Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 29, issue 2, 2019, pp. 82-90
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GROWTH INHIBITION AND OXIDATIVE STRESS IN THE FRESHWATER CILIATE *PARAMECIUM* SP EXPOSED TO COPPER OXIDE NANOPARTICLES

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Abstract: Copper oxide nanoparticles (CuO-NPs) are widely used in various industrial and commercial applications, but little is known about their potential hazard in freshwater ciliate. In this study we investigated the effects of CuO-NPs in an alternative model *Paramecium* sp. which is perfectly adapted with toxicological studies. *Paramecium* cells were exposed in triplicate to control, 30, 60, 90 and 120 mg CuO L-1, and the growth kinetics was followed for 120 hours, enzymatic activities of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione-S-Transferase (GST) and Glutathione content (GSH) were investigated to evaluate antioxidative response to copper oxide nanoparticles.
Our results showed a dose-dependent inhibition of cell growth, on another hand, the monitoring of biomarkers revealed induction of SOD, CAT, and GST activities, in parallel a significant decrease on GSH content compared to control.

Keywords: *Paramecium* sp, growth kinetic, oxidative stress, CuO, toxicity

INTRODUCTION

Nanotechnology utilizes nanoparticles (NPs) that are unique not only due to their minute size (smaller than 100 nm) but also because of their size-dependent characteristics. NPs are being spotlighted as a center stage phenomenon for future industry (Arnaldi, 2014, Zhang et al., 2015). The production and number of applications of engineered NPs are increasing rapidly worldwide. Current applications include the use of NPs in consumer products, construction materials, medical and pharmaceutical industries, agriculture, and information technology (Karen et al., 2009). Metal oxide NPs are an important category of manufactured NPs, accounting for about one third of the consumer products nanotechnology market. For instance, copper oxide (CuO) NPs are used in a host of uses, such as gas sensors, catalysts, superconductors, and ceramic pigments (Zhu et al., 2004; Marino et al., 2007), antifouling agents in paints (Perreault et al., 2012) for ships and offshore oil platforms and applications in antimicrobial textiles (Ren et al., 2009; Dastjerdi, 2010; Delgado et al., 2011).

Nanoparticles are also found widely in nature, and natural sources include ash, desert dusts, aerosols and metal oxide particles. Some plants synthesize NPs that are used to reduce metal uptake in contaminated soils, and anaerobic bacteria may use them in respiration (Bernhardt et al., 2010)

In recent years, a growing number of toxicity studies provide evidence about potential hazards to the human health or the environment of different nanomaterials, including metal oxides, fullerenes, and carbon nanotubes (Kahru and Dubourguier, 2010). Although the mechanisms of nanotoxicity are still poorly understood and numerous reports attribute the toxicity of NPs to their small size, other nanomaterial characteristics such as particle morphology, particle composition, surface area, and surface chemistry have also been implicated on (Handy et al., 2008; Fubini et al., 2010).

Multiple organisms are used in ecotoxicology studies of NPs, including bacteria, fungi, algae, and crustaceans, among others (Kahru and Dubourguier, 2010). Surprisingly, freshwater protist species provide a good model for study on how environmental toxicity differs among species because of their ubiquitous global distribution and special sensitivity to environmental contaminants. Protozoa are single cell organisms that do not possess a protective cell wall. As a consequence, NPs could enter protozoan cells more easily than bacterial and algal cells and interact directly with the cellular structures and organelles (Mortimer et al., 2014; Zou et al., 2013). The ciliated protists have been widely studied by eco-toxicologists not only because of their role in the regulation of microbial populations through the ingestion and digestion of bacteria but also because of their high sensitivity to chemical materials; hence, they are often used as indicator species of environmental pollution (Bick, 1972). For all these reasons, ciliates, especially *Paramecium* species, have been exploited as excellent bioindicators of pollution or bioassays to evaluate the effects of toxic compounds. (Kozai et al., 2011; Amamra et al., 2015).

The aim of the present study was to evaluate the cytotoxic effects of copper nanoparticles CuO-NPs at different sublethal concentrations on population growth

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and some biomarkers of oxidative stress of the ciliate protozoan *Paramecium* sp.

MATERIALS AND METHODS

Test Organisms

The biological model used in our study is a unicellular microorganism *Paramecium* sp. Cells were cultured in a synthetic culture medium (pH 6.5) and 28±2°C as described previously by (Azzouz et al., 2011) (Moumni et al., 2016). The culture was maintained into the oven (memmert 400) and the cells were transplanted each three days in a new medium for keeping the youthful state of the culture.

Test Chemical

CuO nanoparticles was obtained from Laboratory of Chemestry (LCMI) Badji Mokhtar University, Annaba, had an average size of 30 nm.

Treatment

Paramecium sp were incubated with the tested Copper NPs concentrations in aliquots of 10 ml of culture medium, the retained concentrations were 30, 60, 90 and 120 mg/l.

For growth kinetics, the NPs treatment was performed before the transplantation of *paramecium* cells (at t=0). For the enzymatic essays, the treatment was carried at the end of the experimental growth phase (t=96H). (Wong, 1999., Amamra et al., 2015).

Parameter measurement

Growth kinetics

The growth kinetics study was etablished by the daily cell counting during five days (120h), after fixation with a Lugol solution at 1% under optic microscope type LEICA DM 1000. (Azzouz et al., 2011).

Response Percentage

The response percentage was calculated to evaluate the toxicity of xenobiotics via the inhibition of cell growth after 96H of exposure. Positive values indicate an inhibition of growth while values indicate a stimulation of growth. (Wong et al., 1999).

The assessment of the response percentage is calculated according to the following formula:

Response Percentage =(Nc-Ne) /Nc× 100

Determination Of The Number, The Time And The Velocity Of Generation

Based on the data, the number (n), the time (k) and the velocity of generation (g) were calculated by the following formula:

$N = (\log N_t - \log N_0) / \log 2$

$k = n/t$

$g = 1/k$

Determination Of Superoxide Dismutase (Sod)

Superoxide Dismutase (SOD) activity was measured by the method described by Paoletti and Mocali (1990). The principle of the assay is based on the measurement of SOD activity using the auto-oxidation of β-mercaptoethanol in the presence of EDTA / MnCl2 as a generator of superoxide anions (O2. -) during the reaction.

These anions will cause the oxidation of NADH. The SOD then competes for the use of O2.-which tends to decrease in the medium thus causing the inhibition of the oxidation of NADH. It is estimated that 50% inhibition corresponds to one unit of enzyme.

0.1 ml of the enzyme extract are added to 1.3 ml of a solution composed of 0.8 ml of NADH (7.5 mM), 0.5 ml of a mixture (V / V) EDTA (200 mM) / MnCl2 (100mM) and 1,6 ml of phosphate buffer pH 7.4.

The reaction is triggered by the addition of 0.1 ml of β-mercaptoethanol (10 mM). Absorbance is measured for 5 min after 0 and 20-min incubation at 340 nm (total SOD). The activity is expressed in Units / mg of protein.

Determination Of Catalase (Cat) Activity

Measuring the Catalase activity (CAT) is performed according to the method of Clairborne (1985) based on the hydrolysis of H2O2 to H2O and O2. For the assessment of enzymatic activity of Catalase, the samples were homogenized in phosphate buffer 1 mL (0.1M, pH 7.5) using an ultrasonic crusher (SONICS, Vibra cell). The homogenate thus obtained was centrifuged at 15 000g for 10 minutes and the supernatant recovered serves as enzyme source. The reaction is initiated by the addition of hydrogen peroxide (500 mM, 30V). Reading is done against a blank prepared with hydrogen peroxide and phosphate buffer. The decrease in absorbance is measured every 15 seconds for one minute at 240 nm in a spectrophotometer (Jenway, 6300). Catalase activity is expressed in µmol H2O2 per minute per mg of protein.

Determination Of Glutathione-S-Transférase (Gst) Activity

Determination of GST activity was performed by the method of Habig et al. (1974) by following the conjugation of GSH with CDNB. After homogenization of the sample in 1 ml of phosphate buffer (0.1M, Ph 6), homogenate was centrifugated at 14000 rpm for 30 min. 1.2 ml of the mixture CDNB (1mM)/GSH (1mM) was added to 0.2 ml of supernatant to start the reaction. The formation of S-2,4-dinitrophenyl glutathione conjugate was evaluated by recording the increase in absorbance at 340 nm for 5 min after every 1 min interval. It was expressed as µmol/min/mg.

Determination Of Glutathione Gsh Content

The rate of GSH was quantified according to the method of Weckbeker and Cory (1988). Cells were homogenized in 1 ml of EDTA (0.02M) and 0.2 ml of sulfosalicylic acid (ASS) was added to 0.8 ml of homogenate. After agitation, the homogenate was centrifuged at 1000 rpm for 5 min. The reaction was initiated by adding to 0.05 ml of supernatant, 1 ml of tris/EDTA buffer (0.02 M, Ph 9.6) and 0.025 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DNTB). The absorbance was measured at 412 nm and the amount of GSH was expressed as µmol mg-1 of proteins.

Statistical Analysis

The obtained results are represented by the average ± Standard Error. Statistical analysis of data is performed using Minitab student t test.

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RESULTS

Effect Of CuO-NPs On Growth Kinetic

The acute toxicity test on *Paramecium* sp. with increasing concentrations was performed during five days. Fig. 1 represents the effect of CuO-NPs on the variation of *paramecium* cells number (control and treated) versus time.

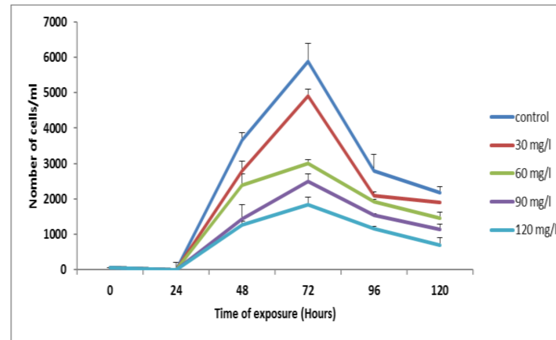

Fig. 1. Effect of CuO-NPs on the growth of *Paramecium* sp.

The selected concentrations inhibited the population growth in a dose-dependent manner especially for the highest concentration (120 mg/l).

In the fifth day of treatment, we denote a difference of nearly 1400 cells between the control and the highest concentration (120 mg/l).

Response Percentage

Response percentage measurement results are presented in Fig. 2.

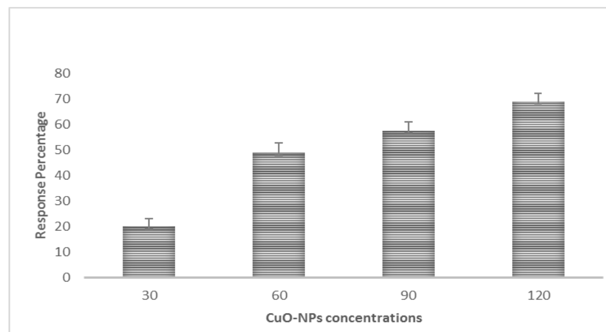

Fig. 2. Effect of CuO-NPs on the response percentage at 96H.

Response percentage is positive in all tested concentrations, it varies from 19% to 68% for 30 mg/l and 120 mg/l.

Therefore, the response percentage confirms the kinetics of growth of the paramecia previously observed.

Table 1.
Effect of CuO-NPs on *Paramecium* sp. generation number, generation time and velocity of generation.

CuO-NPs concentrations (mg/L)	Generation Number (n) ± SE	Generation Time (g) ± SE	Velocity of generation (k) ± SE
0	6.60 ± 0.08	10.90 ± 0.13	0.091 ± 0.01
30	6.28 ± 0.06	11.45 ± 0.11	0.087 ± 0.08
60	5.64 ± 0.03	12.76 ± 0.07	0.078 ± 0.04
90	5.37 ± 0.07	13.39 ± 0.19	0.074 ± 0.01
120	4.92 ± 0.1	14.64 ± 0.38	0.068 ± 0.01

Table 1 illustrates the effect of copper nanoparticles on the number, the velocity and the time of generation.

Exposure to increasing concentrations of CuO-NPs caused a decrease of number and velocity of generation in a dose dependant manner.

However; the generation time gradually increased with increasing concentrations of CuO-NPs.

Superoxide Dismutase (Sod) Activity

The results concerning the variation of SOD activity are represented in Fig 6, exposure of *Paramecium* sp to CuO-NPs revealed a strong induction of the SOD activity appeared with concentrations (90 and 120 mg/L), which had significant ($p \leq 0.05$) and very highly significant ($p \leq 0.001$) increase when compared to the control.

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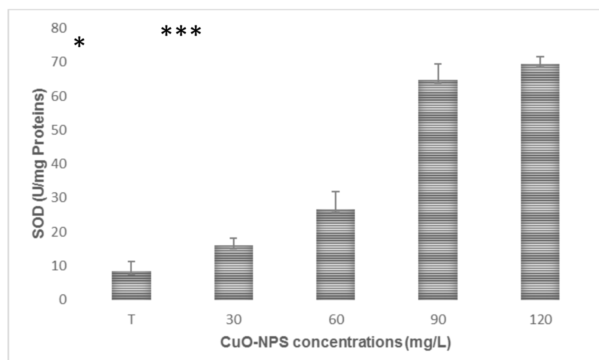

Fig. 3. Effects of CuO-NPs on the SOD activity in *Paramecium* sp. Each value is average ± standard error of three replicates (* P≤ 0.05 *** P ≤0.001).

Catalase (Cat) Activity

The results concerning the variation of CAT activity is represented in Fig 3. After 4 hours of treatment, a significant dose-dependent manner induction of Catalase activity compared with the control (p≤ 0.05), it increased from 5.5544 µmol/min/mg proteins in control to 16.5522 µmol/min/mg proteins for the highest concentration.

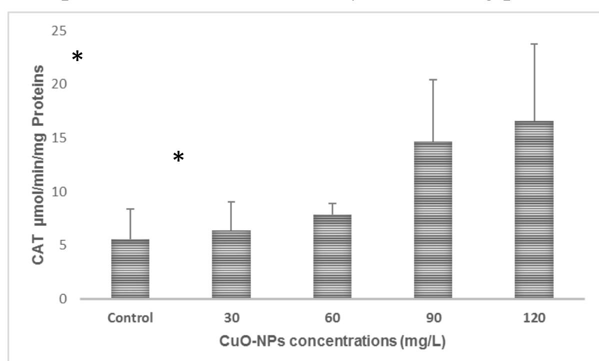

Fig. 4. Effect of CuO-NPs on the CAT activity in *Paramecium* sp. Each value is average ± standard error of three replicates (* P≤ 0.05)

Glutathion-S-Transférase Gst Activity

The results indicated a strong induction of the GST activity (Fig 4), the greatest enzymatic activity appeared with the highest concentration of CuO-NPs (120 mg/l) which had significant increase (p≤0.05) when compared to the control.

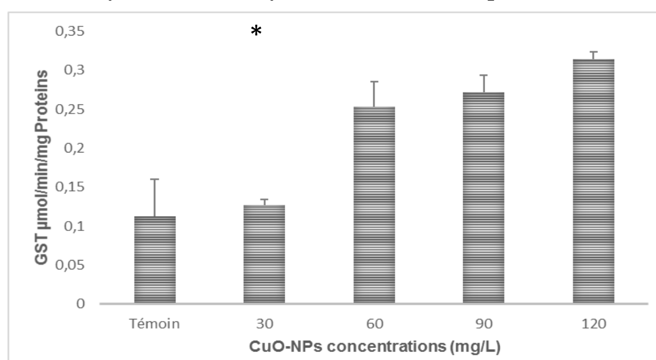

Fig. 5. Effects of CuO-NPs on the GST activity in *Paramecium* sp. Each value is average ± standard error of three replicates (* P≤ 0.05)

Estimation Of Glutathione (Gsh) Content

CuO-NPs exposure induced a significant dose dependent decrease in rate of glutathione reduced (p≤0.05). (Fig 5), it is of the order of 6.100±1.05 µmol/mg of protein for the control while it is 2.175±0.74 µmol/mg of protein for the highest concentration.

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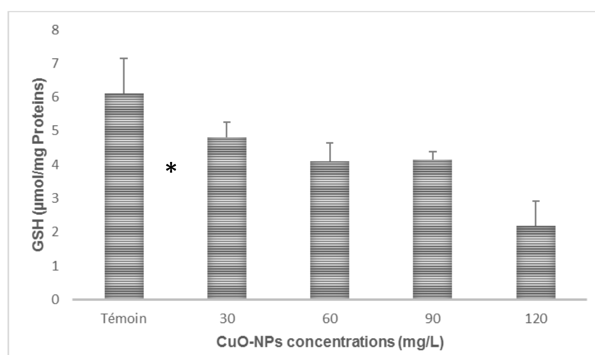

Fig. 6. Effects of CuO-NPs on the rate of GSH in *Paramecium* sp. Each value is average ± standard error of three replicates (* $P < 0.05$)

DISCUSSION

The production, use and commercialization of nanoparticles (NPs) has rapidly increased in recent years (Mwaanga et al., 2014). It is projected that this will inevitably lead to an increase of the release of NPs into aquatic systems (Keller et al., 2010). This will lead to concomitant increase in the expo-sure of aquatic organisms to NPs, which may result into adverse. effects on these biota (Lin et al., 2010). Several NPs have been shown to induce a variety of toxic effects in aquatic organisms (Aruoja et al., 2009). Exposure of organisms to sublethal concentrations of toxic NPs has been observed to result in changes at cellular and biochemical levels that manifested before any physiological effects were observed at the whole organism, these biochemical changes (biomarkers) can help explain the underlying mechanisms of toxicity (Klaper et al., 2009), and have potential for use as early warning signals for ecologically relevant effects (Forbes et al., 2006).

Fresh water protozoan ciliates like Paramecia are the most commonly used ciliated and they are considered as excellent bioindicators of toxicity stress and chemical pollution. (Djekoun et al., 2015)

It is also an advantageous eukaryotic model system for mechanistic studies, as it contains many genes conserved in several eukaryotes (including humans), differently from other widely used unicellular model organisms. Lastly, as protists have highly developed systems for internalisation of nanoscale (100 nm or less) particles (Frankel, 2000), they are very good model organisms for nanotoxicology (Holbrook et al., 2008)

In this work we used *Paramecium* sp as a model cell to study the impact of copper oxide nanoparticles on reproduction and stress antioxidant systems.

In the first time, we were interested at the effect of CuO-NPs on population growth. Our result indicated an inhibition in the growth of paramecia especially for the highest concentration, this inhibition can be explained that the toxicity of metal oxide NPs to unicellular organisms (e.g., bacteria and ciliates) is ascribed, at least in part, to interactions between the NPs and the cell surface. Many studies reported that direct spatial contact between NPs and cell surface is necessary for manifestation of the cytotoxicity, and their interaction is central to the cytotoxicity of NPs. An apparent mechanism relies on direct damages, of NPs to cell surface (cell wall or cell membrane), which can result in death of the cell. Prolonged contact between the cell and the NPs likely alters the cellular surface properties or integrity. (Li et al., 2012). Similar result is reported in the study of Azzouz et al. (2011) and Benbouzid et al. (2012) that demonstrated an inhibition in cell growth and proliferation of paramecia exposed to increase concentrations of Amistar xtra and Phosphoramidate respectively.

On the other hand, positive values of the response percentage confirm the toxic effects of the increasing concentrations of copper oxide nanoparticles. Moreover, the growth rate tests revealed that increasing concentrations provokes an important decrease in the number and velocity of generations by increasing the generation time, which mean that CuO-NPs has affected the multiplication of paramecia in a concentration dependent manner (Rao et al., 2007). Our results are in agreement with studies of Amamra et al. 2015 and Moumni et al. (2016) who reported the effects of Cypermethrin and Cycloxydim respectively on the growth of *Paramecium* tetraurelia.

ROS are chemical species that are produced as by-products of cellular oxygen metabolism, which occurs via mitochondrial respiration in eukaryotic cells. ROS include the superoxide anion, hydrogen peroxide and the hydroxyl radical. The abnormal accumulation of ROS is called oxidative stress and can lead to serious cellular damage. Environmental toxicants induce oxidative stress and alterations in the cellular redox balance. Oxidative stress in turn plays an important role in many types of cellular injury, some of which can result in DNA damage and apoptotic cell death. Eukaryotic organisms have evolved a comprehensive range of proteins to detoxify ROS and repair oxidative damage to DNA, lipids and proteins. These antioxidants include enzymatic scavengers such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST) and the peroxiredoxins, as well as non-enzymatic factors such as glutathione (GSH) and vitamins (Franco et al., 2009; kim et al., 2012).

Superoxide dismutase (SOD) is an essential metalloenzyme to the antioxidant defence system as it catalyzes the dismutation of the superoxide radical ($O2^{-}$) to form hydrogen peroxide (H2O2). (Disner et al., 2017). In our study, SOD activity is found to be

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increased in dose dependent manner especially for the two highest concentrations (90 and 120 mg/L)

This observation is in agreement with the results obtained by Ashouri et al. (2015) who recorded an increase in SOD activity in common carp Cyprinus carpio intoxicated with selenium nanoparticles. Cherait (2014) and Khebbeb (2015) also elucidated a significant increase in SOD activity in Saccharomyces cerevisiae exposed to Nifedipine and ZnO-NPs respectively.

CAT activity consists of a hydrogen peroxide (H_2O_2) transformation in water and molecular oxygen (O_2) (Vander et al., 2003; Brown et al. 2004). Our results showed an increase in catalase activity in these ciliates at the presence of CuO-NPs, probably due to increased antioxidant activity in *Paramecium* cells. The same results are observed by Djekoun et al. (2015) and Khaldi et al. (2016) that presented an increase in CAT activity in *Paramecium* sp exposed to Thiram and ZnO-NPs respectively. Saddick et al. (2017) have also recorded induction in CAT activity in Oreochromis niloticus and Tilapia zillii treated with zinc nanoparticles

It is known that GST is detoxifying enzyme that catalyse the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing fewer toxic forms (Hayes et al., 1995). The significant increase of GST activity may indicate a high rate of xenobiotic conjugation with glutathione (Madoni, 2000). These results are in line with those of Benbouzid et al. (2014) recorded a significant increase of GST activity in *Paramecium* aurelia treated with Phosphoramidate, the same result was observed by Kim et al. (2010) in Daphnia magna exposed to TiO2-NPs.

GSH plays an important role in non-enzymatic antioxidant system, since it acts as a reductant in conjugation with xenobiotics (Kanak et al., 2014; Amr et al., 2015). In the present study, the concentration of GSH in *Paramecium* sp decreased significatively compared to control. GSH may be used by GST for conjugation of electrophilic compounds during exposure (Salazar-Medina et al., 2010; Mwaanga et al., 2014). In addition, some GSH may be used directly in the formation of specific thiol complexes with metal ions released from the NPs (Radwan et al., 2010). These results confirmed by Wang et al. (2015) who recorded a depletion of the GSH content in the intestines of juvenile Epinephelus coioides treated with copper nanoparticles, Ali et al. (2012) also recorded a decrease in GSH content in freshwater snail Lymnaea luteola L treated with zinc oxide nanoparticles. Similary, GSH was significantly decreased In Helix aspersa exposed to TiO2 microparticles (Khene et al., 2017).

CONCLUSION

In toxicological studies, a single bioassay cannot provide the full picture of the effects in an organism. Physiological and biochemical assays were used to provide information for nanoparticles cytotoxicity. The results obtained in this work revealed that CuO-NPs tested caused perturbations in the physiological and biochemical state of *Paramecium* sp. The effects are manifested by the inhibition of growth accompanied with the induction of oxidative damage supported by the increase of the antioxidant enzymes such as SOD, CAT and GST and depletion in GSH content.

AUTHOR CONTRIBUTIONS

Conceptualization: Chiraz BOURAIOU; Houria BERREBAH; Mohamed Réda DJEBAR.. Methodology: Myriam DJEKOUN; Tayeb BOUARROUDJ. Data collection: BOURAIOU Chiraz. Data validation and processing: Houria BERREBAH. Writing—original draft preparation: Chiraz BOURAIOU; Lyes KHENE; Amina YAHYAOUI; Houssem KAHLI. Writing—review and editing: Chiraz BOURAIOU; Houria BERREBAH; Mohamed Réda DJEBAR.

FUNDING

This work was carried out as part of a doctorate in the Cellular Toxicology Laboratory University Badji Mokhtar Annaba Algeria and which was funded by the Ministry of Higher Education and Scientific Research-Algeria.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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