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Reproductive injury convinced by zinc oxide nanoparticles and *Senecio glaucus* plant actions via oxidative damage, hormonal disturbance, and histopathological change with the hopeful prophylactic effect of gallic acid in male rats

Ali, Abdel Aziz El Sheikh[1]; Nabil, Hassan Moustafa[2]; Nouran, Mohamed EL-Shafey[1]

[1]Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt.

[2]Organic Division, Chemistry Department, Faculty of Science, Zagazig University, Egypt.

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Abstract:

This study intended to estimate the lethal effects of either alone or combined treatments of zinc oxide nanoparticles (ZnO NPs) and mureer or *Senecio glaucus* L. plant (SP) on testis via oxidative, biochemical, and histological studies, and to assess the promising protective effect of gallic acid (GA) in rats. Rats were allocated into eight groups with orally treated for 30 days as follows: Control, GA (100mg/kg), ZnO NPs (150mg/kg), SP (400mg/kg), GA+ZnO NPs (100,150mg/kg), GA+SP (100,400mg/kg), ZnO NPs+SP (150,400mg/kg), and GA+ZnO NPs+SP (100,150,400mg/kg). Our data said that ZnO NPs and SP reduced testosterone concentration and raised some parameters, such as (Acidic phosphatase and lactate dehydrogenase activities). They also declined glutathione–S–transferase activity and elevated malondialdehyde levels. Moreover, they caused testicular necrosis and sloughing in its tubules. Our data also detected that the toxic effect of combined treatment was harder than the effect of alone one. Inversely, GA amended testicular damage. Lastly, this study endorsed that the alone or mixed usages of ZnO NPs and SP may be used as alternative insecticides in a pest control program of the agricultural applications and GA may be used as a protective source against ecological testicular-toxins.

Introduction

Rodents are one of the greatest risks pests in Egypt. They persuade harmful impacts on cultivated crops and protected harvests. The recurrent use of chemical rodenticides in pest control programs may be reflected as having a negative effect on the environment. Hence, it must be used unconventional insecticides to control pests, such as nanoparticles and natural plants that act safer than on living organisms. Nanotechnology has newly displayed an inventive science, which could be exploited in many mandatory applications in varied fields, including agriculture, medicine, electricity, and industry (Thiruvengadam *et al*., 2018 and Saratale *et al*., 2018). Generally, the nanostructures have strange physiognomies with a small-scale size of only around (1–100 nm) and a wide surface area to interact with any outward surface. Hence, nanoparticles (NPs) have already become fleeting within intracellular membranes and plentiful biological barriers disturbing the biomolecules in the cellular membranes (Jesus *et al*., 2019). With a full application of NPs, nanotoxicology

has concerned a significant devoutness in environmental science. Due to the easiness permitting of NPs through the blood-testis barrier, they convinced testicular function moans (Stanton, 2016). Many academics have shown that NPs effectively stimulated compensations in the male reproductive system, resulting in the reduction of testosterone (TST) synthesis, impaired spermatogenesis, decreased libido, and eventually infertility. These changes may take a cytotoxic route via oxidative damage, necrosis, apoptosis, and proliferation of testicular cells (Brohi *et al*., 2017 and Wang *et al*., 2018).

Amongst the used eminent NPs, zinc oxide nanoparticles (ZnO NPs) are the further most efficient NPs, which are entered in various profitable materials, such as sensors, sunscreens, batteries, food storing, ceramics, electronics, insecticides, and drug delivery. Therefore, they can urge many deleterious effects in diverse organs, especially in the testis organ (Singh, 2019). Due to their rapid distribution, slow or ineffective elimination, their dissolution, and possible long-time tissue accumulation, they can persuade zinc homeostasis imbalance results in a range of remedial signs, such as testicular atrophy, cerebral as well as immune dysfunctions, and reducing drug removal capability of the body (Grüngreiff *et al*., 2016).

Consuming natural plants is extensively used in an unconventional fashion of insecticidal sources that deed as slighter negative effects and a biodegradable manner in the environment. Amongst the well-known native plants, Mureer or *Senecio glaucus* subsp. coronopifolius (Maire) C. Alexander L. (SP) reveals as one member of (Asteraceae family, *Senecio* species) found in the deserts all over the world.

Unfortunately, they caused risky problems in animals, which negatively produced enzymatic disturbances, hematoma, diarrhea, pulmonary difficulties, cardiac abnormalities, and hepatic degeneration (Ren *et al*., 2016). No experimental studies have explored the effect of SP in testis rats; yet, numerous studies have reported there were disparaging influences of *Senecio* plants in many surviving cells (Tundis *et al*., 2011 and Mendonça *et al*., 2019). From ancient criticism, there are many phytochemical compounds, such as saponins, flavonoids, alkaloids, phenolic compounds, coumarin, and others accumulated in intracellular tissues, which are responsible for tissue damage (Parra *et al*., 2017).

In particular, the testis is a principal organ that is responsible for the spermatogenesis process, which produced spermatozoa in the seminiferous tubules. It is more sensitive to exposure to noxious compounds (Staub and Johnson, 2018). From a consideration, male infertility persuades a result of a disturbance in the spermatogenesis process, which is based on a reduction in sperm count, oxidative stress inspiration, and disturbance of sex hormones. Among the conjoint biomarkers for evaluation of testis damage, TST hormone is the main androgen secreted hormone in the blood. It is secreted from the Leydig cells of the testis. Furthermore, it is responsible for the progress of secondary male sex characteristics and its capability of fertilization (Nassar and Leslie, 2021).

Even if the TST hormone may be found in three primary forms: unbound or free, TST, tightly bound TST, and supported TST. Only free and weakly bound TST can bind to the androgen receptor (Rhoden and Morgentaler, 2004). It is most organs exposed to oxidative stress (OS) that is the main cause for the generation of programmed cell death, which is an

abnormal imbalance between oxidants and antioxidants in cellular membranes, leading to lipid peroxidation (LP), DNA damage, and protein interruption in intracellular tissue. The antioxidant defense mechanism encompasses enzymatic and non-enzymatic systems, such as glutathione-S-transferase (GST) protected the cells from reactive oxygen species production (ROS) action (Preiser, 2012).

Afterward, it is compulsory to examine more tangible approaches against persuaded complications while conserving or augmenting its useful effects. Amid these valuable approaches, the use of antioxidants, such as gallic acid (GA). GA, (3,4,5 trihydroxy benzoic acid), is a polyphenol natural product found in roses, oak, grapes, mango, cashew nut, hazelnut, walnut, raspberry, lemon, spinach, gallnut, and green tea. It is speedily absorbed through the gastrointestinal tract into the bloodstream passing through a wide variety of metabolic processes, which is concomitant with its antioxidant effects. Numerous studies have revealed that it has many important activities, including antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer activities. It has the ability to cell recovery from inflammation (Khan *et al*., 2018 and Singla *et al*., 2020). In order to explain the correlation between the structure of GA and protective capability, it has a benzene ring with hydroxyl and carboxyl groups that can evade ROS. Additionally, it can interrelate with ions to lessen the potentials of free radical production. Overall, the hydroxyl group of its structure can pass through the liposome membrane to retort with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical and stimulate repression of LP in testicular cells (Kilic *et al*., 2019).

As an ultimate point, the purpose of the current study was designed to examine the deadly effects of either alone or joined treatments of ZnO NPs and SP through scrutinizing biochemical, oxidative, and histopathological investigations on testis tissue. Moreover, this study scrutinized to appraise the imaginable ameliorative effect of GA against reproductive toxicity fortified by ZnO NPs and SP in male rats.

Materials and methods

Zinc oxide nanoparticles (ZnO NPs) (<50 nm) (BET), gallic acid (GA), and sodium carboxymethyl cellulose (Na-CMC) salt were purchased from (Sigma Aldrich, St. Louis, Missouri, USA). 70% of ethanol solvent was obtained from (EL-Naser Company, Egypt). Malondialdehyde and glutathione-S-transferase kits were coming from (Biodiagnostic Company, Egypt). A testosterone kit also was bought from (Ezo Company, Asia). LDH kit was credited from (Egyptian Company for Biotechnology, S.A.E, Egypt). Other chemicals and reagents were secondhand from high-quality kinds of biochemical analysis.

1. The characterization and preparation of ZnO NPs nanoparticles:

The description of ZnO NPs can be measured to ascertain their size and morphology using the transmission electron microscopy (TEM) (JEOL-JEM-1230, National Research Center) equipped with a digital CCD camera at 100 kV. ZnO NPs suspension was equipped by using 0.5% Na-CMC sonicated for 20 min. in a bath sonicator and vortexed for 1min before every administration in Plant Protection Research Institute, El-Sharkia.

2. Preparation of ethanolic extract of mureer plant:

The whole portions of the renewed plant of mureer were harvested in April from Cairo- Ismailia Road, Egypt with Taxon identifier (183639) on NCBI (National Center for

Biotechnology Information) database that was nominated by Dr. Abdel-Halim Abdel-Mogaly, Botanist, Herbarium of Horticultural Research Institute, Egypt. After grinding dried plant parts (leaves, stems, roots, and flowers) in the air, the achieved powder was used to prepare the ethanolic extract by the maceration method for 72 h. At the end of the maceration period, the liquid part was filtered with a Whatman paper and gotten out of the excess solvent at 60 °C through a rotary evaporator. Then, it was entirely dehydrated in an aired oven at 45 °C. The crude extract was retained at -20 °C for the experiment (Bahrin *et al*., 2018).

2.1. Estimation of phytochemical components of plant:

The primary phytochemical screening test was carried out the entire plant for the recognition of various constituents using typical methods, such as saponins, alkaloids, flavonoids, and phenolic compounds that were measured as the approaches, Hiai *et al*., 1976; Yubin *et al*., 2014; Zhishen *et al*., 1999 and Chun *et al*., 2003, respectively.

2.2. Calculation of median lethal dose (LD_{50}) of ethanolic plant extract:

It was performed according to Karber (1931). It was used an oral single dose of (2500, 5000, 10000, 2000, 40000, and 80000 mg/kg) of an ethanolic extract of the plant to determine LD_{50} from this 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.

3. Experimental protocol:

An experimental trial was conducted on forty male *Wistar* albino rats (*Rattus norvegicus*). They were weighed (180-220 g) and had 7 weeks age-old. It was made according to the approval of the Animal Ethics Committee of Zagazig University under "Principles of Laboratory Animal Care". The rats were housed in cages in a persistent temperature (23±2 °C), humidity (60±10%), and a light/dark (12 hrs.: 12 hrs.) cycle. After a week of acclimation, the rats were allocated into eight groups containing 5 rats in each one as follows: **a.** Control group: rats were received (0.5% Na–CMC with 5 ml/kg, b.wt) as a vehicle (Dhiyaaldeen *et al*., 2014). **b.** GA-treated group: rats were received (100 mg/kg, b.wt.) of GA (Mansouri *et al*., 2013) dangling in 0.5% Na-CMC (Sen *et al*., 2013). **c.** ZnO NPs-treated group: rats were received (150 mg/kg, b.wt) of ZnO NPs, which was adjourned in 0.5% Na-CMC (Srivastav *et al*., 2016). **d.** SP-treated group: rats were received (400 mg/kg, b.wt) of SP chosen through LD_{50} experiment in this study. **e.** GA+ZnO NPs-treated group: rats were received (100 and 150 mg/kg, b.wt), respectively. 6) GA+SP-treated group: rats were received (100 and 400 mg/kg, b.wt), respectively. **f.** ZnO NPs+SP-treated group: rats were received (150 and 400 mg/kg, b.wt), respectively. 8) GA+ZnO NPs+SP-treated group: rats were received (100, 150 and 400 mg/kg, b.wt), respectively. GA was managed at the pretreatment of other substances. The administration of all poisonous and ameliorating agents was set for one month by gavage administration and suspended in 0.5% Na-CMC at volume (5 ml/kg) (Plate 1).

After this period, the rats were assassinated by cervical dislocation. The serum was collected for total lipid analysis and testis tissue was sensibly dissected that was divided into two parts as follows: a small portion of testis tissue was homogenized and centrifuged at 3000 rpm for 10 min to do biochemical and antioxidant studies. Another part was stored at 10% neutral

buffered formalin for histological studies.

4. Estimation of biochemical parameters:

4.1. Biochemical analyses:

4.1.1. Estimation of testicular testosterone concentration (TST) bioassay:

The TST ELISA kit could be a competitive ELISA (Enzyme-linked immunosorbent assay) that was designed for the accurate and quantitative method. It was purchased from Ezo Company according to the procedures of Abraham (1981). A Supernatant of testicular homogenate was reacted with TST-HRP conjugate in the wells, where TST in the sample competed with the added TST-HRP antibody binding. After incubation, the wells were washed to remove unbound material and TMB substrate was then added catalyzed by HRP to produce a blue color. The reaction was ended with the addition of a stop solution, which was stopped the development of color and produced a color change from blue to yellow. The intensity of the signal was inversely proportional to the amount of TST in the sample and the intensity was measured at 450 nm.

4.1.2. Estimation of testicular acidic phosphatase activity (ACP) bioassay:

Testicular ACP activity was evaluated according to Kind and King (1954) method that phenyl phosphate phenol was reacted with sample acidic phosphatase in the presence of pH 4.9. Then, the liberated phenol was colorimetrically measured in the presence of 4-aminophenazone and potassium ferricyanide to appear a defined color at 510 nm.

4.1.3. Estimation of testicular lactate dehydrogenase activity (LDH) bioassay:

Testicular LDH activity catalyzes the change of lactate to pyruvate. The enzyme was assessed according to Zimmerman and Hennery (1979). 20 µl of testicular homogenate was added to 1ml of working solution, mixed, and read at an initial absorbance after 30 sec. Then, it must be read again after 1, 2, and 3 min. It was estimated the difference absorbance per min.

5. Estimation of prooxidant/antioxidant status biomarkers:

5.1. Estimation of testicular malondialdehyde (MDA) level:

The MDA level of the testicular homogenate was determined according to the method of Ohkawa *et al.* (1979), which was based on thiobarbituric acid (TBA). It reacted with MDA content in a sample at an acidic medium and a boiling water bath for 30 min to make TBA reactive product. The absorbance of the obtained pink product can be measured at 534 nm.

5.2. Estimation of testicular glutathione-S-transferase (GST) activity:

GST activity of the testicular homogenate was estimated according to the method of Habig *et al*. (1974) using 1-chloro2,4-dinitrobenzene as an electrophilic substrate that joined to GSH to the participation of the enzyme, created a colored GSH-substrate complex, and detected at 340 nm. The proportion of the increase was directly proportional to the GST activity in the sample.

6. Histopathological studies:

Testis specimens were fixed using 10% neutral buffered formaldehyde. After proper fixation, the specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylol, impregnated, and embedded in paraffin wax. 5-µm thick sections were cut using a rotatory microtome. Testis sections were stained with routine hematoxylin and eosin (H&E.,) stain for studying the general histological structure of the testis (Bancroft and Layton, 2012).

7. Statistical analysis:

The quantitative variables were estimated by applying the statistical software package SPSS (IBM Corp. SPSS, 2011).They were described as a mean±standard deviation (Mean±SD) using one-way ANOVA followed by Tukey's post hoc test for the comparison between several groups. The level of significance was documented at $P<0.05$.

Results and discussion

The existing study targeted to investigate the toxic impacts of either alone or united actions of ZnO NPs and SP on testes and to assess the cautious effect of GA against their noxious effects in male rats.

1. The size and shape of ZnO NPs estimation:

Our results showed that the size and morphology of ZnO NPs were estimated using TEM that seemed as 43±1 nm and the morphology of particles was noticed as spherical crystals (Figure 1).

2. Phytochemical constituent screening of SP:

Moreover, our data presented that SP enclosed an extraordinary amount of saponins, mediocre amount of phenolic compounds, and small amount of flavonoid and alkaloid compounds, (Table 1) that were the main cause for convincing its hurtful influences of plant extract on testis tissue and persuaded tissue damage when gathered.

3. Toxicity trial of ethanolic extract of SP (LD_{50}):

Table (2) displayed that LD_{50} of SP in rats was obtained (60.000 mg/kg), so our study selected a defined dose (400 mg/kg) or (1/150 LD_{50}) used in it and made our investigations in rats. It did not give any mortality for 24 hrs.

4. Estimation of biochemical parameters:

Table (3) exhibited that either alone or combined treatments of ZnO NPs and SP may be persuaded reproductive-toxicity and the pretreatment of GA caused a testicular-protective effect through the studying of testicular function biomarkers: testicular ACP activity (U/g tissue), testicular TST concentration (ng/g protein), and testicular LDH activity (U/mg protein).

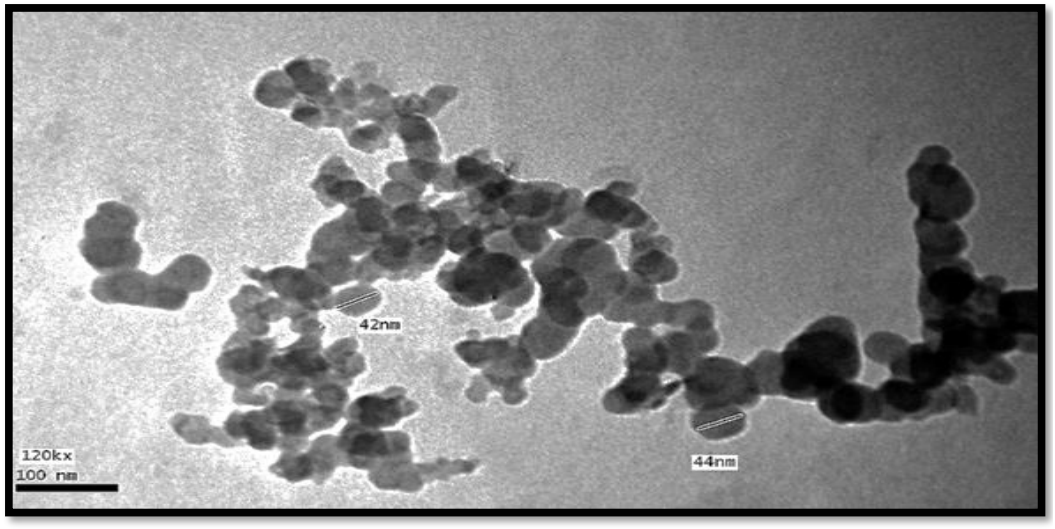

Figure (1): Transmission electron microscopy (TEM) image of ZnO NPs showing the morphology and size of the particles in Milli-Q water. Particles were scanned by TEM at 120kV.

Table (1): Phytochemical constituents of *Senecio glaucus* plant (SP).

Analysis	Quantity
Saponins	+++
Alkaloids	+
Total phenolic	++
Total flavonoids	+

+++: high amount, ++:medium, +: low.

Table (2): Determination of median lethal dose (LD_{50}) of ethanolic extract of *Senecio glaucus* plant (SP) in rats.

Groups	Doses (mg/kg)	Number of animals (N)	Dead animals	Md (Mortality difference)	Dd (Dead difference)
1	0	4	0	0	0
2	2.500	4	0	0	2.500
2	5.000	4	0	0	2.500
4	10.000	4	0	0	5.000
5	20.000	4	0	0	10.000
6	40.000	4	0	0	20.000
7	80.000	4	4	2	40.000
Sum					∑ 80.000

$LD_{50} = D_{100} - \sum \frac{Md\ xDd}{N} = 80.000 - \frac{80.000}{4} = 60.000$ mg/kg.

Table (3): Influence of Zinc oxide nanoparticles (ZnO NPs), *Senecio glaucus* L. plant (SP), and gallic acid (GA) on the biochemical parameters [testicular acidic phosphatase activity (ACP) (U/g tissue), testicular lactate dehydrogenase activity (LDH) (U/mg protein), and testicular testosterone concentration (TST) (ng/g protein)].

Groups	ACP (U/g tissue)	LDH (U/mg protein)	TST (ng/g protein)
Control	**30.152±1.52**	**1162.89±1.93**	**50.33±1.73**
GA	**25.71±1.31**$^{***\ g}$	**1154.24±1.57** $^{***\ g}$	**62.55±1.28**$^{***\ g}$
ZnO NPs	**95.45±1.29** ^{***a}	**1531.72±1.53**$^{***\ a}$	**37.71±1.88**$^{***\ a}$
SP	**46.31±1.05** ***	**1263.19±2.87** ***	**31.57±0.89*****
GA+ZnO NPs	**61.93±1.47**$^{***\ d}$	**1512.98±1.97**$^{***\ d}$	**41.58±1.02**$^{***\ d}$
GA+SP	**42.34±1.19**$^{***\ e}$	**1255.49±2.09**$^{***\ e}$	**36.52±0.78**$^{***\ e}$
ZnO NPs+SP	**151.09±0.85**$^{***\ b,c}$	**1763.93±2.56** $^{***\ b,c}$	**15.77±1.22**$^{***\ b,c}$
GA+ Zn NPs+SP	**126.25±0.95**$^{***\ f}$	**1722.95±1.47** $^{***\ f}$	**23.06±1.12**$^{***\ f}$

Values were given as mean±SD, (n=5 rats per group). Statistical analysis was done by 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}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].

On a hand, it was evident from the data, testicular ACP activity and testicular LDH activity were significantly increased in either alone or combined treatments of ZnO NPs and SP compared to the control group ($P \leq 0.001$). Correspondingly, there was an improvement in GA-treated group in the values of ACP and LDH activities compared to the control group due to its antioxidant activity. On the other hand, the pretreatment of GA to ZnO NPs and SP significantly reduced in these parameters relative to either alone or combined treatments 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) ($P \leq 0.001$).

Furthermore, our data also reported that the testicular TST concentration was significantly decreased in either alone or collective treatments of ZnO NPs and SP

compared to the control group (P≤0.001). Likewise, there was an enhancement in GA-treated group in the value of TST concentration relative to the control group due to its special endocrine excitation characters. In addition, the alterations in all tested parameters of the combined treatment of ZnO NPs and SP were stronger than the alterations in the alone treatment of them. Moreover, the changes in these parameters of ZnO NPs-treated group were severer than the changes in SP-treated group.

In contrast, the pretreatment of GA to ZnO NPs and SP significantly increased in the TST concentration relative to either alone or combined treatments 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) (P≤0.001).

5. Estimation of testicular antioxidant/oxidative status biomarkers:

Table (4) presented that either alone or combined treatments of ZnO NPs and SP could persuade OS *invivo* and provoke the antioxidant outcome of GA against oxidative damage that was discussing the effect of MDA level (nmol/g tissue) and GST activity (U/g protein) in testicular tissue. Our records observed that they caused a significant increase in MDA level; however, they induced a significant decrease in GST activity compared to the control group (P<0.001). Moreover, there was no significant change between the control and GA-treated groups in both parameters. Furthermore, the shifts in these biomarkers of the combined treatment of ZnO NPs and SP (ZnO NPs+SP-treated group) were more powerful than the shifts in the alone treatment of them (ZnO NPs-treated group and SP-treated group). Moreover, the deviations in these biomarkers of ZnO NPs-treated group were more sturdily than the deviations in SP-treated group.

Table (4): Influence of Zinc oxide nanoparticles (ZnO NPs), *Senecio glaucus* L. plant (SP), and Gallic acid (GA) on testicular oxidative biomarkers [malondialdehyde (MDA) (nmol/g tissue) and Glutathione-S-transferases (GST) (U/g protein)].

Groups	MDA (nmol/g tissue)	GST (U/g protein)
Control	41.69±1.43	1.25±0.07
GA	39.10±1.18 $^{n.s.\ g}$	1.47±0.16 $^{n.s.\ g}$
ZnO NPs	101.13±0.91$^{***\ a}$	0.36±0.06 $^{***\ a}$
SP	51.64±0.79***	0.41±0.05 ***
GA+ZnO NPs	81.48±0.77^{***d}	0.64±0.04 $^{***\ d}$
GA+SP	40.99±1.29$^{***\ e}$	0.60±0.03 ^{***e}
ZnO NPs+SP	103.26±1.76$^{***\ b,c}$	0.26±0.04 $^{***\ b,c}$
GA+Zn NPs+SP	91.31±1.11^{***f}	0.50±0.08 $^{***\ f}$

Values were given as mean±SD, (n=5 rats per group). Statistical analysis was done by 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}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].

Indifference, the pretreatment of GA to both compounds caused a significant reduction in MDA level, but it induced a significant rise in GST activity relative to either alone or combined treatments 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) ($P \leq 0.001$).

Inclusively, our data observed that the deadly impacts of mixed treatment of ZnO NPs and SP were more strappingly than the alone treatment on the level of the biochemical and antioxidant/oxidant status parameters. Correspondingly, our records showed that GA might be acting as an anti-testicular toxic and antioxidant agent against testicular toxicity induced by both compounds.

6. Histopathological studies:

Figure (2:a-h) illustrated that the histological investigation of testis tissue in treated groups. Our data showed that the testicular toxic effect of ZnO NPs and SP and the preventive effect of GA against them. Microscopically, there was a normal histological structure of testis tissue comprising from spermatogonia in the control group, including spermatocyte cells surrounded by spermatocyte membrane, which secreted spermatozoa and Leydig cells in interstitial areas, (Figure 2:a). Additionally, a healthy structure of testicular tissue appeared in GA–treated group similar to the control group (Figure 2:b). Meticulously, on a hand, the sloughing in the seminiferous tubules, the appearance of necrotic cells, the degeneration of germinal epithelium, the widening in the interstitial tissues and vacuolization in the germ cells occurred in ZnO NPs-treated group, (Figure 2:c). Correspondingly, a slight sloughing of seminiferous tubules from the basement membrane and an appearance of a small hemorrhage region transpired in SP-treated group, (Figure 2:d).

On the other hand, a mild sloughing of seminiferous tubules, a moderate degeneration in some tubules, and an appearance of vacuolization in the germ cell existed in GA+ZnO NPs-treated group, (Figure 2:e). Interestingly, GA+SP-treated group seemed to have any change in testis tissue close to the control group, (Figure 2:f). The combined group of ZnO NPs and SP persuaded extensive necrosis in the spermatogenic cells, a reduction in the germinal cells, interstitial capillary congestion, a thickening of the wall of blood vessels, and a sloughing in the seminiferous tubules, (Figure 2:g). However, the pretreatment of GA to them in GA+ZnO NPs+SP-treated group caused a simple sloughing in tubules, (Figure 2:h).

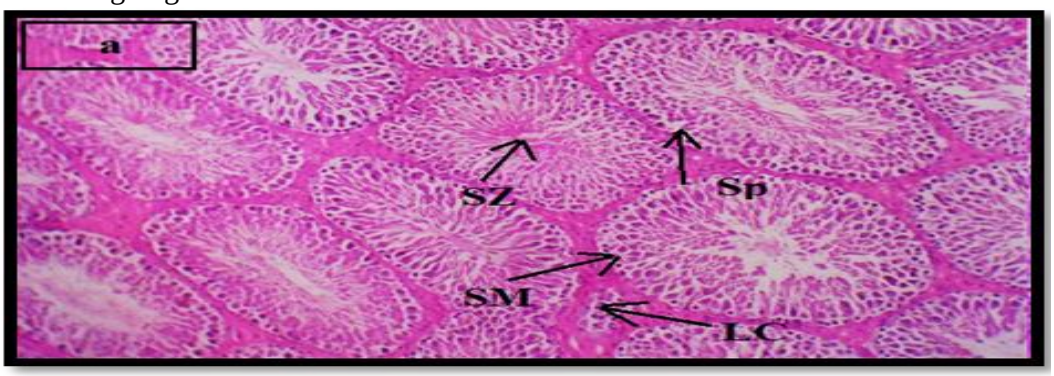

Figure 2(a): Control group showing the normal histological structure of testis tissue: spermatocyte cells (Sp), spermatocyte membrane (SM), spermatozoa (SZ), and Leydig cells (LC) (H&E.,×200).

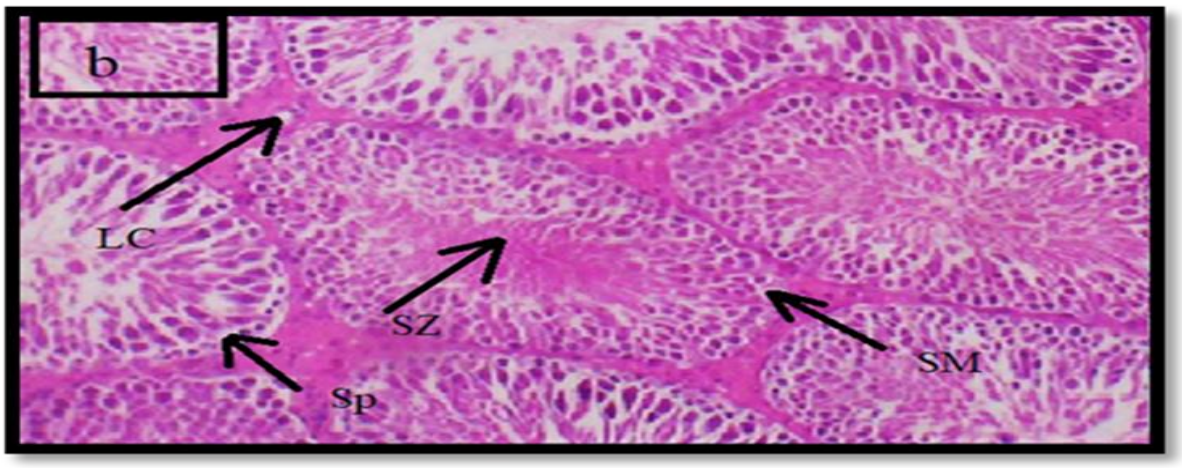

Figure 2(b): GA-treated group showing a healthy normal structure of testicular tissue similar to the control group: spermatocytes (Sp), spermatocyte membrane (SM), spermatozoa (SZ), and Leydig cells (LC) (H&E.,×200).

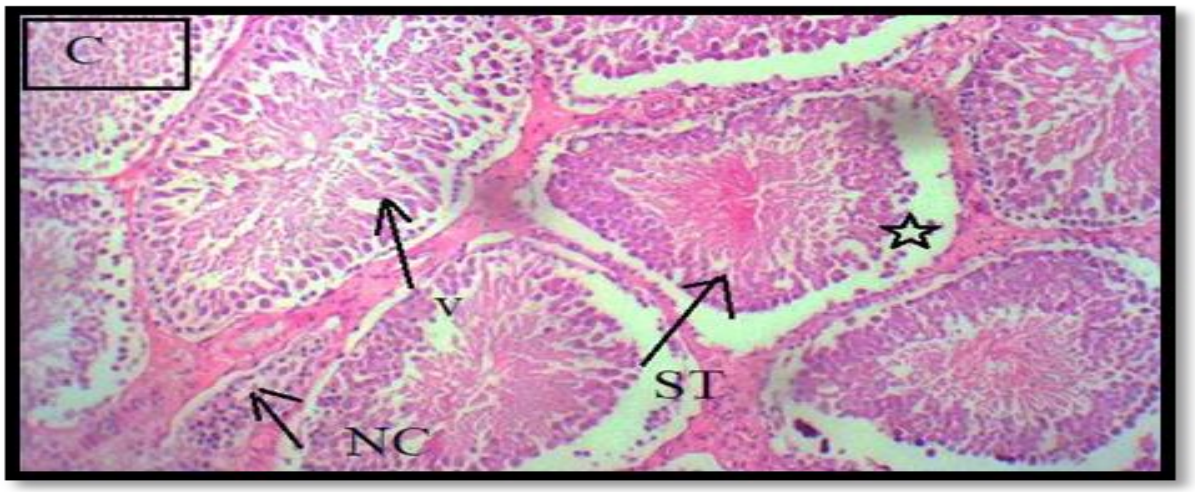

Figure 2(c): ZnO NPs-treated group showing an appearance of necrotic cells (NC), degeneration of germinal epithelium with sloughing of seminiferous tubules from basement membranes (ST), widening of interstitial tissue (star), and appearance of vacuoles (V) (H&E.,×200).

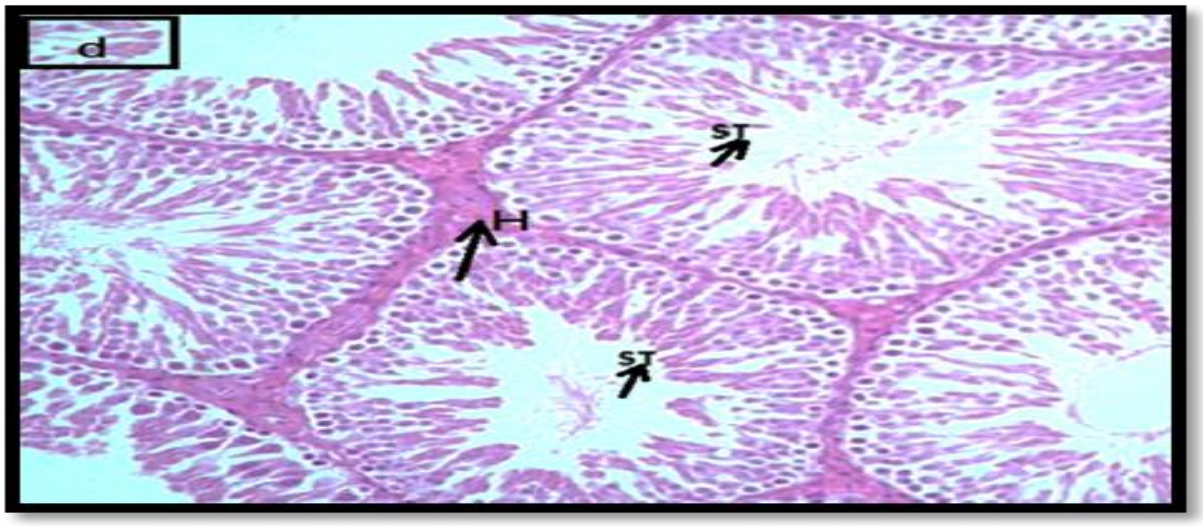

Figure 2(d): SP-treated group showing slight sloughing of seminiferous tubules from basement membranes (ST) and small hemorrhage area (H) (H&E.,×200).

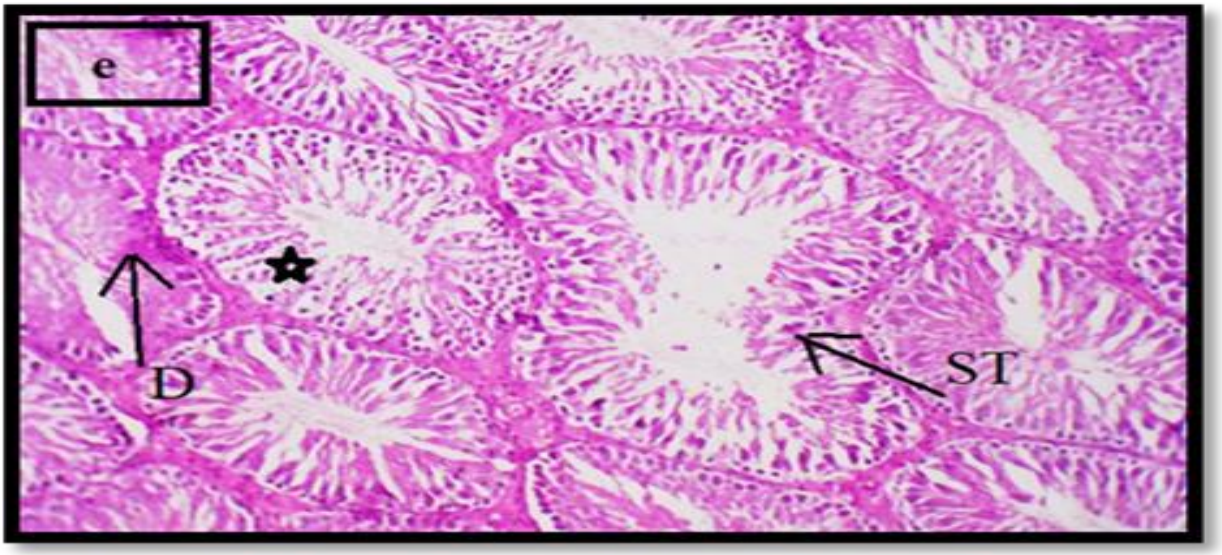

Figure 2(e): GA+ZnO-NPs-treated group showing a mild sloughing of seminiferous tubules organizations (ST), moderate degeneration in some tubules (D), and an appearance of vacuolization (star) (H&E.,×200).

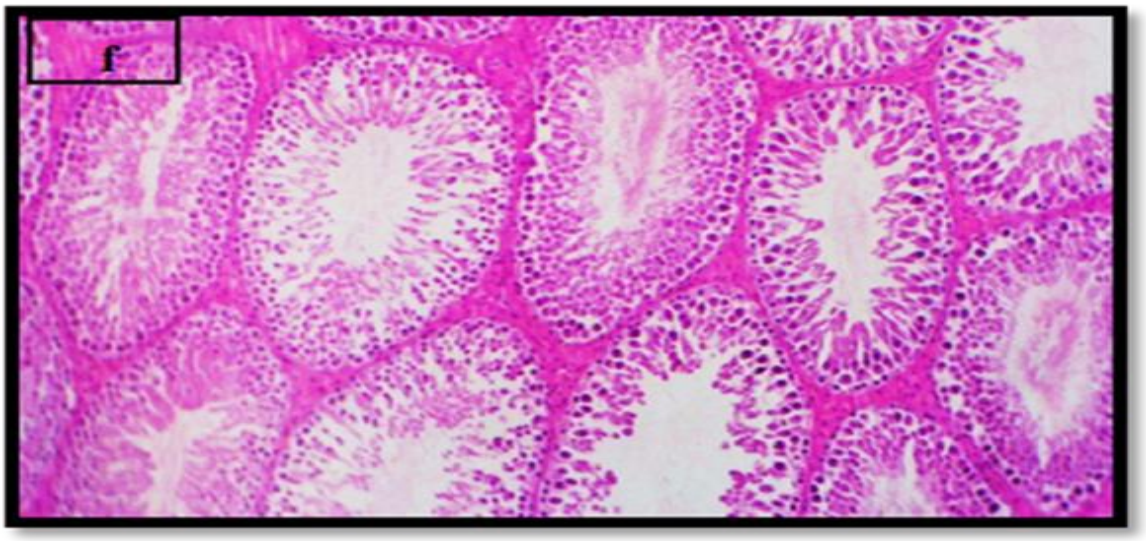

Figure 2(f): GA+SP-treated group showing a closely similar normal structure of testis tissue (H&E.,×200).

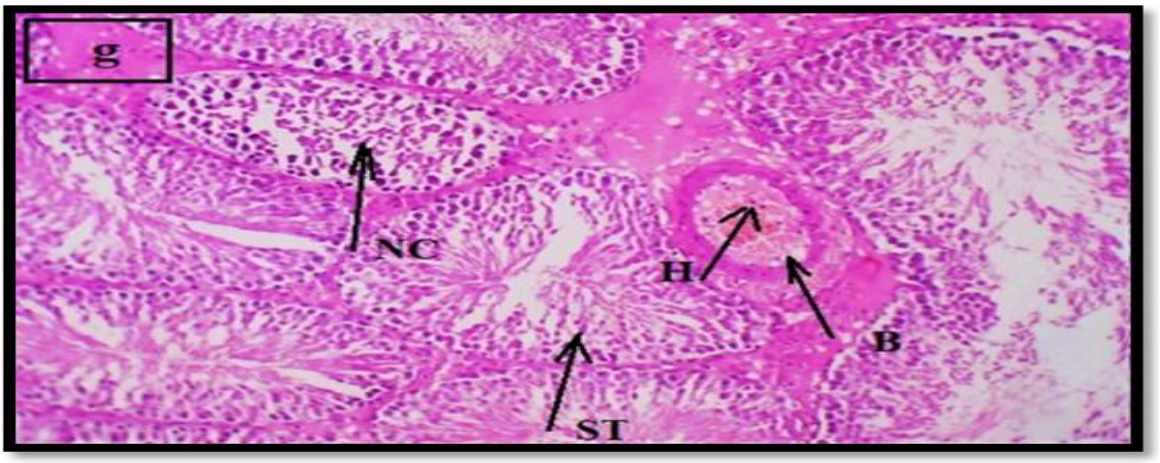

Figure 2(g): ZnONPs+SP-treated group showing extensive necrosis of spermatogenic cells (NC), reduction in germinal cells along with interstitial capillary hemorrhage (H), widening of interstitial tissue with thickening of the wall of blood vessels (B), and sloughing of seminiferous tubules (ST) (H&E.,×200).

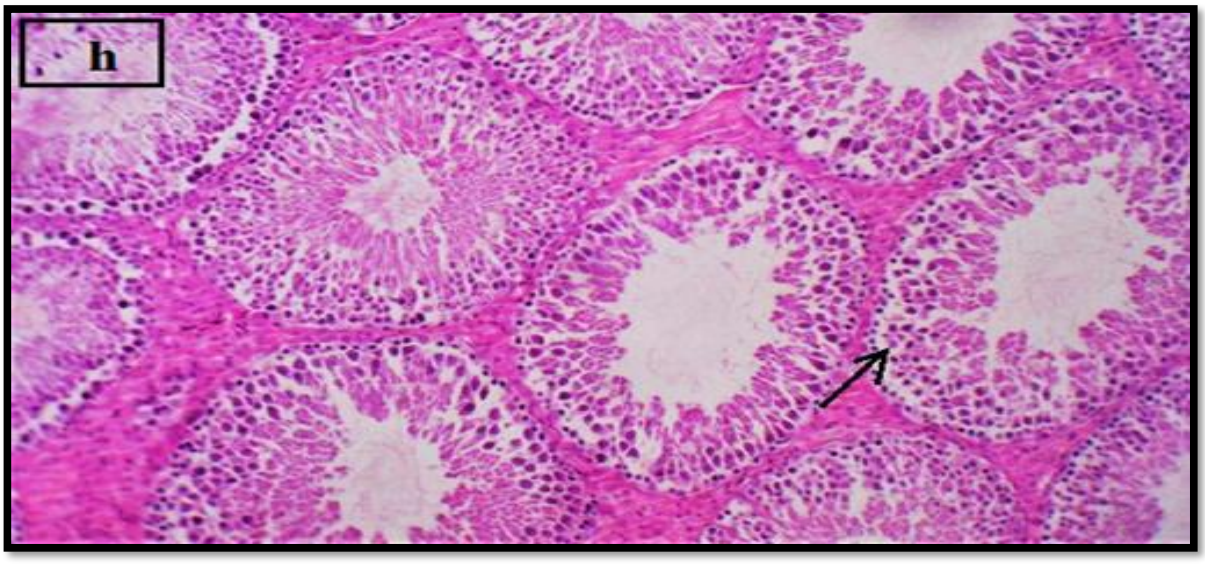

Figure 2(h): GA+ZnO NPs+SP-treated group showing close to the normal structure of testis (arrow) (H&E.,×200).

Figure 2(a-h): Photomicrograph of influence of Zinc oxide nanoparticles (ZnO NPs), Mureer or *Senecio glaucus* L. plant (SP), and gallic acid (GA) on the histopathological studies of testis tissue in treated groups.

In order to evaluate testicular toxicity, TST hormone is the main biomarker, which must be measured. It may be found as a free form or binding to a carrier molecule (albumin or globulin) that protected the integrity of the testis. Moreover, this protein controls TST secretion, which is the conversion of TST to active metabolites stimulating the binding of luteinizing hormone (LH) to its receptors to initiate the intracellular signaling pathway. Thus, this is responsible for the production of TST from the Leydig cells. Total TST is the best biomarker for testis integrity estimation (Kelly and Jones, 2013). In light of such results, the repetition in TST synthesis leads to a loss of the ability of fertility and sperm production. Furthermore, the deficit in TST level inhibits germ cell production from the transitory meiotic stage of division (Holdcraft and Braun, 2004).

Generally, the sperm supply and androgen metabolic enzymes have become the principal functions of testis relevant to the spermatogenesis process, but testicular damage is caused by secreting LDH enzymes outside the tissue and produced the deficiency in TST exudation. Hence, it has already produced hypogonadism parameters, such as prolonged sexual development and shrinking testes that are allied to a drop in the energy content and increased body fat accumulation inside them (Strauss *et al*., 2009).

Nevertheless, the existing information regarding about ACP enzyme is a very vital enzyme that is responsible for hydrolyzing phosphoric esters of various phosphate-containing compounds at acid pH. It is involved in several metabolic processes, such as production, transport, and utilization of inorganic phosphate that is the principle of cell growth and differentiation. It is found in the lysosome of the Leydig

cells, which performs the synthesis of proteins carried by sex hormones. Unfortunately, ACP enzyme is released in large amounts into semen when there is reproductive harmfulness, such as prostate cancer. It caused thickening of the basement membrane of seminiferous tubules, tubular atrophy, and arrest of cell maturation (Akimoto *et al*., 1997). Apart from the previous approaches and explanations, ACP activity and TST concentration are the chief biomarkers for a guesstimate of the reproductive toxicity of any toxic material antagonizing to the androgen receptor that can change the glycosylation of gonadotropins leading to the turbulences in the levels (Sikka and Naz, 1999).

Our results were in the same line with, Hong *et al*. (2016) who reported that NPs treatment caused a reduction in TST level, damaged spermatogenesis, and induced infertility in mice. In addition, they were in harmony with Zhang *et al*., (2020) who showed that NPs management persuaded an inhibition in TST production, DNA damage, and stimulated levels of mRNA expression of apoptotic genes in genital cells. Thus, they impaired the quality of sperm parameters, including a reduction in sperm count, motility, and vitality. Our observations were in agreement with Sharafutdinova *et al.* (2018) who presented that titanium dioxide nanoparticles instigated many morphological changes in spermatocyte constituents and sperm numbers. Furthermore, our results about the toxic effect of plant extract were according with, Lienou *et al*. (2012) who described that *Senecio* plant caused peritubular testicular fibrosis, reduced sperm number, disrupted regulation of LH, depressed TST synthesis, destruction of prenatal follicles, increased atresia, and reduced number of primordial follicles in female rats. Likewise, Diallo *et al*. (2015) revealed that feeding on a hydroalcoholic extract of *Ageratum conyzoides* plant (Asteraceae) induced many physiological disorders in the gentile cells of rats.

When testes may suffer dramatically from OS due to induction of LP and caused leakage of LDH from the mitochondrial membrane altering both spermatogenic and steroidogenic functions. These events instigated a lack of blood supply in testis (Aitken and Roman, 2008). OS can be modulated a steroidogenic capacity of Leydig cells disrupting the ability of germinal epithelium to distinguish spermatozoa, which may be arisen infertility (Hales *et al*., 2005). Generally, MDA is the product of LP that impairs antioxidant capacity in testicular tissue. Furthermore, zinc balance disturbance causes a reduction in the sulfhydryl groups of cysteine residues, leading to a conformational modification in the protein assembly causing GST activity disorder (Ho *et al*., 2003).

From the aforementioned report, ROS are the main cause for the induction of inflammation and necrosis associated with NF-κB pathway activation that caused the production of the inflammatory cascade. Moreover, testicular damage can drop the expression of connexin43 protein because of apoptosis in Sertoli and spermatogenic cells, which may be a reason for a reduction in the height of epithelial cells and tubules diameter. Thus, this process has already caused the breakdown of DNA, chromatin condensation, and protein destruction involving histological alterations (Jiménez-Lamana *et al*., 2014). Finally, of this part, Lone *et al*., (2014) explained the negative impact of plant extract in the testis due to the accumulation of chemical constituents

of the *Senecio* plant, which is responsible for its cytotoxicity effect.

In contrast, to explain the positive role of GA in this study, antioxidants alleviated prostate and testicular injuries because of suppressing the inflammatory and apoptotic mechanisms promoting normal spermatogenesis (Shahin and Mohamed, 2017). Confirming our results, the histological structure of the testis was recovered, which was evidence of the protective effect of GA against either alone or mixed treatments of ZnO NPs and SP. It restores testicular degeneration, atrophy, and tubular disarrangement. The antioxidant power is dependent on the potential phenolic compound, which can prevent OS-mediated testicular damage by the capture of ROS. Furthermore, GA has the capability to stimulate the hypothalamic-pituitary-gonadal axis that improves the testicular function for the production of sperms (Mazloom *et al*., 2017).

Moreover, the optimistic effect of GA may be attributed to its proficiency in the generation of intracellular free zinc ions by persuading the secretion of metallothionein enzyme to incite nuclear factor-erythroid2-related factor2 (Nrf2) transcription factor that is responsible for stimulating the synthetic pathway of glutathione (Cortese *et al*., 2008).

Our findings were in the same line with, Oyagbemi *et al*. (2016) who showed that GA significantly balanced TST concentration and induced an improvement in the testicular atrophy disturbed by cyclophosphamide treatment in rats. Moreover, our outcomes were parallel to, Mehraban *et al*. (2020) who revealed that GA attenuated the disturbance in DNA sperm, marked testicular atrophy, and caused a significant decline in MDA levels after cyclophosphamide administration (Plate 1).

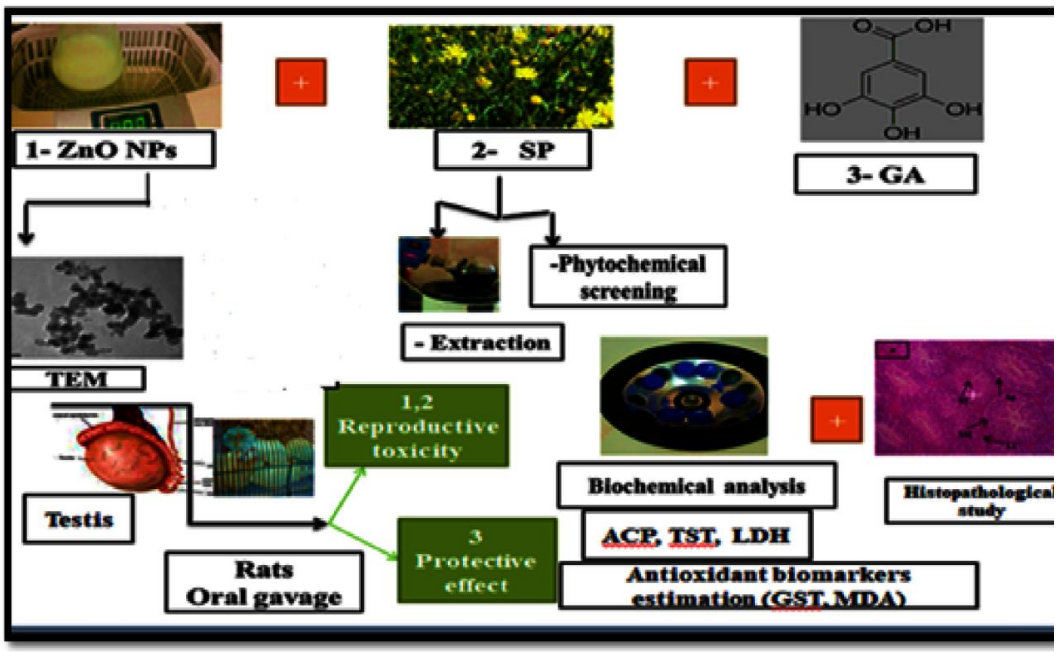

Plate (1): Graphic abstract

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