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HEPATIC OXIDATIVE AND MOLECULAR EXAMINATIONS OF LIVER INJURY INDUCED BY ZINC OXIDE NANOPARTICLES AND MUREER EXTRACT VIA APOPTOSIS INDUCTION WITH THE AMELIORATIVE EFFECT OF GALLIC ACID IN RATS

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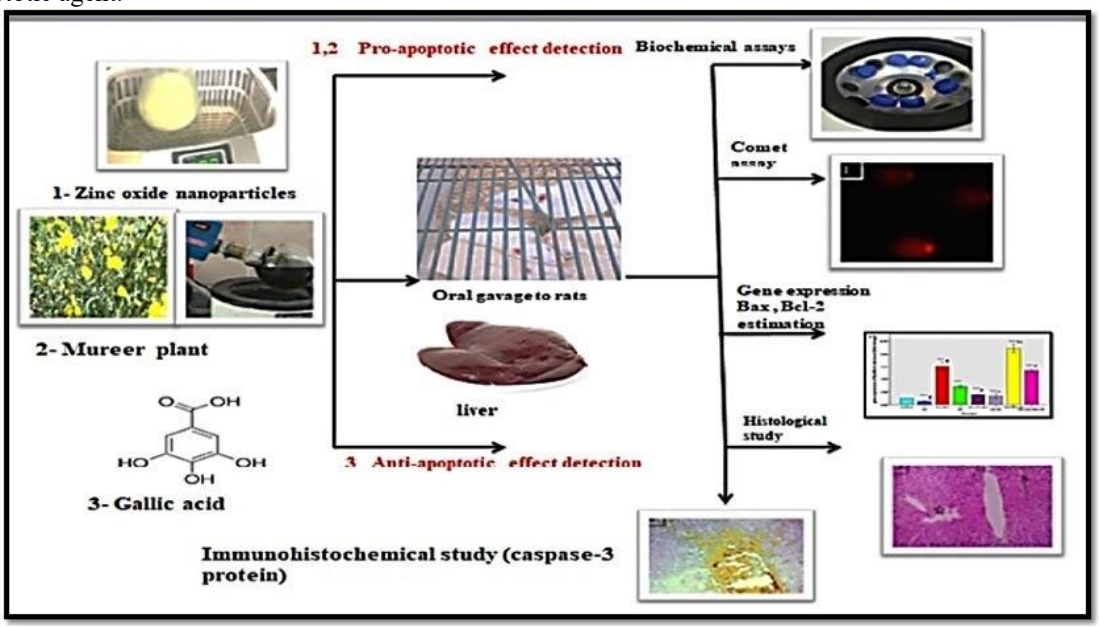
ABSTRACT

The recent study targeted to estimate the plain appliance of liver damage induced by either alone or combined treatments of zinc oxide nanoparticles (ZnO NPs) and mureer or *Senecio glaucus* L. plant (SP) via studying biochemical, histological, and genetic tests for 30 days, and to evaluate the prophylactic action of gallic acid (GA) in rats. Forty rats were orally treated and equally estranged into 8 groups with five rats in each group: 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). This study tested DNA content via comet assay, mRNA expression of an anti-apoptotic gene (Bcl-2) and a pro-apoptotic gene (Bax) via real-time qPCR, ($P < 0.001$), and caspase-3 expression via immunohistochemical study. Outcomes revealed that alone and combined treated groups of ZnO NPs and SP significantly altered enzyme activity and incited oxidative damage. They made DNA breakup, raised Bax and Bax/Bcl-2 ratio levels, dwindled Bcl-2 level, overexpressed caspase-3, and then initiated histopathological variants. The deadly effect of combined treatment was more than the effect of alone treatment. In contrast, it displayed that GA moderated this injury. Lastly, it clinched that ZnO NPs and SP act as pro-apoptotic agents; yet, GA acts as an anti-apoptotic agent.



Graphic abstract

Key words: Zinc oxide nanoparticles, mureer extract, gallic acid, oxidative stress, apoptosis mechanism, liver tissue, rats.

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INTRODUCTION

Nanotechnology is an ingenious science that currently engages in a wide-range multiplicity of chastisements, including agriculture, manufacturing, and medicine. Furthermore, nanoparticles (NPs) are widely used as numerous lucrative merchandise in the arcade. Bio-distribution tryouts exposed that the liver, kidney, and spleen might be the goal structures on behalf of designing NPs, which were absorbed through gastric area. Consequently, some detrimental effects of NPs on mammals have been established (Souto *et al*., 2019; Alsaba *et al*., 2020). Amongst the distinguished familiar NPs, zinc oxide nanoparticles (ZnO NPs) could become the supreme broadly used NPs in treasured discoveries, such as suntan lotion, insecticides, unguents, greasepaints, cement merchandises, antennas, and drug delivery. Awkwardly, they can ionize into zinc ions in the liver membrane and befall zinc homeostasis uproar, causing many modifications in the membrane portions (Xu *et al*., 2018; Mohammed *et al*., 2019). The action of these materials can lead to their toxicity. Due to having NPs of precise idiosyncrasy with an inconsequential bulk within (1–100) nm, they have an all-embracing surface area, which convinces the passageway of nanostructures through intracellular tissues. This feature is the core domicile of their metabolism in the body because of the inspiration of programmed cell death or apoptosis contrivance (APOP) (Huang *et al*., 2017). Numerous maneuvers have presented that NPs trigger noxious effects in the liver tissue. There is a prosperity of supportive sanction, producing oxidative stress (OS) as one of the foremost intentions for NPs snags (Jeevanandam *et al*., 2018). Due to OS generation, they galvanize many maladies in biotic amalgams in the cell, initiating a distraction in the regulation of intracellular signaling pathways and cell incarnation. Overall, it is deliberated as inequality among the productions of reactive oxygen species (ROS) and enzymatic antioxidants. Thus, it argues mitochondrial depolarization, creating APOP (Fu and Chung, 2018).

Meanwhile, there are abundant signaling alleyways, regulating this biological process, such as intrinsic and extrinsic alleyways. They lead to DNA mutilation, protein cleavage, immune response vicissitudes, and gene expression renovation. It is renowned that the intrinsic signaling alleyway is regulated by key genes of the Bcl-2 family. They have an authoritative protagonist in the rheostat of APOP. They also are blamable for intensification in the mitochondrial membrane permeability, including B-cell lymphoma2 (Bcl-2) and Bcl-2-associated x (Bax) genes. Bcl-2 has an anti-apoptotic outcome, yet Bax is an illustrious pro-apoptotic outcome. The assembly between both Bcl-2 family affiliates in the cytosol and mitochondria adjusts the persistence or loss of the cell (Elmore, 2007). Bcl-2 gene can impasse to Bax gene, leading to undermining of the mitochondrial veracity. The expression ratio of Bax/Bcl-2 portrays cell APOP. Moreover, an apoptotic stimulus may be enthused by the stimulation in the caspase (cysteine–aspartic acid protease) family proteins. They also may be romped a decisive role in the implementation of APOP (Shakeri *et al*., 2017).

To use a supplementary maintainable custom of bio-insecticide, natural plants are broadly used because of causing trivial damaging effects from chemical ones toward living entities. Among them, Mureer or [*Senecio glaucus* L.] plant (SP), (Asteraceae family-*Senecio* species), is a flowering plant found in dry and warm places in many regions (Norton *et al*., 2009). Besides, *Senecio* florae utilizes as herbal product in outmoded remedies (Panter *et al*., 2018). No above-mentioned polls explored the effect of these plants in rats; however, foregoing studies have recounted that they impelled insecticidal and antimicrobial accomplishments in diverse beings convinced cellular decease (Ruiz-Vásquez *et al*., 2017; Soares *et al*., 2019). As an upshot of having several chemical residents, such as coumarin, alkaloid, flavonoid, and saponin composites, their accretion may be aggravated many undesirable impressions inside living tissues (Yu *et al*., 2018).

The convention of antioxidants acts a brigadier protagonist in an anti-hepatotoxic standpoint, removing ROS and deactivation of lipid peroxidation (LP). Thus, one of the prevailing antioxidants that are used in the protection and/or the treatment of hepatic grumbles, is gallic acid (GA). GA, (3,4,5-trihydroxy benzoic acid), is naturally found inside gallnuts, strawberry, and oak bark. Literature data stated that it might be a polyphenol antioxidant, which is driven as an antitumor, anti-carcinogenic, and anti-inflammatory adjudicator. GA and its offshoots may enhance the fabrication of antioxidant enzymes, such as glutathione peroxidase, catalase, and glutathione-S-transferase (Kahkeshani *et al*., 2019; Schimites *et al*., 2020). With apprehension to a sympathizer of the liaison between GA edifice and its hepatoprotective activity, GA has a benzene ring with functional groups that can capture ROS and conjoin with metals to overcome the jeopardy of the origination of free radicals (Sroka and Cisowski, 2003).

Finally, the foremost unprejudiced of the prevailing enquiry was preferred to gauge the dreadful effects of either alone or combined treatments of ZnO NPs and SP in hepatocellular tissue via valuing the foremost molecular and biochemical tools of hepatic destruction over the estimation of the echelons of mRNA expression of APOP genes using real-time polymerase chain reaction manner, DNA integrity, biochemical estimation, protein expression of caspase-3, and histological investigation. Still, not at all many erstwhile reports have explored the cytoprotective stimulus of GA against liver destruction. Thus, our study also might bid

an innovative ghoul about the hepatoprotective impending of GA, discussing the molecular mechanism of its liver fortification against the cytotoxicity prompted by ZnO NPs and SP, using rats as mockups.

MATERIALS AND METHODS

Chemicals and reagents: Zinc oxide nanoparticles **(**ZnO NPs) (<50 nm) (BET), sodium carboxymethyl cellulose (Na-CMC) salt, gallic acid (GA), and a secondary antibody of caspase-3 were procured from Sigma Aldrich Corporation St.Louis, Missouri, USA. 70% of ethanol solvent was attained from El-Naser Corporation, Egypt for plant extraction. Furthermore, malondialdehyde (MDA) and glutathione-S-transferase (GST) kits could be gained from (Bio-diagnostic Corporation, Egypt). Additionally, the RNAeasy mini kit was bought from (Qiagen Corporation, Germany). Moreover, the QuantiTect SYBR Green PCR kit and a reverse transcriptase enzyme were acquired from (Invitrogen Corporation, Germany) for gene expression analysis. The primers of the used genes were gotten from (Macrogen Corporation, Asia). Similarly, the primary antibody of caspase-3 was come from (Santa Cruz Corporation, USA). Finally, high-quality constituents were used for biochemical studies.

Extraction of plant and estimation of phytochemical constituents: Leaves, stems, roots, and flowers of SP were collected, dehydrated in the laboratory, and deeply extracted in 70% ethanol for 3 days in glass cruses. At the culmination of the drenching epoch, the elucidation was sieved and concerted. Ethanol was recovered using a rotary evaporator (IKA-WERK, RV10, China) at 60°C and then oven-dried to concentrate at 45°C to obtain the greenish extract (Bahrin *et al*., 2018). The preliminary screening trial of SP was done using a desiccated plant by archetypal tactics, such as saponins, alkaloids, flavonoids, and phenolic compounds (Hiai *et al*., 1976; Yubin *et al*., 2014; Zhishen *et al*., 1999; Chun *et al*., 2003), respectively to know the main compounds inside it.

Dose preparation of all used compounds: Stock solutions of ZnO NPs, SP, and GA were prepared using 0.5% Na-CMC as a suspension for medicating, which was ultra-sonicated for 20 min by an ultrasonic homogenizer and prepared for the volume (5 ml/kg, BW for a rat).

Scheming of the median lethal dose (LD_{50}) of an ethanolic extract of SP: A median lethal dose (LD_{50}) experiment was done according to the technique (Karber,1931). It was used to twenty-eight rats with (0, 2500, 5000, 10000, 2000, 40000, and 80000 mg/kg) doses for an ethanolic extract and calculated according the equation: $LD_{50}=LD_{100}-\Sigma(Dd\times Md)/n$, n=Total number of animals in a group, Dd=The difference between two successive doses of administering extract, Md=The average number of dead animals in two successive doses, and LD_{100} =The lethal dose causing 100% death of all tested animals.

The experimental design: All experimental procedures were dopted according to the global procedures of Ethics of the Institutional Animal Care and Use Committee (Zagazig University, Egypt) as certification number (No.ZU-IACUC/1/F/42/2019). Forty male albino rats (*Rattus norvegicus*), (180-220 g BW, 6-7 weeks age-old), were used afterwards a 1-week retro of adaptation. They were reared in the animal House of the Faculty of Medicine, Zagazig University, Egypt in 2019 according to RCD system. They were allowed a customary pellet feeding regime and tap water *ad libitum* in plastic birdcages during the trial period. They also were retained at a tenacious temperature (23±2°C), moisture (60±10%), and a light/dark (12 h: 12 h) round.

The experiment was allocated into eight groups, which were comprised from five rats in each group:

A) Control group: rats received 0.5% Na-CMC as a vehicle (5 ml/kg of 0.5% Na-CMC/rat) (Dhiyaaldeen *et al*., 2014).

B) GA-treated group: rats received (100 mg/kg of GA) (Mansouri *et al*., 2013), suspending in 0.5% Na-CMC (Sen *et al*., 2013).

C) ZnO NPs-treated group: rats received (150 mg/kg of ZnO NPs), suspending in 0.5% Na-CMC (Srivastav *et al*., 2016).

D) SP-treated group: rats received (400 mg/kg of SP).

E) GA+ZnO NPs-treated group: rats received (100 mg/kg of GA plus 150 mg/kg of ZnO NPs) in a volume of (1ml/200g, BW).

F) GA+SP-treated group: rats received (100 mg/kg of GA plus 400 mg/kg of SP) in a volume of (1ml/200g, BW).

G) ZnO NPs+SP-treated group: rats received (150 mg/kg of ZnO NPs plus 400 mg/kg of SP) in a volume of (1ml/200g, BW).

H) GA+ZnO NPs+SP-treated group: rats received (100 mg/kg of GA plus 150 mg/kg of ZnO NPs plus 400 mg/kg of SP) in a volume of (1ml/200g, BW).

GA supplement was administrated before the handling of other substances about 10 min. The administration of all toxic and ameliorating agents was prearranged for 30 days (three times per week) via oral administration. At the finish of the era of exposure, animals were surrendered by cervical dislocation after inhalation with ether. Then, the liver tissue was instantaneously expurgated from 5 rats in each group and distributed into 3 fragments: (1) The fragment was rinsed

in ice-cold saline, pulverized, and centrifuged at 3,000 rpm for 20 min at 4°C. The supernatant was placid, relocated into vials, and warehoused at (−80°C) for succeeding biochemical and antioxidant investigations. (2) The fragment was preserved at 10% neutral buffered formalin for histological and immunohistochemistry assessments. (3) The fragment was conserved at (-80 °C) for doing molecular studies.

Biochemical investigation

Estimation of liver function biomarkers: Liver function biomarkers, [aminotransferase (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], and alkaline phosphatase (ALP) enzymes, and total protein (TP) were scrutinized using manual ingredients according to (Reitman and Frankel, 1957; Belfield and Goldberg, 1971; Gornall *et al*., 1949), respectively.

Estimation of prooxidant/antioxidant status biomarkers

Assessment of hepatic malondialdehyde level (MDA): MDA level in liver homogenate was done allowing to (Ohkawa *et al*.,1979) based on thiobarbituric acid (TBA) that was reacted with MDA content in the sample at an acidic medium in a boiling water bath. The absorbance of the assimilated rosy product could be assessed at 534 nm permitting to (Satoh,1978).

Assessment of hepatic Glutathione-S-transferase activity (GST): The activity of GST in liver homogenate may be dogged affording to (Habig *et al*.,1974), which was acquired a colored product and perceived at 340 nm.

Molecular investigation

Estimation of hepatic DNA Fragmentation using comet assay: The comet test (single-cell gel electrophoresis) is an expansively used procedure, entering in gaging and scrutinizing DNA fracture of tissues bestowing to (Blasiak *et al*., 2004) with negligible amendments to undo DNA double strands underneath basic surroundings. Tissues of each group were homogenized, diversified with low-melting-point agarose, and pipetted to glide with a normal one. They were engaged at 4°C in a gloomy location. They also were dejected to lysis solution and then transferred to electrophoresis run at 4°C. For the conception of DNA mutilation, explanations were observed using ethidium bromide staining on a fluorescent microscope (Zeiss epifluorescence microscope). The magnitude of DNA immigration was dogged for each sample by conquering the appearance magnified at ×200.

Estimation of apoptotic genes using quantitative real-time polymerase chain reaction (qRT-PCR) bioassay: For entire RNA extraction from liver tissue after collapsing in the solution of tissue lyser, the RNAeasy mini kit was used. The magnitude and pureness of the extracted RNA were noticed using a Nanodrop spectrophotometer. Upon extraction of RNA, 1 µg of RNA was reverse-transcribed using a reverse transcriptase enzyme permitting the manufacturer's way. The qRT-PCR assay was used to estimate the amount of expressed mRNA levels genes (Bcl-2, Bax, and β-actin). β-actin is an internal housekeeping control to normalize the expression of other genes. The relative quantification was attained by reckoning the upsurge in the fluorescence bright as because of binding to SYBR Green dye through the usage of Step One thermal cycler kits (qRT-PCR) rendering to the industrialist's protocol. Then, the amplification reaction was done in an ultimate volume of 25 µl, which was included 1 µl of cDNA, 12.5 µl of SYBR Green Master Mix, 11 µl of RNase water, and 0.5 µl of each primer to examine the relative amount of the qRT-PCR analysis. The nominated primers were checked from the BLAST tool for *Rattus norvegicus* organism for perceiving the annealing temperature used in it. The succeeding primers were used: Forward/Reverse—with accession number (NM_), such as:1)β-actin [Forward 5′-CTAAGGCCAACCGTGAAAAG-3′(20bp)/Reverse5′-ACCAGAGGCATACAGGGACA-3′(20bp)], NM_031144.3 (149bp for amplicon), 2)Bcl-2[Forward5′-CGGGAGAACAGGGTATGA-3′(18bp)/ Reverse5′ CAGGCTGGAAGGAGAAGAT-3′ (19bp)], NM_016993.1(130bp for amplicon), and 3) Bax [Forward 5′-ACGCATCCACCAAGAAGC-3′ (18bp)/ Reverse 5′-GCCACACGGAAGAAGACC-3′(18bp), NM_017059.2 (104 bp for amplicon). The amplification circumstances were boosted as follows: pre-denaturation at 94°C for 5 min tailed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 5 min. For relative quantitation of gene expression (fold change), mRNA expression level of ambition genes was designed, sustaining to the comparative threshold cycle formulation of **Livak and Schmittgen** (Livak and Schmittgen, 2001). Records were achieved at triplicate epochs. To notice the threshold cycle (Ct) values of the augmented product, the qRT-PCR was calculated as the threshold cycle of PCR. The alteration in the threshold cycles for objective genes and β-actin gene was calculated to attain the relative threshold cycle (Ct),using the ensuing formula: $\Delta Ct=Ct_{target}-Ct_{reference}$, $\Delta\Delta Ct=\Delta Ct_{\text{ test sample}}-\Delta Ct_{\text{control sample}}$, Relative expression: $2^{-\Delta\Delta Ct}$.

Histopathological examination: Samplings were static using neutral buffered formaldehyde. After apt fixation, the samplings were dehydrated in ascending rankings of ethyl alcohol and implanted in paraffin wax. 4-µm thick sectors were sullied with hematoxylin and eosin (H&E.,) and witnessed under a light microscope (Bancroft and Layton,2012).

Immunohistochemistry examination: For the recognition of pro-apoptotic protein (caspase-3), the paraffin-embedded livers were censored into a 4-µm part and mounted on positively charged glides. The immunohistochemical antiphon was done using the peroxidase/anti-peroxidase (PAP), conferring to the scheme of (Ramos-Vara *et al*., 2008). The parts were incubated with primary antibodies and secondary antibodies (dilution, 1:2000), and PAP complex (dilution, 1:200) in different steps with washing by phosphate buffer. After immunostaining, they were informally tarnished with (H&E.,) and identified underneath a light microscope.

Statistical analysis: Statistics were pigeonholed as a mean±standard deviation (mean±SD) by one-way ANOVA test, using statistical software package SPSS for Windows 20.0 (IBM, Chicago, IL,USA). It could be undertaken a comparison between the biochemical statistics, trailing by Tukey's post hoc test for the judgment between manifold groups. The significance level was done next to P<0.05 (IBM Corp SPSS,2011).

RESULTS

Phytochemical elements' study of SP: Our upshots exposed that SP has involved numerous phytochemical constituents, such as saponins, alkaloids, total phenolic, and total flavonoids.

Determination of LD_{50} of ethanolic extract of SP in rats: Our results showed that the LD_{50} of SP was 60.000 mg/kg BW and this study nominated a distinct dose of 400 mg/kg of SP (1/150 LD_{50}) to assess the negative possessions in liver tissue. It did not obtain any loss over 24 h.

Impact of ZnO NPs, SP, and GA on hepatic function biomarkers: Our statistics displayed that either alone or combined treated groups of ZnO NPs and SP prompted hepatotoxicity. They created a significant rise in AST activity and TP concentration; yet, they incited a significant lessening in ALP activity compared to the control group ($p < 0.001$, **Table 1**). Congruently, our results indicated that there was no significant modification between the control group and GA-treated group in all valued factors.

Besides, an alone treated group of ZnO NPs or SP instigated a significant upsurge in ALT activity compared to the control group ($p < 0.001$); nevertheless, a combined treated group of ZnO NPs and SP did not cause a statistically dissimilarity compared to the control group due to averting the construction of this enzyme from the liver. In addition, the shifts of these factors in the combined treatment of ZnO NPs and SP were more persuasive than the shifts in the alone treatment of them. Further, the disparities in these issues of ZnO NPs-treated group were stronger than the disparities in SP-treated group.

In dissimilarity, our observations documented that the co-administration of GA with either alone or combined treated groups of ZnO NPs and SP appeared a perfection in these valued parameters ($p < 0.001$, **Table 2**). It influenced a significant decline in the activities of ALT, AST, TP concentration and committed a significant rise in ALP activity relative to either alone or combined treated groups of ZnO NPs and SP as follows: (GA+ZnO NPs-treated group relative to ZnO NPs-treated group, GA+SP-treated group relative to SP-treated group, and GA+ZnO NPs+SP-treated group relative to ZnO NPs+SP-treated group).

Table 1. Impact of ZnO NPs, SP and GA on AST activity (U/g tissue), ALT activity (U/g tissue), ALP activity (U/g tissue), and TP concentration (mg/100 mg tissue) in liver tissue.

Groups	AST (U/g tissue)	ALT (U/g tissue)	ALP (U/g tissue)	TP (mg/100 mg tissue)
Control	**21.28±0.96**	**531.19± 0.69**	**1.51±0.02**	**34.31±1.29**
GA	**21.37±1.29** $^{n.s.g}$	**531.29±0.96**$^{n.s.g}$	**1.52±0.02** $^{n.s.g}$	**34.61± 0.70** $^{n.s.g}$
ZnO NPs	**48.19±0.88**^{***a}	**589.51± 1.69**^{***a}	**0.55±0.03**^{***a}	**121.27± 1.53**^{***a}
SP	**36.95±0.65*****	**580.91± 1.32** ***	**0.85±0.03*****	**162.19± 1.87*****
GA+ZnO NPs	**28.66±0.85**^{***d}	**553.07± 1.54** $^{*** d}$	**1.17±0.01**^{***d}	**111.91± 1.33**^{***d}
GA+SP	**26.81±0.88**$^{*** e}$	**531.67± 1.36** $^{n.s. e}$	**0.94±0.02**^{***e}	**144.05±2.29**^{***e}
ZnO NPs+SP	**18.18±1.75**$^{n.s. b, c}$	**590.84± 1.24** $^{*** b, c}$	**0.37±0.02**$^{*** b, c}$	**132.86± 2.16**$^{*** b, c}$
GA+ ZnO NPs+ SP	**19.41±0.85**$^{n.s. f}$	**553.95± 0.93** ^{***f}	**1.02±0.01**^{***f}	**125.21±2.13**^{***f}

Compared to the control group,***:($P < 0.001$) or highly significant and $^{n.s.}$: P is non-significant. a,b,c,d,e,f,g letters epitomize the relationships between treated groups at $P < 0.05$: [^{a}ZnO NPs relative to SP, ^{b}ZnO NPs+SP relative to ZnO NPs, ^{c}ZnO NPs+SP relative to SP, ^{d}GA+ZnO NPs relative to ZnO NPs, ^{e}GA+SP relative to SP, ^{f}GA+ZnO NPs+SP relative to ZnO NPs+SP, ^{g}GA relative to control].

Impact of ZnO NPs, SP, and GA on hepatic antioxidant/oxidant balance status biomarkers: The assessment of antioxidant/oxidant balance status factors was perceived ($p< 0.001$, **Table 2**). Our fallouts exposed

that either alone or combined treated groups of ZnO NPs and SP encouraged a significant rise in MDA level; nonetheless, they swayed a significant decline in GST activity compared to the control group. Still, a combined group of ZnO NPs and SP caused the highest elevation in the level of MDA and the premier lessening in the activity of GST enzyme relative to alone treated groups of them. Furthermore, our statistics naked that a non-significant link may be found between the control and GA-treated group in GST activity, while there was a significant liaison between them at the MDA level.

In unlikeness, our records validated that the administration of GA with either alone or combined treatments of ZnO NPs and SP meaningfully abridged MDA level and augmented GST activity relative to either alone or combined treated groups of them as follows: (GA+ZnO NPs-treated group relative to ZnO NPs-treated group, GA+SP-treated group relative to SP-treated group, and GA+ZnO NPs+SP-treated group relative to ZnO NPs+SP-treated group).

Table 2. Impact of ZnO NPs, SP and GA on hepatic antioxidant/oxidant balance status biomarkers: MDA level (nmol/g tissue) and GST activity (U/g protein).

Groups	MDA (nmol/g tissue)	GST (U/g protein)
Control	**38.24±1.56**	**1.23 ±0.08**
GA	**27.88±2.02** ^{***g}	**1.40±0.07** $^{n.s.\ g}$
ZnO NPs	**100.89±2.89** $^{***\ a}$	**0.29±0.04** ^{***a}
SP	**78.50±1.93** ***	**0.40±0.03** ***
GA+ ZnO NPs	**49.36 ±1.64** ^{***d}	**0.54±0.07** ^{***d}
GA+ SP	**49.45±1.54** ^{***e}	**0.60±0.04** ^{***e}
ZnO NPs+ SP	**112.35±1.88** $^{***\ b,c}$	**0.26±0.03** $^{***\ b,c}$
GA+ ZnO NPs+ SP	**66.29±1.69** $^{***\ f}$	**0.50±0.07** ^{***f}

Compared to the control group, ***:($P < 0.001$) or highly significant and $^{n.s.}$:P is non-significant. a,b,c,d,e,f,g letters epitomize the relationships between treated groups at $P < 0.05$: [^{a}ZnO NPs relative to SP, ^{b}ZnO NPs+SP relative to ZnO NPs, ^{c}ZnO NPs+SP relative to SP, ^{d}GA+ZnO NPs relative to ZnO NPs, ^{e}GA+SP relative to SP, ^{f}GA+ZnO NPs+SP relative to ZnO NPs+SP, ^{g}GA relative to control].

Impact of ZnO NPs, SP and GA on DNA integrity via comet bioassay: To extra sightsee the consequence of oxidative DNA fragmentation over comet bioassay, our significances detected that the percentage of DNA fragmentation (%DNA fragmentation) meaningfully amplified in either alone or combined treated groups of ZnO NPs and SP paralleled to the control group ($p<0.001$, Figure 1). Furthermore, our records proved that %DNA fragmentation of a combined treated group of ZnO NPs and SP was upper than that of the alone treated groups. Furthermore, the %DNA fragmentation of ZnO NPs-treated group was more than that of SP-treated group. Besides, our registers exhibited that there was a non-magnificent amendment between the control and GA-treated groups in this stricture.

Contrariwise, our facts perceived that the pretreatment of GA with either alone or combined groups of two substances (ZnO NPs and SP) provoked a significant drop in %DNA fragmentation relative to either alone or combined treated groups of them as follows: (GA+ZnO NPs-treated group relative to ZnO NPs-treated group, GA+SP-treated group relative to SP-treated group, and GA+ZnO NPs+SP-treated group relative to ZnO NPs+SP-treated group). Additionally, there was an augmentation in GA+SP-treated group relative to GA+ZnO NPs-treated group in this parameter.

Hepatic DNA appeared as an intact DNA state in the control group and GA-treated group that have (intact nuclei) in the form of (0-class) (Figure 2:a,b). On a hand, the alone treated groups of ZnO NPs or SP exhibited far-reaching DNA damage in the form of (III- and IV-classes) (Figure 2:c,d). In contrast, the co-administration of GA with ZnO NPs or SP produced a sensible appearance of DNA damage in the form of (II- and III-classes) (Figure 2:e,f). Consistently, a combined treated group of ZnO NPs and SP induced a very resilient DNA damage in the form of (IV-class) (Figure 2:g). On the other hand, the pretreatment of GA to a combined treated group of ZnO NPs and SP encouraged a slight form of DNA damage in the form of (III-class) (Figure 2:h). These results indicated that the reversibility magnitude of GA persuaded against DNA fragmentation, inducing by ZnO NPs and SP and instigated an escalation in the relative DNA fragmentation.

Impact of ZnO NPs, SP and GA on gene expression of apoptotic genes: Our records displayed that either alone or combined of ZnO NPs- and SP-treated groups affected mRNA expression (fold change) levels of APOP genes (Bax plus Bcl-2) in the hepatocellular tissue. Our documents indicated that they initiated a significant upsurge in the mRNA Bax level; nonetheless, Bcl-2

mRNA level induced a significant decrease paralleled to the control group ($p< 0.001$, Figure 3:a,b). Likewise, the mRNA Bax level of a combined treated group of ZnO NPs and SP was more than the level of the alone treated groups of them ($p<0.001$,Figure3:a). Similarly, the mRNA level of Bcl-2 of a combined treated group of ZnO NPs and SP was lesser than the level of an alone treated group of them ($p< 0.001$, Figure 3:b). Furthermore, the amendments of mRNA levels of APOP genes of ZnO NPs-treated group were upper than the amendments of the group of SP ($p< 0.001$, Figure 3:a,b).

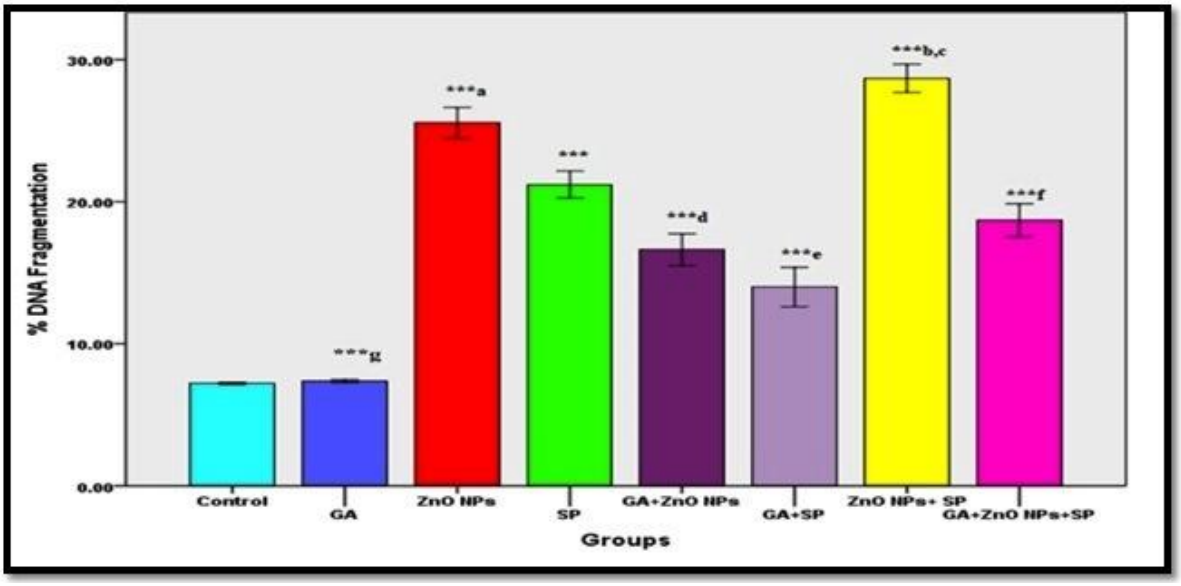

Figure 1: The impact of ZnO NPs, SP and GA on DNA content integrity via alkaline comet bioassay. Values were donated as mean±SD, (n=5 rats per group). Compared to the control group, ***:($P < 0.001$) or highly significant and $^{n.s.}$:P is non-significant. a,b,c,d,e,f,g letters epitomize the relationships between treated groups at $P < 0.05$: [^{a}ZnO NPs relative to SP, ^{b}ZnO NPs+SP relative to ZnO NPs,^{c}ZnO NPs+SP relative to SP, ^{d}GA+ZnO NPs relative to ZnO NPs, ^{e}GA+SP relative to SP, ^{f}GA+ZnO NPs+SP relative to ZnO NPs+SP, ^{g}GA relative to control].

Moreover, Figure 2(a-h) noticed that the classes comet tail of treated groups, depending on the length of the tail.

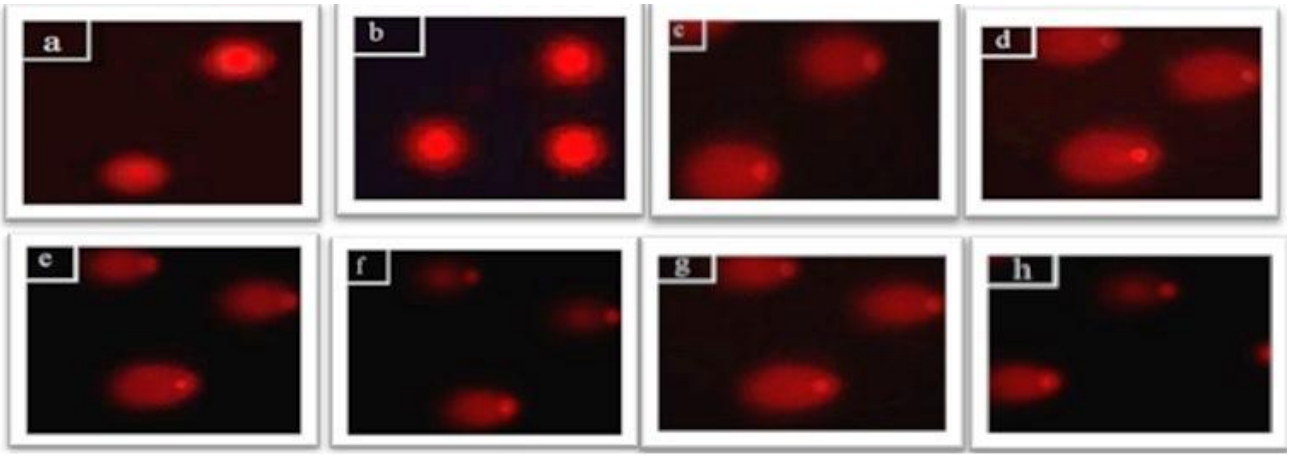

Figure 2(a-h): The impact of ZnO NPs, SP and GA on the DNA content integrity via comet bioassay in the liver tissue (comet tail classes). a,b) Control group and GA-treated group showing 0 class. c,d) ZnO NPs-treated group and SP-treated group showing III and IV classes. e,f)GA+ZnO NPs-treated group and GA+SP-treated group showing II and III classes. g) ZnO NPs+SP-treated group showing IV class. h) GA+ZnO NPs+SP-treated group showing III class (x200).

Moreover, our aftermaths indicated that either alone or combined groups of ZnO NPs and SP significantly augmented in Bax/Bcl-2 ratio mRNA expression level paralleled to the control group. Moreover, this parameter of a combined group was greater than the level of alone treatment ($p<0.001$, Figure 3:c). Harmoniously, the shifts of this factor in ZnO NPs-treated group were bigger than the shifts in SP-treated group ($p<0.001$, Figure 3:a,c).

Contrariwise, our registers divulged that the addition of GA to either alone or combined treated groups of ZnO NPs and SP meaningfully dropped mRNA expression levels of Bax and Bax/Bcl-2 ratio; hitherto, it meaningfully amplified mRNA expression level of Bcl-2 relative to either alone or combined treated groups of them as follows: (GA+ZnO NPs-treated group relative to ZnO NPs-treated group, GA+SP-treated group relative to SP-treated group, and GA+ZnO NPs+SP-treated group relative to ZnO NPs+SP-treated group) ($p<0.001$, Figure 3:a,b,c). Furthermore, an addition of the antioxidant (GA) to both ingredients prompted a significant up-regulation in the fold change of the Bcl-2 gene paralleled to the control group because of its antioxidant activity in a hepatic cell.

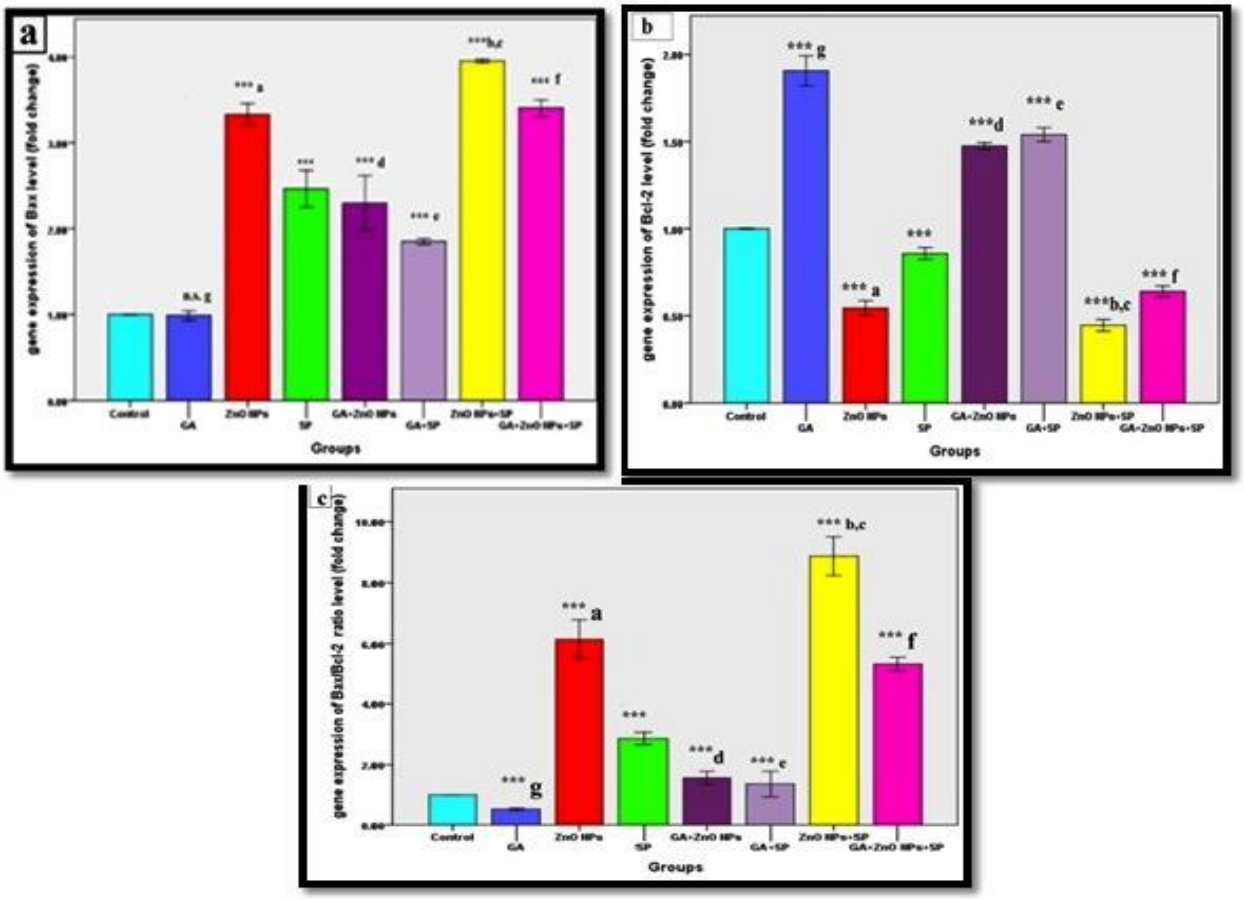

Figure 3(a-c): The impact of ZnO NPs, SP and GA on mRNA expressed levels of the APOP genes (Bax and Bcl-2) in hepatic tissue using qRT–PCR. Notes: Figure3: a,b, and c): Representative images of Bax, Bcl-2, and Bax/Bcl-2 ratio expressions. The intensity of the signal was normalized by β-actin gene to normalize the data. Values were donated as mean±SD, (n=5 rats each group). Values were denoted as mean±SD, (n=5 rats per group).***:($P < 0.001$) or highly significant and $^{n.s.}$: P is non-significant. a,b,c,d,e,f,g letters epitomize the relationships between treated groups at $P < 0.05$: [^{a}ZnO NPs relative to SP, ^{b}ZnO NPs+SP relative to ZnO NPs, ^{c}ZnO NPs+SP relative to SP, ^{d}GA+ZnO NPs relative to ZnO NPs, ^{e}GA+SP relative to SP, ^{f}GA+ZnO NPs+SP relative to ZnO NPs+SP, ^{g}GA relative to control].

Impact of ZnO NPs, SP and GA on histopathological examination in liver tissue: Our histological scrutiny of liver tissue explained in (Figure 4:a-h). The typical parenchymal manner of lobules, comprising from a central vein bounded by forcefully crowded cords of polygonal hepatocytes, round vesicular nuclei, and acidophilic cytoplasm appeared in the control group (Figure 4:a). A well hepatic architecture of hepatic

lobules happened in GA-treated group (Figure 4:b). A presence of apoptotic cells and congestion of blood vessels acted in ZnO NPs-treated group

(Figure 4:c). Besides, dilation of the sinusoids and advent of the apoptotic cells ensued in SP-treated group (Figure 4:d).In divergence, the pre-administration of GA of both treatments prompted perfection in the damaged construction. Moderate hydropic collapses, slight deterioration of the parenchymal cells, and trifling blood vessel congestion acted in GA+ZnO NPs-treated group (Figure 4:e).Trivial dilation of the sinusoids and slender appearance of the apoptotic cells arose in GA+SP-treated group (Figure 4:f). Dilation of the blood vessels, hefty area of the

apoptotic cells, and all-embracing hydropic parenchyma transpired in ZnO NPs+SP-treated group (Figure 4:g). Nevertheless, judicious dilation of the sinusoidal vessels and lean appearance of the apoptotic cells occurred in GA+ZnO NPs+SP-treated group (Figure 4:h). Hence, our documents publicized that GA brought a good effect on the depraved influences induced by ZnO NPs and SP and recuperated liver mutilation.

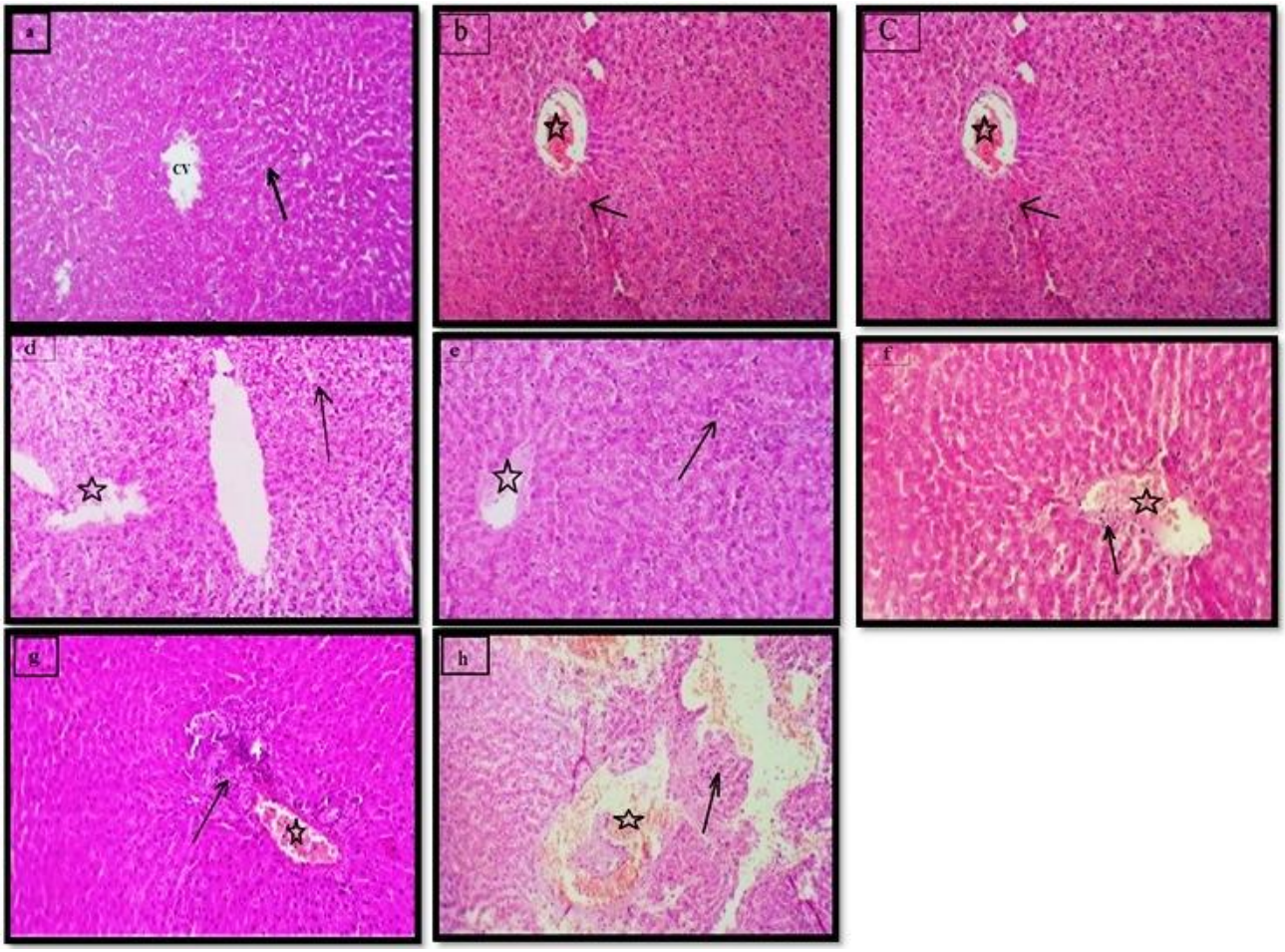

Figure 4(a-h): The impact of ZnO NPs, SP and GA on liver tissue in different groups: a) Control group involving the usual construction of hepatic lobules entailing a central vein (CV) bordered by the firmly packed cords of polygonal hepatocytes with round vesicular nuclei plus acidophilic cytoplasm (arrow). b) GA-treated group involving the well hepatic manner of hepatic lobules: a central vein (CV) bordered by cords of hepatocytes (arrow) (H&E.,x200). c) ZnO NPs-treated group involving the atypical hepatic manner of hepatic lobules: a form of apoptotic cells (arrow) and hepatic blood vessel congestion (star). d) SP-treated group involving dilation of sinusoids (star) and attendance of apoptotic cells (arrow). e) GA+ZnO NPs-treated group involving mild hydropic degenerations in the parenchymal cells (arrow) and blood cell congestion (star). f) GA+SP-treated group involving minor dilation of sinusoids (star) and trivial appearance of apoptotic hepatocytes (arrow) (H&E.,x400). g) ZnO NPs+SP-treated group involving more dilation in the blood vessels (star), hefty extents of apoptotic hepatocytes with hydropic degeneration in the parenchyma cells (arrow) (H&E.,x200). h) GA+ZnO NPs+SP-treated group involving modest dilation of blood vessels (star) and minor look of apoptotic hepatocytes (arrow) (H&E.,x400).

Impact of ZnO NPs, SP and GA on the immunohistochemical exploration in liver tissue for pro-apoptotic protein (caspase-3): Our data identified the pro-apoptotic effect of ZnO NPs and SP and the anti-apoptotic effect of GA against them (Figure 5:a-h). A negative immune response of protein appeared in the control group (Figure 5:a). Besides, a negative immune response of caspase-3 protein seemed in GA-treated group (Figure 5:b). Nevertheless, our archives explained that a durable positive immune response of this protein acted in ZnO NPs- and SP-treated groups (Figure 5:c,d). Temporarily, our data showed that the pre-administration of GA was able to confine the pro-apoptotic signal. A restrained positive immune response of this protein was observed in GA+ZnO NPs-treated group and GA+SP-treated group (Figure 5:e,f). Cruelly, a very durable positive immune response of this protein arose in ZnO NPs+SP-treated group (Figure 5:g).Painstakingly, the co-treatment of GA to both treatments persuaded a judicious positive immune response of caspase-3 expression (Figure 5:h).

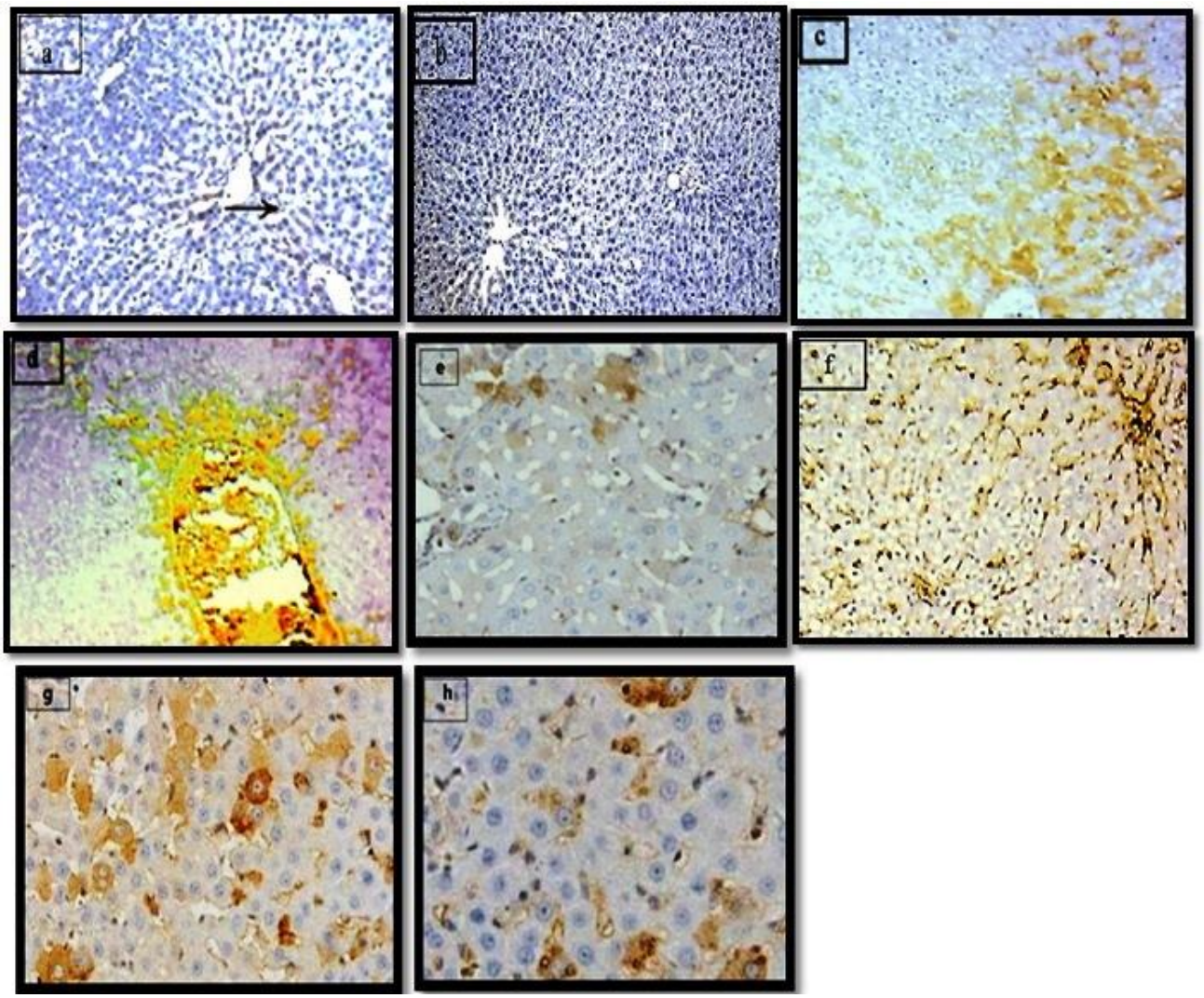

Figure 5(a-h): The impact of ZnO NPs, SP and GA on immunohistochemical staining of caspase-3 protein expression in liver tissue of diverse groups. a,b) Control and GA-treated groups viewing a negative immune response (H&E.,x100). c,d) ZnO NPs-and SP–treated groups viewing a resilient positive immune response in the cytoplasm of hepatocytes with stout apoptotic vicissitudes as dispersion bottomless brown color pigment (H&E.,x200). e,f) GA+ZnO NPs- and GA+SP–treated groups viewing a mediocre positive immune response in the cytoplasm of hepatocytes (H&E.,x400,200). g) ZnO NPs+SP-treated group viewing a pure widespread for the positive immune response in the cytoplasm of hepatocytes with robust apoptotic variations as scattering the yawning brown color pigment. h) GA+ZnO NPs+SP–treated group viewing a slight positive immune response (H&E.,x400).

Overall, on all levels of biochemical, antioxidant, and molecular investigations, our records unveiled that the discrepancies of the mixed treatment of ZnO NPs and SP were more than the discrepancies of the alone treatment of them. Likewise, our records disclosed that the pro-apoptotic and hepatotoxic activities of both treatments were more than the activities of the alone treatment of them. Additionally, GA exhibits a compelling anti-apoptotic and anti-hepatotoxic agent.

DISCUSSION

Due to plentiful studies scrutinizing the noxious effects of nanoparticles and natural plants, data on the contributory mechanism remain not utterly implicit. Primarily, this study discerned that the hepatotoxic effects of either alone or combined treatments of ZnO NPs and SP through appraising the biochemical, histopathological, and molecular investigations. On the level of phytochemical inquiry of the plant, our outcomes alleged that SP enclosed various phytochemical composites including, saponin, alkaloid, flavonoid, and phenolic compounds, and mostly containing saponins. The formed toxicity of plant extract in rats was due to the accretion of their chemical elements in liver tissue through metabolism, convincing its mortal impact (Walker *et al*., 1990). Respectively, the accrual of saponins in hepatic tissue persuaded inflammatory rejoinders and triggered fatty liver, leading to liver mutilation (Podolak *et al*.,2010). This result was in the same line with Parra *et al*., 2018 who disclosed that *Senecio* plant induced cell death due to its congregation of saponins and phenolic compounds in living cells (Parra *et al*., 2018).

On the level of biochemical analysis, our results revealed that either alone or combined treated groups caused significant reforms in hepatic parameters paralleled to the control group **($p<0.001$, Table 1)**. From the former echo, aminotransferase activity is related to the integrity of liver parenchymal cells. If the hepatocellular membrane is bruised, transaminase enzymes will leak out from the cytosol due to an upsurge in the penetrability of the cellular membrane. Besides, intensification in the concentration of TP might be ascribed to the failure of normal uptake and defecation of the indignant hepatic parenchyma because of the overproduction of the lytic proteins. Consequently, these dealings have convinced the secretion of pro-inflammatory proteins that can be urged inflammation after proteotoxicity initiation (Mannaa and Abdel-Wahhab,2016).

Furthermore, the moans in ALP activity may be mostly attributed to the fibrotic mechanism in hepatic cells. Its activity is used to parade the rate of the integrity of the liver tissue (Moss,1997). In particular, abnormal zinc (Zn^{2+}) level occurrence distrustfully acts on ALP functions of the liver, which hydrolyzes phosphoric esters of the diverse phosphate-containing compounds in numerous metabolic processes (Gruengreiff *et al*.,2016). Unpardonably, liver destruction could cause a large capacity of metabolic adjustments, which convinces tenderness and hepatic edema. Likewise, zinc deficit can cause LP and oxidative destruction, leading to liver APOP (Su *et al*.,2019).

Then, on the level ofantioxidant/oxidant balance status factors, our marks examined that either alone or combined treated groups of ZnO NPs and SP significantly altered in the valued antioxidant biomarkers paralleled to normal rats ($p<0.001$,**Table 2**). Overall, about the venomous effects of xenobiotics on antioxidant capacity in intracellular membranes, the overproduction of ROS has already affected unsaturated fatty acids in bio-membranes, coaxing a drop in the membrane fluidity and a hubbub in the membrane erection and utility (Rolo *et al*.,2012). Thus, it convinces LP pervasiveness that can connect to up-regulation of the fibrogenic and inflammatory cytokines (Maher and Yamamoto,2010). In order to deliberate the aptitude of the antioxidant defense arrangement in the hepatocellular cells, GST enzyme is a pivotal detoxifying enzyme that can work as a buffer to thiol-disulfide bonds found in glycine, cysteine, and glutamic acid amino acids. It is predominantly involved in the detoxification of electrophilic radicals via, catalyzing the creation of GSH-electrophilic conjugate amalgams. Thus, OS argues protein amendment in arrangements (Cichoż-Lach and Michalak, 2014).

On the level of molecular scrutiny, DNA fragmentation was inspected using comet assay in which the percentage of DNA fragmentation eloquently elevated in either alone or combined treated groups compared to normal rats ($p<0.001$, Figure1). Still, they seemed DNA impairment through the advent of III-and IV-classes of comet tail classes (Figure 2:c,d,g) and transpired an utmost proportion of DNA mutilation. From premature brochures, the integrity of genomic DNA is habitually at risk, which can assure point mutations, electing through the alkaline comet assay. It is a trustworthy cytogenetic procedure for the quantification of the primary mutilation DNA. The length of the comet tail is unswervingly relational to DNA destruction, linking to the commonness of DNA discontinuities (Ho *et al*., 2003).

Similarly, on the level of molecular inquiry, our statistics publicized that either alone or mixed treated groups of ZnO NPs and SP significantly elevated Bax and Bax/Bcl-2 ratio mRNA levels, and diminished Bcl-2 mRNA level paralleled to normal rats ($p< 0.001$, Figure 3:a,b,c). To our familiarity, qRT-PCR is solitary of the most thoughtful tactics for gene expression guesstimate and identifies the relative amount of transcripts (Pistritto *et al*., 2016). From more forgoing information, APOP is convincingly controlled by inhibition or activation of the expression of abundant macromolecules. The ratio of the

innumerable Bcl-2 family members has postulated to prompt a cell to either enhance or inhibit APOP in response to external stimuli. Based on multiple studies, an escalation in the expression level of Bax/Bcl-2 ratio results in the occurrence of APOP (Ghasemi *et al*., 2018).

Likewise, the guesstimate of histological studies necessities to be addressed after using noxious materials in mammalian cells to discover the size of toxicity. On the level of histopathological explanations, ZnO NPs and SP prompted incidence of the apoptotic cells, overcrowding of blood vessels, and expansion of the sinusoids (Figure 4:c,d,g). Likewise, our facts identified that the hepatotoxic possessions of a combined treatment of ZnO NPs and SP were more than an alone treatment of them on the echelons of biochemical, antioxidant, histological, and genomic investigations. Our fallouts were in synchronization with, Zhang *et al*., 2018 and Valentini *et al*., 2019 who described that NPs elicited a weakening in the activity of anti-oxidative enzymes, changes in the aminotransferase activities, and provoked the attendance of inflammation with hemorrhage areas in hepatic architecture, generating DNA damage (Zhang *et al*., 2018; Valentini *et al*., 2019). Also, our proceedings were a bargain with Dimande *et al*., 2007 and Lakshmanan *et al*., 2016 who described that *Senecio* ingestion caused hematoma, deterioration, necrosis, and inflammation in liver tissue in treated organisms (Dimande *et al*., 2007; Lakshmanan *et al*., 2016).

Our documents also verified that ZnO NPs and SP caused a robust positive immune response of caspase-3 expression with the occurrence of a large area of apoptotic tissue (Figure 5:c,d,g). Our data were in the same inclination, Yousef *et al*., 2019 observed that nanoparticle handling instigated a surge in the expression of caspase-3 and a decline in the expression level of Bcl-2 because of the generation of inflammatory reactions and DNA fragmentation incidence (Yousef *et al*., 2019).

In unlikeness, the simultaneous management of GA with either alone or combined treated groups of ZnO NPs and SP restored the changes in liver function factors relative to the control group and presented a defensive consequence against forfeiture hepatocellular tissue (**Table 2**). Compatibly, it significantly dropped the level of MDA and augmented GST activity in hepatic tissue relative to either alone or combined treated groups (**Table 3**). Remarkably, yet again, the linked treatment with GA improved oxidative damage and presented a bettering effect against loss of the antioxidant/oxidant status balance convinced by ZnO NPs and SP components. Furthermore, this study reported that GA already has alleviated DNA breakup (Figure 1,$p<0.001$). Additionally, DNA damage has appeared as II-and III-classes of the comet tail in treated groups (Figure 2:e,f,h). These results proved that the protective magnitude of GA against DNA fragmentation of hepatic injury due to deterioration in the oxidative injury stimulation.

Our verdicts about the positive outcome of GA against other dealings were in relationship with prior studies, Reckziegel *et al*., 2016 and BenSaad *et al*., 2017 who informed that GA could be regulated the modulation of antioxidant/pro-oxidant disturbance by snowballing the activity of antioxidant enzymes and reducing MDA level that boosted repossession of the bruised hepatic manner (Reckziegel *et al*., 2016; BenSaad *et al*., 2017).

On the glassy of transcript expression, our penalties presented that the addition of GA could constrain apoptotic appliance stimulation, which significantly reduced Bax and Bax/Bcl-2 ratio expression levels and significantly elevated Bc-l2 expression level relative to either alone or mixed treated groups of ZnO NPs and SP ($p < 0.001$, Figure 3:a,b,c). Thus, our records concluded that the earlier treatment of GA suppressed the apoptotic contrivance and reduced the hepatic utility. The antioxidant power of GA against liver mutilation may be permitted to the supposition of the hydroxyl and carboxyl groups to capture ROS (Maurya *et al*., 2014). From the prehistoric echo, GA persuades an upsurge in the intracellular concentration of free zinc ions that can fortify metallothionein enzymes to relieve the nuclear factor-erythroid2-related factor2 (Nrf2) transcription factor. It also catalyzes the expression of Gclc gene that can provide a code for glutamate-cysteine ligase and prompt a synthetic alleyway of glutathione enzyme (Cortese *et al*., 2008).

Moreover, our chronicles exemplified that GA already has unveiled perfection in the dented architecture, such as a mild deterioration of parenchymal cells and a trivial appearance of apoptotic hepatocytes (Figure 4:e,f,h). Thus, our records showed that GA might be convinced of an accommodating effect against the venomous influences prompted by ZnO NPs and SP and mended liver mutilating. Furthermore, our data also displayed that the intensification in Bax/Bcl-2 ratio because of the generation of the intrinsic APOP corridor, impelling in a mitochondrial membrane potential (MMP) failure and activated other downstream dealings of the apoptotic cascade (Eastman and Barry, 2009).

Still, our results reported that GA incited moderation against genetic impairment and apoptotic event stimulation that generated a mediocre positive immune response of caspase-3 expression relative to either alone or both treatments of ZnO NPs and SP (Figure 5:e,f,h). Likewise, our digits were in covenant with, Sun *et al*., 2017 who cleared that the amelioration effect of GA against tissue reparation through impeding a discharge of cytochrome c from mitochondria and retreating of caspase-3 cascade initiation, leading to convalesce protein destruction, gene expression amendment, and hampering of DNA hurt (Sun *et al*., 2017). Further, Zhang *et al*., 2019 broadcasted that a prophylactic consequence of GA significantly lessened Bax mRNA expression level and enlarged Bcl-2 mRNA

level against cisplatin-provoked injuriousness (Zhang *et al*., 2019).

Finally, our study concluded that ZnO NPs and SP treatments persuaded biochemical and genetic alterations during induction of apoptotic cascade event and GA convinced a prophylactic action due to its anti-apoptotic action, controlling in the liver damage. We indorsed that using of the alone or combined treatments of ZnO NPs and SP as pro-apoptotic agents and GA as anti-apoptotic agent against the toxic materials in the market.

Conclusion: Convincingly, our records clinched that ZnO NPs and SP actions elicited a significant variation in the levels of hepatic function indices. Moreover, they convinced antioxidant status disturbance relating to APOP mechanism accompanied by histological and genetic restructurings. Moreover, they upturned Bax/Bcl-2 ratio level, impelled DNA disintegration, and immunologically overexpressed caspase-3. Unswervingly, this study found that the immoral impact of mixed treatment of ZnO NPs and SP was more than the impact of alone treatment on the liver. Thus, these prospects will help to elucidate that both substances could be had pro-apoptotic and genotoxic properties. In disparity, our outcomes assumed that GA reduced the fatal impacts persuaded by ZnO NPs and SP, which may be lessened the severity of hepatic injuries and succeeding hepatic function instabilities with the mitigation of the genetic and oxidant prominences in liver cells. Thus, this study confirmed that GA acts as an anti-apoptotic and genoprotective agent in hepatocellular tissue. As an ultimate point, we recommend using GA as a supplement through the consumption of risky sources in the marketplace claims.

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