearance of brown staining. e) GA+ZnO NPs-treated group showing a mild positive immune reaction of caspase-3 expression. f) GA+SP-treated group showing a moderate positive immune reaction of caspase-3 expression. g) ZnONPs+SP-treated group showing a very strong positive immune reaction of caspase-3 expression with the appearance of brown staining (H&E., x200). h) GA+ZnONPs+SP-treated group showing a mild positive immune reaction of caspase-3 expression (H&E., x200).

The immune system is a noticeably complex network that shows an energetic role in preserving immune homeostasis. Spleen displays a forceful role in the beginning immune response, stimulating inflammation. Formerly, it is a normal physiological response to any damage. Furthermore, apoptosis produces intermolecular destruction in the macromolecules. Unfortunately, it causes impairment in protein expression. Subsequently, a mitochondrial membrane potential is reduced, leading to cell death. Additionally, modified protein serves as a hapten, which may trigger immune responses (Qaraghuli *et al*., 2021). With regard to understanding IL-6 function, it is one of the main pro-inflammatory cytokines, which helps in the variation of CD_{8+}T cells into cytotoxic T cells via NF-κB signaling pathway (Tang *et al*., 2021). Moreover, LDH enzyme is a critical indicator used in toxicology and clinical chemistry to identify organ damage (Farhana and Lappin, 2022). Besides, data were in agreement with, (Ibrahim *et al*., 2018; Li *et al*., 2022) who revealed that NPs dealings produced the discharge of pro-inflammatory cytokines and incited spleen toxicity, liberating LDH from damaged cells.

Furthermore, our findings were in a similar tendency to, (Lakshmanan *et al*., 2016) recorded that *Senecio* ingestion persuaded T-cell, which initiated mild mononuclear infiltration and impelled the liberation of immunomodulatory cytokines. These findings pointed out the risk of the phytochemical elements, saponins can cause hemolysis, forming the complexes with cell membrane cholesterol, leading to an increase in the cell permeability and an induction of the changes in the negatively charged carbohydrate portions due to their amphipathic properties (Verstraeten *et al*., 2020). Moreover, flavonoids are potent inhibitors for several enzymes, such as xanthine oxidase, lipoxygenase and acetylcholinesterase (Abou Baker, 2022).

In divergence, no doubt, there is less information about the role of GA against stimulated immunotoxicity. Thus, this study clarified that the inhibitory effect of GA against splenic-toxicity through its anti-inflammatory and antioxidant properties by overcoming inflammation mechanism occurrence (Karimi-Khouzani *et al*., 2017). Data about the pretreatment of GA with the management

of ZnO NPs and/or SP in the spleen cells, which significantly decreased IL-6 level, LDH activity and TL level relative to alone or mixed treatments of ZnO NPs and SP, ($p<0.001$). Correspondingly, our microscopic results showed that the co-administration of GA made a mild lymphoid depletion and amended spleen structure. It could prohibit the immune reaction of caspase-3 expression. Thus, it can recover and alleviate splenic degeneration due to its ability to restore inflammatory biomarkers. These results were in accordance with, (Bustami *et al*., 2018; Zamudio-Cuevas *et al*., 2021) who reported that GA withdrew the release of pro-inflammatory cytokines through reticence of the nuclear factor-κB dependent pathway in the damaged cells after toxin treatment.

CONCLUSION

In supposition, our data concluded that either alone or mixed treatments of ZnO NPs and SP may exploit as pro-inflammatory and pro-apoptotic agents. On the contrary, this study anticipated that GA might be an anti-inflammatory and anti-apoptotic agent.

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