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Hypotaurine effect on the bovine oocyte *in-vitro* maturation and fertilization

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ABSTRACT

The objective of the present research was to examine the impact of various concentrations of hypotaurine (0, 10, 15 and 20 µM /ml) on the bovine oocytes' *in-vitro* maturation and fertilization rates as well as on the enzymatic activity of lipid-peroxidation and antioxidants. Bovine ovaries were harvested from El-Mounib slaughterhouse in Giza Governorate, Egypt. The oocytes were aspirated from medium sized follicles (4-8 mm). The selected oocytes were matured *in-vitro* in TCM-199 media supplemented with different concentrations of hypotaurine. Some of the matured oocytes were evaluated for their maturation rates (nuclear & cytoplasmic maturation), and the maturation media were analyzed to determine the lipid peroxidation levels (Malondialdehyde, MDA), and various antioxidant enzymes, including glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD). The other mature oocytes were *in-vitro* fertilized and examined for the fertilization rate and the different stages of embryonic development. The results represented that hypotaurine at a concentration of 10 µM /ml significantly ($P \leq 0.05$) improved the maturation rate of oocytes (MII), the fertilization rate and the different stages of embryo development. In addition, hypotaurine decreased the level of lipid peroxidation (MDA) significantly ($P \leq 0.05$) and increased the antioxidants levels (CAT, GSH, and SOD), especially at a concentration of 10 µM /ml. In conclusion, hypotaurine improved the bovine oocyte *in-vitro* maturation and fertilization, with the effect being clearly noticeable at a concentration of 10 µM /ml.

Introduction

The success of the *in-vitro* embryonic production (IVEP) system requires competent oocytes and sperm (Rahmatullah *et al*., 2023). The oocyte ability to undergo nuclear and cytoplasmic maturation indicates its *in-vitro* fertilization quality and development successfully into an embryo which is associated with changes in its organelles, metabolites, and RNA during the growth and maturation (Reader *et al*., 2017).

One of the most considerable aspects affecting the IVEP system is oxidative stress (OS). It is caused by the imbalance between prooxidants and antioxidants (Al-Gubory *et al*., 2010) either due to the elevated levels of prooxidants (Reactive oxygen species, ROS) or reduced antioxidant defense mechanism (Ruder *et al*., 2009; Burton and Jauniaux, 2011). The Reactive oxygen species (ROS) generation is a common phenomenon in *in-vitro* culture conditions (Gocher and Narayan, 2020). Reactive oxygen species trigger the oocyte intracellular damage and affect its capacity for further embryonic development (Sidi *et al*., 2022). It can pass through oocyte membranes and alter the different cellular molecules, leading to alterations in mitochondria (Kowaltowski and Vercesi, 1999), meiotic arrest (Nakamura *et al*., 2002), and embryonic block (Hashimoto *et al*., 2000). The sources of ROS can be endogenous, including NADPH oxidases and oxidative phosphorylation, or exogenous, including light, oxygen tension, ionizing radiation, and metal ions (Chen *et al*., 2020).

Antioxidants are ROS scavengers by either inhibiting their formation or countering their effects which results in maintaining the cell oxidant/antioxidant balance (Khazaei and Aghaz, 2017; Zarbakhsh, 2021). Hypotaurine is a sulfur-containing and non-peptide amino acid (Wan *et al*., 2020). It is a food material and is found at high levels in male and female reproductive tracts (Kochakian, 1976; Kaye, 1986; Tevatia *et al*., 2015). It is secreted by oviduct epithelial cell monolayers in cows (Guerin *et al*., 1995). Hypotaurine has a powerful antioxidant activity involved in various biological functions incorporating immunomodulation, cell proliferation, and inhibition of oxidative stress (Bouckenooghe *et al*., 2006; Agnello *et al*., 2013). It inhibits lipid-peroxidation (Barnett and Bavister, 1992) and decreases the oxygen tension during embryo culture (McKiernan and Bavister, 1990), improving the cleavage environment. Furthermore, hypotaurine has an effective role in sperm functions, including capacitation, motility, and fertilizing ability (Bucak *et al*. 2013).

The antioxidant activity of hypotaurine occurs through 2 main mechanisms; it reacts *in-vitro* with the superoxide anions to form new peroxytaurine molecules (Rahmatullah *et al*., 2023) and reacts with the hydrogen-peroxide forming Taurine (Grove and Karpowicz, 2017). Furthermore, it can eliminate intracellular free radicals, improve enzymatic antioxidant activity, regulate membrane permeability to positive ions (Ca^{2+}), and protect phospholipid layer integrity (Zhang *et al*. 2021).

To the authors' knowledge, few studies on the impact of hypotaurine supplementation on oocyte maturation and embryo development are available. Therefore, the present study aimed to supplement hypotaurine in the *in-vitro* maturation media of bovine oocytes and determine its impact on oocyte cytoplasmic and nuclear maturation, fertilization rate, and the *in-vitro* embryo production as well as oxidative stress and lipid peroxidation.

Materials and methods

The different experiments and sample manipulations were conducted following the regulations of the Faculty of Veterinary Medicine Ethics Committee, Benha University (BUFVTM01-05-29).

The study took place in the Department of Theriogenology (IVF-unit), Animal Reproduction Research Institute, El Haram, Giza, from November 2023 to March 2024.

Chemicals and media

The media was prepared according to a standard protocol with aseptic techniques. only tested chemicals for cell culture used after filtration through a 0.22 µm pore size filter (Durapure® membrane filter, Ireland) and pH calibrated at 38.5°C with 5% CO_2 in a humidified atmosphere for a minimum of 2 hours. All chemicals and reagents used in the media were obtained from Sigma-Aldrich Company (Saint Louis, MO, USA), unless otherwise specified.

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Samples

Cattle ovaries were obtained from the El-Mounib abattoir in Giza Governorate, Egypt. The ovaries were harvested directly after slaughtering and transferred to the lab within 2 hours in a warm saline solution (37°C) supplemented with 0.1 mg/ mL streptomycin and 100 U/mL Penicillin (Pang *et al*., 2019). In the lab, the ovaries were trimmed, washed 3–5 times with sterile warm saline (37°C) (Zhao *et al*., 2024), and kept at this temperature till aspiration.

Oocyte recovery and selection

The oocytes were collected through the aspiration of follicles (4–8 mm diameter) using an 18-gauge needle connected to a sterile 10 ml syringe containing 0.5 ml aspiration media (Modified Dulbecco's phosphate-buffered saline, M-DPBS, enriched with bovine serum albumin 6 mg/ml and gentamicin 50 mg/ml) (Yousaf and Chohan, 2003). The follicular fluid sediments were examined in a clear 60 mm petri-dish under a stereomicroscope for oocyte selection (Zhao *et al*., 2024). The cumulus-oocyte complexes (COCs) with at least 4 layers of homogenous, and compact cumulus cells were selected then washed with the maturation media (Bahgat *et al*., 2023; Zhao *et al*., 2024).

In-vitro maturation

The selected oocyte underwent maturation in TCM-199 with Earle's salts enriched with 10% inactivated FCS, 5µg/ml FSH, 1µg/ml Estradiol-17β, 10 µg/ml LH, 100 IU/ml of penicillin,100 mg/ml streptomycin, and 2.2 mg/ml sodium pyruvate (Bahgat *et al*., 2023). The maturation media supplemented with various concentrations of hypotaurine (0, 10, 15 and 20 µM) to make 4 experimental groups. The oocytes were cultured into a disposable NUNC© culture dish at a rate of 15-20 oocytes/well in 100µL media, overlaid with sterile mineral oil, then incubated for 18–20 h at 5% CO_2, 38.5°C, and maximum humidity (Bahgat *et al*., 2023; Zhao *et al*., 2024).

Assessment of the nuclear and cytoplasmic maturation

According to Mostagir *et al*. (2019), the oocyte maturation rates were determined by assessing the cumulus cell expansion under the stereomicroscope (Olympus, SZX2-ILLTQ) and selected the COCs characterized by multiple layers (at least 4 layers) of compact, granular, and homogenous cumulus (Granulosa) cells surrounding a uniform cytoplasm (Zhao *et al*., 2024). The oocyte maturation was considered through the complete or moderate cumulus expansion and the first polar body extrusion into the peri-vitelline space (Figure 1).

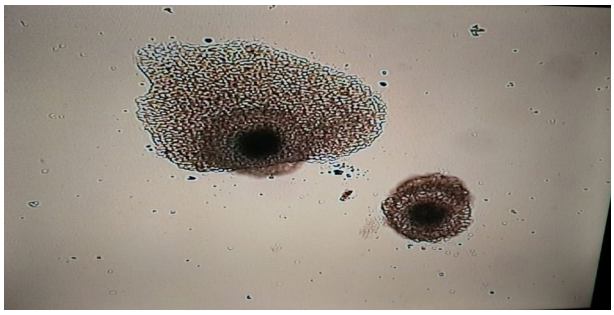

Figure 1. The cumulus expansion after oocyte maturation.

Oocyte staining by Aceto-Orcein stain

The matured oocyte cumulus cells were denuded by low-speed Vortex for two to three minutes and gentle pipetting for full cumulus cell removal followed by washing in phosphate-buffered saline (PBS) (Kumar *et al*., 2018; Pang *et al*., 2019; Zolini *et al*., 2019) . According to Masoud *et al*. (2023), the denuded oocytes were stained with the aceto-Orcein stain in 3 steps; 1- The fixation step: transferring the oocytes to 1% hypotonic solution of sodium citrate for three minutes, after which, on a clean glass slide 5-10 oocytes were placed and covered with a coverslip and fixed for 24 h in Carnoy's fixative (Ethanol: Acetic acid, 3:1 respectively). 2-The staining step: the fixed oocytes were dipped in a jar of 1% aceto-orcein stain in 45% acetic acid for 30 minutes. 3- The washing step: after staining the oocyte slides were washed with a washing solution (20% glycerol and 20% acetic acid in distilled water) and assessed under a phase-contrast microscope to evaluate the status of nuclear maturation based on nuclear-chromatin configuration. The stained oocytes by aceto-Orcein stain can represent the nuclear-maturation stage of the oocyte by defining the nucleus and its filamentous chromatin (El-Raey, 2014; Kumar *et al*., 2018).

Assessment of the effect of hypotaurine impact on antioxidant enzymatic activities and Lipid peroxidation

The effect of hypotaurine on ROS and lipid-peroxidation levels was assessed by estimating the levels of the MDA, catalase, GSH, and SOD levels after oocyte maturation. The tested sample was a cell lysate which was prepared from the maturation media after the oocyte centrifugation in accordance with the kit's instructions.

Determination of Malondialdehyde (MDA) level

The level of MDA was measured using the MDA- ELISA Kit (Cat No. MBS260816). Briefly, the tested sample was centrifuged, and the supernatant absorbance was spectrophotometrically (Spectronic 601-reader, Milton Roy) measured at 535 nm. According to Olszewska-Słonina (2011), the concentration of Thiobarbituric Acid-Reactive Substances TBARS was calculated and recorded as nmol/L. The standard curve was obtained using the stock solution of 10mM MDA formulated from tetramethoxypropane (Sigma-Aldrich).

Determination of Catalase level

The level of catalase was detected by a catalase activity assay kit (BioVision, Inc., K773-100, Milpitas). The activity of the catalase enzyme was calculated as the H_2O_2 amounts that are reduced/minute/ml and are reported as µU/mg of cell lysate protein. Using a microplate reader (Micro Scan MS5608A, ECIL), the optical density was recorded depending on Pandey and Chaube (2014).

Determination of GSH level

The concentration of GSH was determined depending on Curnow *et al*. (2010). The rate of absorbance was detected at room temperature at 412 nm (García-Martínez *et al*., 2020). Using glutathione standards, the concentration of thiol and sulfhydryl groups were recorded, and the end-results were represented in nmol/g protein.

Briefly, Dithionitrobenzoic acid (DTNB) and thiol groups react to produce a colorful anion (absorbance peak at 412 nm). A stock solution (10 nM DTNB in absolute ethanol) was added to 25µl of the tested sample and 1 ml of the Tris-EDTA buffer (25 nM Tris-base, 20 mM ethylenediamine tetra-acetic acid / pH 8.2). The mixture was then incubated at ambient temperature for 15 minutes, followed by a second absorbance estimation at 412 nm and using DTNB as a blank (Costa *et al*., 2006).

Determination of SOD level

According to Engelbrecht *et al*. (2023), the SOD level was estimated using a Fructosamine assay Kit (Labtest Diagnóstica S.A. Brazil) at 37°C in CO_2 incubator then the absorbance rate was assessed at 530 nm.

Shortly, two test tubes were prepared. The first included the tested material and 2N HCL then incubated at 37°C for two minutes before adding 1 M NaOH (1:1:2). In the second tube, the kit reagent was allocated and incubated at 37°C for 2 minutes then add the mixture from first tube to the second one (1:5) incubated for 10 minutes at 37 before measuring the absorbance rate at 530 nm.

In-vitro Fertilization

Preparation of IVF culture droplets

The matured-oocytes were fertilized in modified Tyrode's albumin lactate pyruvate medium (TALP) media enriched with 20 µg/ml heparin and 6 mg/ml BSA, fatty acid free, fraction-V. Briefly, several fertilization drops (50 µl/drop) of the TALP were placed in a sterile disposable 60 ml culture dish, covered by mineral oil, and incubated for about two hours before sperm-oocytes co-incubation at 38.5°C in the CO_2 incubator with 5% CO_2 and maximum humidity (Rahman *et al*., 2020). The Mature oocytes were washed in the TALP medium and transferred to the fertilization drops (10 oocytes /drop) (Elmetwally *et al*., 2022).

Sperm preparation

Cryopreserved semen straw was thawed for 30 sec at 37°C water bath (Najafi *et al*., 2014; Bahgat *et al*., 2023), and thawed semen underwent the swim-up technique according to Elmetwally *et al*. (2022) to select the most motile sperm for the fertilization process. Briefly, the thawed semen sample was inserted in the bottom of a tube containing 1 ml sperm-TALP medium, andmedium and incubated for 30 minutes at 37°C in an inclining position. After the incubation, the upper layer of the media was collected into a tube containing 3 ml of sperm-TALP medium followed by 2 times centrifugation (500xg) for 10 min (Parrish *et al*., 1986). After the supernatant discarding, the sperm pellet was mixed with 1 ml of fertilization media, gently mixed and evaluated to check the motility (60-80%) and diluted till a concentration of 2 million sperm /ml. 20 µl of prepared semen was added to each fertilization drop and co-incubated with the oocytes for at least 18 h (Elmetwally *et al*., 2022). The penetration rate was assessed through visualizing the sperm tail through the oocyte zona pellucida (Figure 2) then the fertilization determined by the appearance of the 2 pronuclei (2PN).

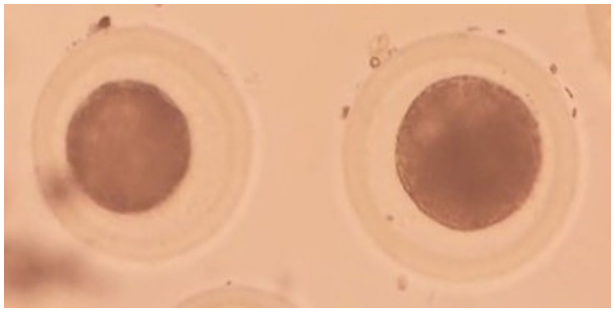

Figure 2. Penetrated oocytes.

In-vitro culture (IVC) and embryo development

The fertilized oocytes were cultured on TCM -199. The culture dish was prepared by allocating several drops of TCM-199 (50µl/drop) in a sterile 60 ml petri dish, covered with mineral oil, and incubated at 38.5oC with 5% CO_2 and maximum humidity (Elmetwally *et al*., 2022). The fertilized oocytes were washed in a culture media and cultured at a rate of 5 oocytes/ drop. The embryos were hanged over in a fresh culture medium every 48 hrs until day 7 of culture (Elmetwally *et al*., 2022). The percentage of cleaved oocytes was recorded 48 hrs post-insemination, while the rate of the blastocyst formation was reported after 5-7 days post-insemination (Bahgat *et al*., 2023).

Statistical analysis

The data were statistically analyzed using SPSS software (ver. 25). One-way ANOVA with Duncan's Multiple Range tests was used to determine the difference between groups. The significant difference was set at P ≤0.05.

Results

Effect of hypotaurine on cattle oocytes' nuclear maturation rates

As presented in Table1, the percentages of GVBD and MI significantly (P≤0.05) increased with the hypotaurine concentrations of 10 and 15 µM, while the percentages of MII increased (P≤0.05) with the concentrations of 10 µM only compared to the control.

Table 1. Effect of different concentrations of hypotaurine on nuclear maturation rates of cattle oocytes.

Group	Nuclear status		
	GV (%)	GVBD& MI (%)	MII (%)
Control	10.433±1.43	18.07±5.81^{a}	22.95±4.60^{a}
10 µM	12.11±2.74	45.06±5.12^{b}	45.06±5.12^{b}
15 µM	7.33±4.13	46.40±13.24^{b}	35.40±9.71^{ab}
20 µM	4.94±2.48	32.66±8.10^{ab}	27.72±5.70^{ab}

Data are presented as mean ±SEM. Values with different superscripts within the same column differed significantly at p ≤ 0.05. GV: Germinal vesicle; GVBD: Germinal vesicle breakdown (GVBD); MI: Metaphase I; MII: Metaphase II.

Effect of hypotaurine on the penetration and fertilization rates of cattle oocytes

A significant (P≤ 0.05) increase has been recorded in the fertilization rate with the hypotaurine concentration of 10 µM compared to the control (Table 2).

Table 2. Effect of hypotaurine on the penetration and fertilization rates of cattle oocytes

Group	Penetration rate (%)	Fertilization rate (%)
Control	50.56±5.30	28.41±6.74^{a}
10 µM	63.19±5.18	54.45±6.19^{b}
15 µM	57.18±3.62	47.61±1.21^{ab}
20 µM	51.50±14.00	31.28±7.35^{a}

Data are presented as mean ±SEM. Values with different superscripts within the same column differed significantly at p ≤ 0.05.

Effect of hypotaurine on the embryonal development of bovine oocytes.

As shown in Table 3, the hypotaurine with the concentration of 10 µM significantly (p≤ 0.05) increased the different stages of embryo development including the 2-8-cell and Morula stages as opposed to the control.

Table 3. Effect of hypotaurine on the embryo development of bovine oocytes.

Group	2-8 cell stage (%)	Morula stage (%)	Blastocyst stage (%)
Control	13.89±2.78^{a}	5.55±2.78^{a}	0.00±0.00
10 µM	39.45±6.83^{c}	23.89±5.80^{b}	11.11±7.35
15 µM	28.97±2.41^{bc}	18.65±3.25^{ab}	5.16±2.60
20 µM	22.22±2.78^{ab}	5.55±2.78^{a}	2.78±2.78

Data are presented as mean ±SEM. Values with different superscripts within the same column differed significantly at p ≤ 0.05.

Effect of hypotaurine on lipid peroxidation and oxidative stress of the in-vitro matured bovine oocytes

As presented in Table 4, Significant ($p \leq 0.05$) improvements in the levels of CAT, GSH, and SOD has been recorded with hypotaurine at the concentration of 15 µM compared to the control.

Table 4. Effect of hypotaurine on lipid peroxidation & oxidative stress of the *in-vitro* matured bovine oocytes.

Groups	MDA nmol/ml	CAT U/L	GSH mM/L	SOD U/ml
Control	6.86±1.28	20.60±1.86^{a}	2.95±0.71^{a}	19.34±3.50^{a}
10 µM	8.25±1.85	29.78±4.55^{ab}	6.35±1.76^{ab}	24.39±1.89^{ab}
15 µM	11.01±1.16	34.44±3.70^{b}	10.15±1.75^{b}	30.81±2.79^{b}
20 µM	6.96±1.21	23.67±2.95^{ab}	5.95±1.18^{ab}	18.39±1.39^{a}

Data are presented as Mean±SD. Values with different superscripts within the same column differed significantly at $p \leq 0.05$. The experiment was replicated for at least three times / group.

Discussion

In-vitro maturation is one of the most critical steps of *in-vitro* embryo production (IVEP), during which the oocytes acquire the essential characteristics for subsequent embryonic development (Hatirnaz *et al*., 2018). The IVEP system is associated with various sources of free radicals which can accumulate, result in damage and developmental arrest or apoptosis (Chen *et al*., 2020). Reactive-oxygen species (ROS) is the most common type of the free radicals which produced in a high level during IVEP and adversely affects the oocyte competence for further embryonic development (Prescott and Bottle *et al*., 2016; Sidi *et al*., 2022). So, embryo culture media have been formulated to include different antioxidants to overcome the overproduction of ROS (Guérin, *et al*., 2001). Hypotaurine is a well-known scavenger especially for hydroxyl radicals (•OH) that reduces lipid-peroxidation in spermatozoa (Alvarez and Storey, 1983; Aruoma *et al*., 1988).

The present study tried to reduce the level and the effect of different ROS by incorporating hypotaurine in the *in-vitro* maturation of media of bovine oocytes. Hypotaurine has been implicated in the protection of gametes and embryos (Agnello *et al*., 2013). It has been found in serum and follicular fluids at a low level compared to that in the tubal fluid (Guerin *et al*., 1995). The secretion of hypotaurine by oviductal epithelial cells occurs through tubal cell monolayers, and is spontaneous in some species as Cows, goats, and sows (Ouhibi *et al*., 1989) and induced in others as rabbits (Guerin *et al*., 1995). In all species, hypotaurine is secreted in the gametes and embryos environment before and after the time of fertilization (Guerin, 1995). On the other hand, hypotaurine is considered a low molecular weight antioxidant; many reports showed that the addition of low molecular weight antioxidant compounds to IVM media enhanced the oocyte cytoplasmic maturation and embryo development by promoting the synthesis of antioxidant enzymes (Khazaei and Aghaz, 2017).

Fellman and Roth (1985) have argued that hypotaurine act as an in-vivo scavenger. Despite *in-vitro,* appears to suppress weakly iron-dependent ' site-specific OH damage to the sugar deoxyribose, hypotaurine is an excellent free radical scavenger especially hydroxyl radical ('OH) and hypochlorous acid (HOCl) and can interact with iron-ion-dependent formation of OH (Aruoma *et al*., 1988). The hypotaurine antioxidative function has been found to be effective at a concentration of 10 µM (Aruoma *et al*, 1988).

Few records are available about the effect of hypotaurine on maturation and fertilization rates of oocytes in bovine species. The present study revealed that hypotaurine (mainly at concentrations of 10 and 15µM) has a positive improving effect on the bovine maturation and fertilization rates of oocytes as well as different stages of embryonic development. In addition, it increased the levels of antioxidants in the oocyte maturation media. Similar results have been recorded in sheep in which the hypotaurine increased the viability of the gametes and the *in-vitro* fertilization rate by reducing oxygen free radicals (García-Álvarez et-al., 2015; Rahmatullah *et al*., 2023). In the same manner, hypotaurine improved the development of the *in-vitro* produced embryos to the 8-cell stage in mice (Wakayama *et al*., 1996). Moreover, it has been found that the IVF media supplementation with a mixture of hypotaurine & Heparin leads to normal stable fertilization and blastocyst formation rates in the bovine (Miller et-al., 1994; Kang *et al*., 2015). In addition, Gonçalves *et al*. (2014) recorded a higher rate of 2PN in the *in-vitro* matured bovine oocytes after supplementation of media mixture of hypotaurine, penicillamine, epinephrine (PHE) in addition to heparin. In the same respect, Susko-Parrish *et al*. (1990) and Kang *et al*. (2015) reported a positive improving effect of hypotaurine in the PHE Mixture on the penetration and cleavage rates of bovine oocytes. However, hypotaurine did not affect the rate of oocyte maturation in goats (Gocher and Narayan, 2020). Cryopreservation medium supplementation with hypotaurine for human sperm represented a reduction in apoptotic-sperm percentage after thawing (Brugnon *et al*., 2013). Moreover, an improvement in the development of 1 and 2-cell stage of porcine embryos to the blastocyst stage was recorded by enrichment the embryo culture medium with hypotaurine (Petters and Reed, 1991). In high oxygen tension culture conditions, hypotaurine addition to embryo culture media showed a very effective role in improvement of porcine embryo development (Chen *et al*., 2020).

Conclusion

Hypotaurine (mainly at concentrations of 10 and 15µM) has a prominent effect on bovine oocyte maturation and fertilization rates as well as the different stages of embryonic development. In addition, it has a marked antioxidant effect represented in increasing the levels of CAT, GSH, and SOD.

Conflict of interest

The authors have no conflict of interest to declare.

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