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In vitro protective effects of *Helichrysum cymosum* (L.) D.Don (Asteraceae) on TM4 Sertoli cell

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Original Research

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Abstract:

Helichrysum cymosum (L.) D.Don phytocompounds play an integral role in treating various diseases due to their bioactive properties. This study evaluated the protective effects of two *H. cymosum* extracts (aqueous and 70% ethanol) on cell viability, morphological changes, antioxidant enzyme activities (SOD, GSH and TAC) and ROS production on TM4 Sertoli cells. Results showed no significant effect on cell viability after 24 h but significantly decreased after 48 h ($p < 0.05$). Cell structure integrity was maintained at all concentrations compared to the positive control (10% DMSO). Antioxidant enzyme activities SOD, GSH and TAC had good scavenging properties, although no statistically difference was recorded. ROS production was maintained with both extracts, albeit with no significant differences. *H. cymosum* extracts may contain potent bioactive components such as flavonoids that work synergistically to mitigate OS-induced infertility in males. Further investigations including *in vivo* experiments, are recommended to validate their use in herbal supplements and drug discovery.

Keywords: Antioxidant capacity; Asteraceae; Cytotoxicity; Glutathione; *Helichrysum cymosum* (L.) D.Don; Phytocompounds; Superoxide dismutase

1. Introduction

Infertility is a global health challenge which affects 40-50% of couples in the world [(Kaltsas, 2023)]. A couple is considered infertile if they are unable to conceive over one year after having regular and unprotected sex (Nantia et al., 2009; Takalani et al., 2023). The problem of infertility has historically been associated with the females, however, there is increasing evidence of male infertility in literature (Abdillahi et al., 2012; Sylvest et al., 2018; Agarwal et al., 2021). Sexual dysfunctions have been reported in 25%-63% of women and 10%-52% of men (Dutta et al., 2018). The majority of male infertility cases are linked to idiopathic sperm disorders, sexual dysfunction and anatomical sperm abnormalities (Abdillahi et al., 2012; Semenya et al., 2013; Dutta et al., 2018); however, environmental, physiological and genetic factors have also been implicated (Bansal et al., 2011; Brehm et al., 2019). Sertoli cells are known to play a key role in spermatogenesis by regulating and supporting germ cell development (Monsees et al., 2000; Opuwari, 2009; Chang et al., 2017; Olabiyi et al., 2020). The pathogenesis of male reproductive dysfunction involves complex endogenous pathways (Petricca et al., 2023), and the involvement of oxidative stress (OS) has been linked to the disproportionate production of reactive oxygen species (ROS) and the antioxidant defense mechanism (ADM) of the body (Chang et al., 2008; Kaltsas, 2023; Petricca et al., 2023). OS can lead to cellular damage if the levels of ROS (free radicals, peroxides, etc.) production are not brought under control by the body's ADM (Bansal et al., 2011). Such damage could affect the semen (the sperm protein, lipid and DNA) (Adewoyin et al., 2017; Koshevoy et al., 2021; Kumbhare et al., 2023) resulting in infertility com-

plications. Studies have shown that OS can be modulated by the use of oral antioxidants to enhance the body's defense mechanism (Kumbhare et al., 2023; Liew et al., 2024; Siddique et al., 2024). Antioxidants have the capacity to lessen OS-induced damages through a number of mechanisms (Kaltsas, 2023; Bajaj et al., 2024).

The current treatment and management of male infertility involves the use of injectable and oral medications. The injectable medications include follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG), gonadotropin-releasing hormone (GnRH) (Mansour, 2023; Alexander et al., 2024; Fink et al., 2024), while oral medications like clomiphene citrate, anastrazole, clomiphene-tamoxifen combination, etc. have also been reported (Chua et al., 2013; Panner Selvam et al., 2023). These known medications are known to be generally effective for treating male infertility, they are generally costly, have many side effects and require prolonged use. These factors necessitate the search for alternative treatment options such as herbal therapies (Ho et al., 2011; Dutta et al., 2018), or combination therapies (Nantia et al., 2009).

Herbal therapies are used in different parts of the world to treat different disease conditions and enhance general health (Malviya et al., 2016), and most contain abundant compounds with potent antioxidant properties which play a major role in alleviating the OS induced in different disease conditions (Olabiyi et al., 2020; Liew et al., 2024).

The Asteraceae family is one of the most common group of herbs used in traditional medicine and food in different parts of the world, and provide nutritional and therapeutic benefits (Kazeminia et al., 2022; Amirahmadi et al., 2022). The family encompass 1600 genera and 25000 species including the genus *Helichrysum* (Amirahmadi et al., 2022). *H. cymosum*, commonly known as gold carpet or yellow-tipped strawflower in English, goute tapyt in Afrikaans, and impepho in isiXhosa or isiZulu, belongs to the Asteraceae family, genus Helichrysum, tribe Inuleae and subtribe Gnaphalinae encompass approximately $500-600$ species of medicinal herb that have been established in different parts of the world including Asia, Australia, Europe and Africa. Species of this genus are branched, aromatic, perennial shrubs with yellow flowers having diverse therapeutic potentials exhibited by important secondary metabolites such asphenolic acids, flavonoids, diterpenes, coumarins, chal-cones, polyacetylenes, sterols, pyrenes acylphloroglu-cinols, humulone derivatives, phthalides and sesquiter-penes (Lourens et al., 2008; Nkemzi et al., 2022).

H. cymosum is distributed along the eastern coastline of South Africa, from KwaZulu-Natal through to the Western Cape and known to possess great medicinal value (Van Vuuren et al., 2006; Lourens et al., 2008; Philander, 2011; Matanzima, 2014; Giovanelli et al., 2018; Maroyi, 2019). The known major traditional uses of *H. cymosum* include treatment of cough and cold, pains, infected wounds, headache and "goodwill to ancestors". The minor medicinal applications include blocked nose, immunity boost, cardiovascular problems, diarrhea, dizziness, eye problems, flatulence, improvement of appetite, influenza, as insect repellent, for insomnia, kidney problems, laxative, menstrual pain, pertussis, pulmonary problems, skin infections, urinary problems, varicose veins, vomiting, and weak bones (Lourens et al., 2008; Giovanelli et al., 2018). Some of the bioactive compounds identified in *H. cymosum* include helihumulone, helichromanochalcone, 5-hydroxy-8-methoxy7-prenyloxyflavanone, α-pinene, Δ-3-carene β-caryophyllene, 1,8-cineole, *trans*-caryophyllene, and (*Z*)-β-ocimene (Van Vuuren et al., 2006; Maroyi, 2019). These compounds have demonstrated diverse biological activities like antioxidant, antifungal, anti-inflammatory, antiviral, antimalarial, and cytotoxicity (Lourens et al., 2008; Jadalla et al., 2022; Panner Selvam et al., 2023; Nkemzi et al., 2024). It is expected that phytoconstituents that cause significant reduction in testosterone, LH, and FSH levels as well as those that impair testicular steroidogenesis, could be useful sources of male contraceptives (Abdillahi et al., 2012; Dike et al., 2023). The anti-infertility potentials of some *Helichrysum* species like *H. odoratisssimum* have been reported (Watcho et al., 2019), but there are no studies reported in the literature on the reproductive benefits of the Helichrysum species of South Africa. Thus, the current study seeks to explore the protective effects of *H. cymosum* on Sertoli cell functions as evaluated by cytotoxicity, ROS generation, total antioxidant capacity, SOD and GSH activities.

2. Methodology

2.1 Ethic approval and plant collection

All experimental protocols and procedures of the study were approved by the Research Ethics Committee (REC) of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, South Africa (ethics approval number CPUT/HWS-REC2021/H1).

H. cymosum shoots were harvested in the garden of the Cape Peninsula University of Technology, Bellville campus, Cape Town, South Africa. The plant was authenticated by a botanist (P. Dryfhout) with voucher number 3708 and stored at the herbarium at the Department of Horticultural Sciences, Cape Peninsula University of Technology, Bellville, Cape Town, South Africa.

2.2 Extract preparation

The shoots of *H. cymosum* were washed thoroughly, cleaned, and dried in an oven at 40 °C. The extraction protocol was adapted from Aladejana et al. (2020) with some modifications. The plants were then crushed to powder using an electric grinder. Subsequently, 200 g of the crushed samples were soaked in 2.5 L of 70% ethanol and distilled water, respectively, and stirred for 48 hours. Both the ethanol and aqueous extracts were then filtered using a funnel and Whatman No.1 filtered paper. The ethanol extract was concentrated at 70 °C using a rotary vacuum evaporator while the aqueous extract was freeze-dried. The concentrated extracts were stored at 4 °C in the refrigerator until required. The quantification of the bioactive constituents of both extracts using high-pressure liquid chromatography (HPLC) and the ultra-performance liquid chromatograph (UPLC) (LCMS) has been reported in our earlier publications

(Nkemzi et al., 2024).

2.3 Cytotoxicity evaluation (MTT assay)

2.3.1 Cell culture maintenance

TM4 mouse Sertoli cell lines were purchased from the American Type Culture Collection (ATCC) and maintained in 25 cm culture dishes in complete culture medium (Dulbecco's Modified Eagle's medium (DMEM) /F12 Ham nutrient mixture supplemented with 2.5% fetal bovine serum (FBS), 5% horse serum, 1% penicillin/streptomycin). The cells were incubated at 37 °C, 5% CO_2 in a humidified environment, and sub-cultured after confluence. Test samples of the plant extracts were prepared by reconstituting in 0.1% dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL, then sonicated and stored at 4 °C until used. Different concentrations of the extracts (120, 60, 30, 15, 7.5 and 3.75 µg/mL) were prepared in complete medium DMEM. The negative control was prepared in (0.1% DMSO in DMEM) and positive control in (10% DMSO in DMEM).

2.3.2 Cell viability (MTT assays)

The viability of TM4 cells was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) according to the procedure in (Olabiyi et al., 2020). TM4 cells were seeded in 96 well plates at 4000 cells/well (200 µL aliquots) and left overnight to attach (24 hours) and the media removed. The cells were then exposed to the different concentrations (120, 60, 30, 15, 7.5 and 3.75 µg/mL) of the extracts reconstituted in complete DMEM medium, the negative control containing (0.1% DMSO in DMEM) and the positive control containing (10% DMSO in DMEM) and incubated for 24 or 48 hours. Subsequently, all treatments were aspirated from the wells and 100 µL MTT (1 mg/mL) in a complete medium was added to each well and incubated for 3 hours. After incubation, the supernatant was removed, and 100 µL DMSO added to each well to dissolve the formazan crystals. The absorbance was measured at 560 nm and the background at 750 nm using a plate reader (FLUO Star® Omega, BMG Labtech). The graphs of the percentage cytotoxicity against the concentrations of the extracts were plotted using GraphPad Prism 5. The results were expressed as percentage of control, according to the equation 1:

$$\text{Cell viability}(\%) = \frac{(\text{Absorbance of treated cells})}{(\text{Absorbance of untreated cells})} \times 100 \quad (1)$$

2.3.3 Cell morphology

The effects of the aqueous and 70% ethanol extracts of *H. cymosum* on TM4 cell morphology was performed using 96 well plates. Into each well, 200 µL/well (4000 cells) were seeded and allowed for 24 hours to attach. Later, cells were treated with different extract concentrations (120, 60, 30, 15, 7.5 and 3.75 µg/mL) for 24 and 48 hours, respectively. The morphological changes after treatment were then observed using the Invitrogen™ EVOS™ XL Core imaging system, and micrographs captured at 10 X magnification. More than one image was captured for each concentration.

2.4 Cellular antioxidant enzyme assays

2.4.1 Cell lysate preparation

TM4 cells (1 million cells/ mL) were seeded into a six well plate and allowed overnight (24 h) to attach. The cells were then treated with different concentrations (120, 60, 30, 15, 7.5 and 3.75 µg/mL) of the aqueous and 70% ethanol extracts, respectively. The control wells comprised of negative control (0.1% DMSO in DMEM) and positive control (10% DMSO in DMEM). All treated plates were incubated for 24 h and 48 h, respectively. After that the supernatant was removed, rinsed with PBS followed by scrapping of cells and addition of 1 mL of SDS (1.0%) to all the wells and transferred to Eppendorf tubes and sonicated in cool ice for 10 seconds. After sonication, samples were centrifuged for 10 mins at 2500 rmp to obtain the lysates and stored at −20 °C for subsequent use (Lowry et al., 1951; Bara et al., 2018).

2.4.2 Glutathione (GSH) levels

The level of reduced GSH was determined based on the oxidation of GSH by 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) according to the procedure by Ellman (1959), modified by Polycarp et al. (2016) with minor modifications. Briefly, GSH standard concentrations (20, 40, 60, 80 and 100 µM) were prepared from reduced L-glutamine (GSH) stock (1 mM) and DBTN reagent (1 mM) was prepared separately. Test samples and standards (13.3 µL) were each placed into 96 well plates in triplicates. Thereafter, 15.3 µL of PBS and 33.3 µL DBTN reagent were added to initiate the reaction and then shaken for 30 seconds. The reaction mixture was allowed to incubate at room temperature for 5 minutes, and absorbance measured at 412 nm.

2.4.3 Superoxide dismutase activity (SOD)

The SOD activity was measured on cell lysates, which is based on the ability to inhibit auto-oxidation of pyrogallol (Marklund et al., 1974). The analysis was performed according to a modified procedure adapted from (Bara et al., 2018). A volume of 8.3 µL of samples (cell lysates) were placed in triplicates in a 96 well plate, and 238.3 µL DTPA-Tris-HCl buffer {1 mM DTPA and 5 mM TRIS-HCL (pH 8.2)} was added into the sample wells and 246.6 µL to the blank wells. A 3.4 µL of pyrogallol (15 mM) was added to the sample and blank mixtures, excluding the sample blanks. The change in absorbance per minute for 3 minutes was recorded on a plate reader at 420 nm. The result is express as percentage of control according to the equation 2.

$$\%\text{cell viability} = \frac{(\text{Absorbance of treated cells})}{(\text{Absorbance of untreated cells})} \times 100 \quad (2)$$

2.4.4 Total antioxidant capacity (TAC)

The total antioxidant capacity of the *H. cymosum* extracts was measured using the colorimetric assay 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) following the procedure described by Greifová et al. (2022). This method is based on oxidation of colorless reduced ABTS to a blue-green color cation $ABTS^+$ using hydrogen peroxide

in an acidic reaction medium (pH 3.6). Briefly, into a 96 well plate, 20 µL of samples and standards were placed and 200 µL of 0.4 M acetate buffer (0.4 M glacial acetic acid + 0.4 M anhydrous sodium acetate, pH 5.8) was added and first read at 660 nm. Subsequently, 20 µL of 10 mM ABTS prepared by dissolving in 2 mM hydrogen peroxide (30% H_2O_2) in 30 mM acetate buffer (pH 3.6) was added. The mixture was incubated in the dark for 5 minute and absorbance read at 660 nm. The results were expressed as percentage of control.

2.4.5 Intracellular ROS production

Intracellular ROS production in TM4 was determined using the method by Banerjee et al. (2019), involving 2,7-dichlorofluorescein diacetate (DCF-DA), with slight modifications. Briefly, twenty microliters (20 µL) of the sample (cell lysates) were placed in to a 96 well plate in triplicates with 152 µL Hank's buffer and 30 µL of DCF-DA (5 µM). The mixture was incubated at 37 °C for 15 minutes and the wavelength recorded at 485 nm excitation and 520 nm emission, incubated and read for a second time using Spectro star® Omega (supplier) absorbance microplate reader. Results were expressed as percentage ROS production according to the calculation below.

2.5 Statistical analysis

Values were expressed as means ± standard errors of the means of three repeated experiments. Differences between the means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were performed with GraphPad Prism 5. A difference in mean values was considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1 Effects of *H. cymosum* extracts on cell viability

The *H. cymosum* AQ and ETOH extracts were assessed on TM4 Sertoli for cell viability after 24 h and 48 h treatments, to establish a safer concentration as shown in Fig. 1. Cells were exposed to the following concentrations (120, 60, 30, 15, 7.5 and 3.75 µg/mL), and the results showed no significant changes in cell viability after 24 h in all tested concentrations for both AQ and ETOH extracts ($p > 0.05$), compared to the negative control.

Meanwhile, the positive control declined significantly ($p < 0.05$) compared to all other treatments (Fig. 1A and Fig. 1C).

Following 48 h treatment, the AQ extracts exhibited a slightly significant ($p < 0.05$) decrease in cell viability

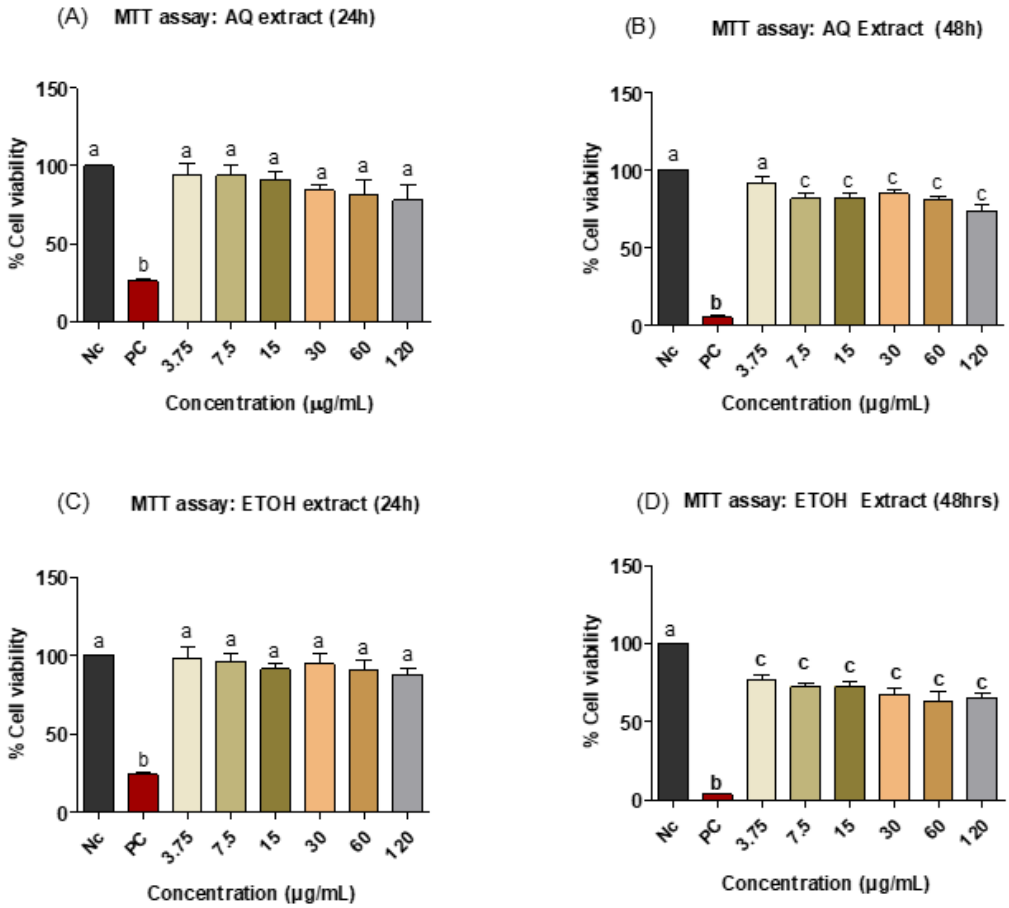

Figure 1. The effect of *H. cymosum* (A and B) aqueous (AQ) and (C and D) 70% ethanol (ETOH) extracts on TM4 Sertoli cells viability treated for 24 h and 48 h period. Results are represented as means ± SEM of three repeated experiments (n = 3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p > 0.05$) compared to the negative control.

was recorded in almost all the tested concentrations compared to the control except the lowest concentration (3.75 µg/mL) (Fig. 1B). However, the percentage cell viability was significantly ($p < 0.05$) higher than the positive control. On other hand, the cells exposed to the ETOH extracts for 48 h showed a significant decrease ($p < 0.05$) in cell viability for all concentrations compared to the negative control (Fig. 1C) and a significant increase ($p < 0.05$) compared to the positive control (Fig. 1D).

The findings showed that overall, both extracts did not significantly reduce the viability of the Sertoli cells at all concentrations and treatment durations (Fig. 1A and Fig. 1C). Previous studies on TM4 Sertoli cells have reported toxicity at a higher concentrations of 100 or 1000 µg/mL after treatment with *Phyllanthus amarus* (Olabiyi et al., 2020). The lack of cytotoxicity observed in the current study may due to the concentrations used being lesser than 1000 µg/mL. The positive control displayed significant reduction in cell viability, demonstrating toxicity effects on the TM4 Sertoli cells interestingly, the changes in the cell morphology (Fig. 6) aligns to the results of cell viability performed in this study.

3.2 Superoxide dismutase activity (SOD)

Superoxide dismutase (SOD) is a vital antioxidant detoxifying enzyme found in cells and acts as a preliminary defense system against ROS by eliminating the superoxide anion and converting it to hydrogen peroxide hence preventing the toxic effects of these free radicals (Ighodaro et al., 2018; Islam et al., 2022). The SOD activity was measured on TM4 Sertoli cells treated with AQ and ETOH extracts for a period of 24 h and 48 h. Both extracts (AQ and ETOH) showed no significant difference at all concentrations compared to the NC and PC after 24 h exposure (Fig. 2A and Fig. 2C). Likewise, the 48 h AQ extracts did not show any significant change at all concentration compared to both controls NC and PC, but the positive control declined by 9% difference in SOD activity compared to the other treatments without any significant difference Fig. 2B. The ETOH extract after 48 h treatment showed no significant difference across all concentration comparatively to the controls (NC). However, a significant difference was observed at (120, 60, 30, 7.5 and 3.75 µg/mL) compared to the PC (Fig. 2D).

Following treatment with the ETOH and AQ extracts of *H. cymosum* (24 h), SOD activity showed no significant changes at all concentrations compared to the controls (Fig. 2). Similarly, the SOD activities for the AQ and ETOH extracts (48 h) did change significantly between the respec-

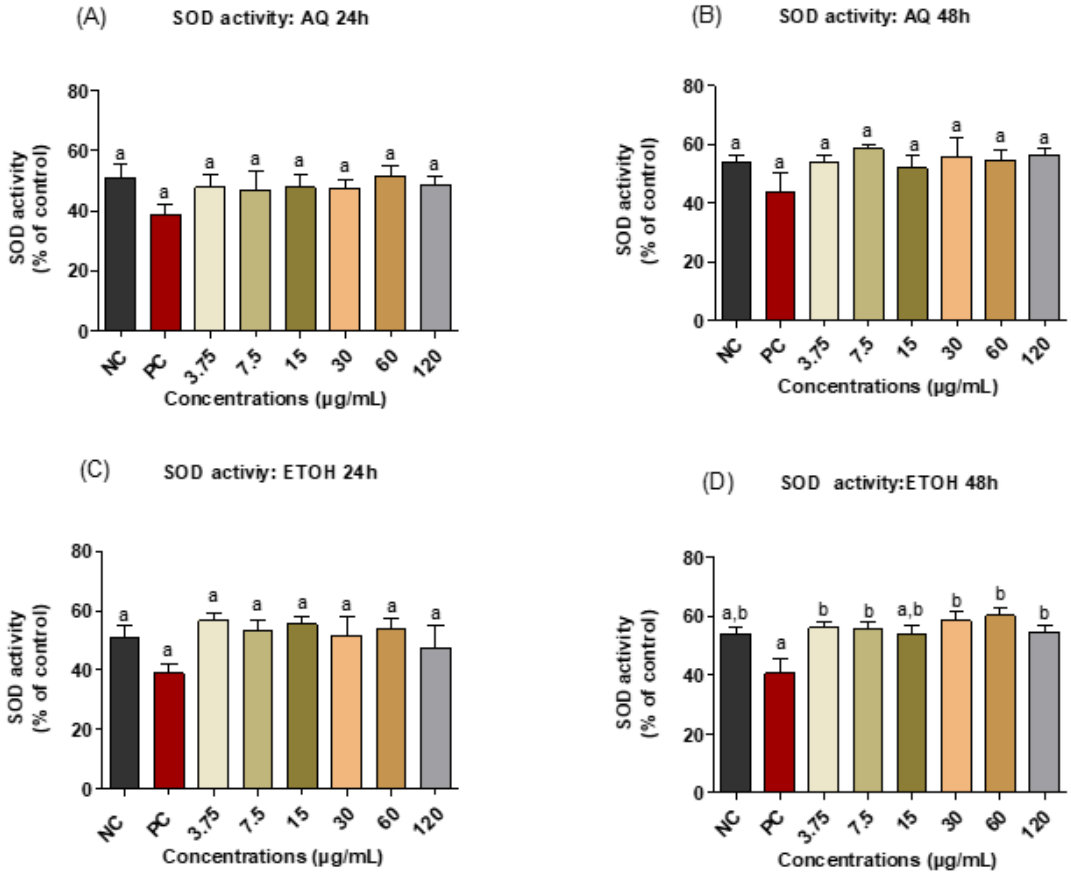

Figure 2. Superoxide dismutase (SOD) enzyme activity after exposure to *H. cymosum* (A and B) aqueous (AQ) and (C and D) 70% ethanol (ETOH) on TM4 Sertoli cells for 24 h and 48 h period respectively. Values represent mean ± SEM of three repeated experiments (n = 3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p > 0.05$) compared to the negative control.

tive concentrations and the controls. Study by Oyedemi et al. (2010) reported an increase SOD inhibition when exposed to plant extract (*S. hennigsii*). Moore et al. (2020) also reported an elevated SOD activity with $50-90\%$ activity after treatment with *Solanum sisymbriifolium* extracts. Hence, findings suggest that for *H. cymosum* to fully activate its catalytic effects requires cofactors such as copper, iron and manganese to exert its full catalytic potential when metabolizing free radical (Zheng et al., 2023).

3.3 Glutathione (GSH)

The levels of reduced GSH were measured after exposure to *H. cymosum* AQ and ETOH for 24 h and 48 h duration (Fig. 3). The AQ extracts showed slight increase at most concentrations for both 24 h and 48 h treatments, with no statistically significant differences ($p > 0.05$) compared to the negative control. However, the positive control exhibited significant ($p < 0.05$) reduction in glutathione levels compared to all tested concentrations (Fig. 3A and Fig. 3B). The ETOH extracts treated for 24 h duration, exhibited no significant difference ($p > 0.05$) in GSH level compared to the negative control, with a significant ($p < 0.05$) increase compared to the positive control (Fig. 3C). However, Fig. 3D shows statistically significant decreases ($p < 0.05$) only at the highest two concentrations (60 and 120 µg/mL) compared to the negative control and no substantial difference at lower concentrations ($p > 0.05$) after 48 h period. The positive control showed statistically significant ($p < 0.05$) reduction in glutathione levels compared to all tested concentrations (Fig. 3C and Fig. 3D).

Glutathione (GSH) is another significant antioxidant which regulates and balances the oxidation and reduction processes in cells, thereby maintaining proper cell functions (Aoyama, 2021; Averill-Bates, 2023). The multifunctional intracellular antioxidant, glutathione (GSH) plays a major role in counteracting oxidative stress, and also participates in several metabolic functions like regulating the cell cycle, calcium homeostasis, enzyme activity as a cofactor, DNA repair, activation of transcription factors, etc. (Van Haaften et al., 2003; Forman et al., 2009; Georgiou-Siafis et al., 2023). The efficacy of glutathione is controlled by a balance between its active (GSH) and inactive (GSSG) forms. The active form facilitates glutathione to carry out its cellular functions whereas the inactive form is produced from neutralized free radicals (Di Giacomo et al., 2023). Therefore, a balance between these two forms of glutathione is important in maintaining cellular health, whereas an imbalance could result in accumulation of free radicals and inflammatory cytokines, cellular dysfunction, rapid aging, risk of chronic degenerative diseases, etc. (Novelli et al.,

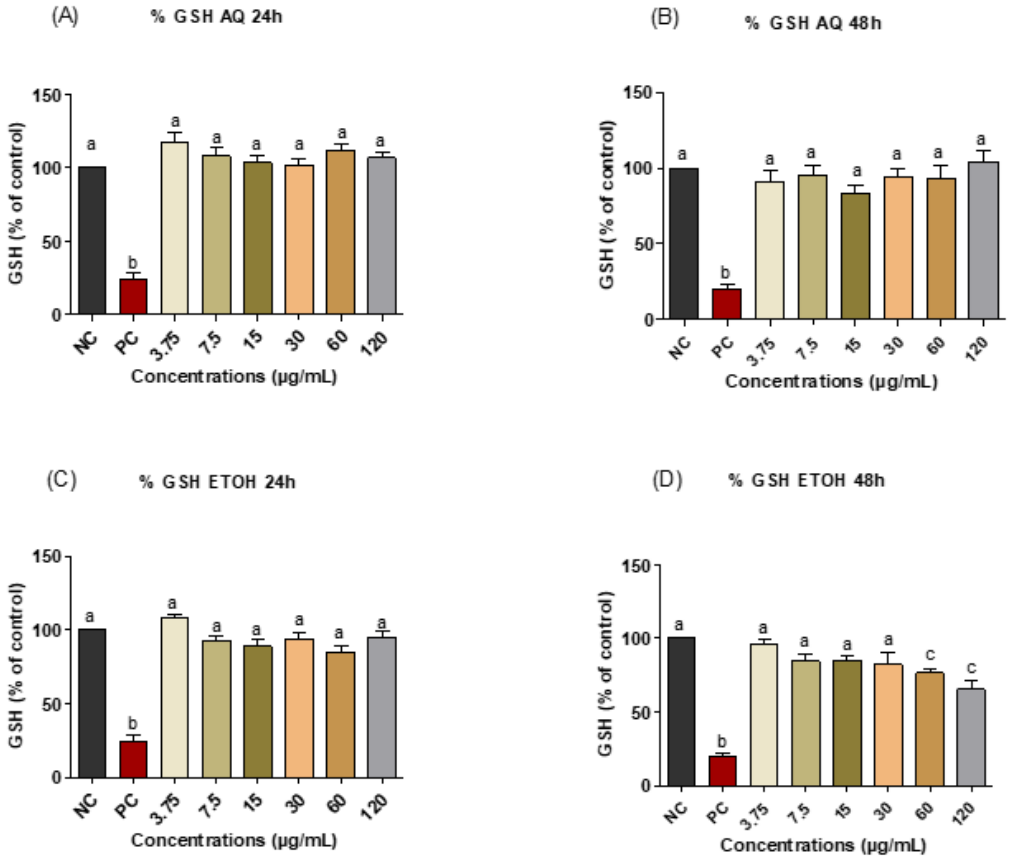

Figure 3. Effect of *H. cymosum* aqueous (A and B) and ethanol (C and D) extracts on Glutathione level after 24 and 48 hours exposure to TM4 Sertoli cells. Values are represented as mean ± SEM of three repeated experiments (n = 3), 10% DMSO in DMEM was used as positive control and 0.1% DMSO in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p > 0.05$) compared to the negative control.

2022; Al-Temimi et al., 2023).

The findings from the present study showed only minor increase in GSH levels for AQ extract 24 h and 48 h (Fig. 3A, Fig. 3B, and Fig. 3C) and the ETOH extract (24 h), as well as a dose-dependent decrease at 48 h in ETOH extract - treated cells (Fig. 3D), with no significant differences noted. Similarly, another study reported significant ($p < 0.05$) increased GSH levels in treatment group of smoker given *Allium sativum* extract compared to smokers and control groups (Savira et al., 2023). Likewise, Falang et al. (2022) reported increased in GSH content in male rats treated with *Zizuphus mauritiana* compound *p*-coumaroyl alphitolic acid (ACA) compared to the control. Despite no significant difference observed in this study, the slight increase in GSH content suggests that the *H. cymosum* extracts is capable of enhancing GSH biosynthesis, however, to conclusively confirm assertion, further investigation needs to be conducted to determine if the extracts (AQ and ETOH) could promote GSH recycling or have an effects on its biosynthesis (Van Haaften et al., 2003; Al-Temimi et al., 2023).

3.4 Evaluation of intracellular ROS production

In the current study, both the AQ and ETOH extracts of *H. cymosum* mitigated the generation of ROS at all tested concentrations for both AQ and ETOH treated for 24 h and 48 h durations respectively, maintaining ROS levels below both the positive control (10% DMSO in DMEM) and negative control (0.1% DMSO in DMEM) (Fig. 4A-Fig. 4D).

The measurement of ROS is also a marker used to determine oxidative stress damage in biological samples. In this study, a decrease in ROS levels was observed (Fig. 4A, Fig. 4B, Fig. 4C and Fig. 4D) compared to the positive control for both extracts following treatment with both aqueous and ethanol extracts for 24 and 48 h, even though the data did not show any significant differences compared to the controls (NC and PC). Nevertheless, our recent study reported significant antioxidant activities of the AQ and ET extracts (Nkemzi et al., 2024), and important metabolites such as flavonoids and polyphenols present in the plant (Jadalla et al., 2022; Nkemzi et al., 2024) in which their action could possibly have an effect on ROS production, however, further study is necessary to compare and confirm the observed results. Our findings align with earlier report that demonstrated increased ROS levels in rats exposed to the drug, valproic acid (VPA), causing valproate-induced oxidative stress which was reversed by the antioxidant action of *Moringa oleifera* (Ertik et al., 2023). Accordingly, Jambor et al. (2022), reported significant reduction ROS production after treatment Lepidus at 5, 10, and 25 µg/mL, these results match the current observation shown by *H. cymosum* treatments on ROS production, although our study showed significant reduction at all concentrations. This indicates that the phytochemicals present in plants could have strong antioxidant effects to reverse oxidative stress. Earlier studies on phytochemical component of these extracts reveals 43 phytochemical derivatives, some of which includes caffeic acid, chlorogenic acid, flavan-3-ols, flavonol, hydoxycinnamic acids, helihumulone, kaempferol and rutin amongst others and exhibited potential antioxidant capacity (Nkemzi et al., 2024). Phytocompounds like flavonoids have been extensively reported to possess antioxidant properties, possibly through scavenging ROS and activating the cellular antioxidant systems (Ramli et al., 2023).

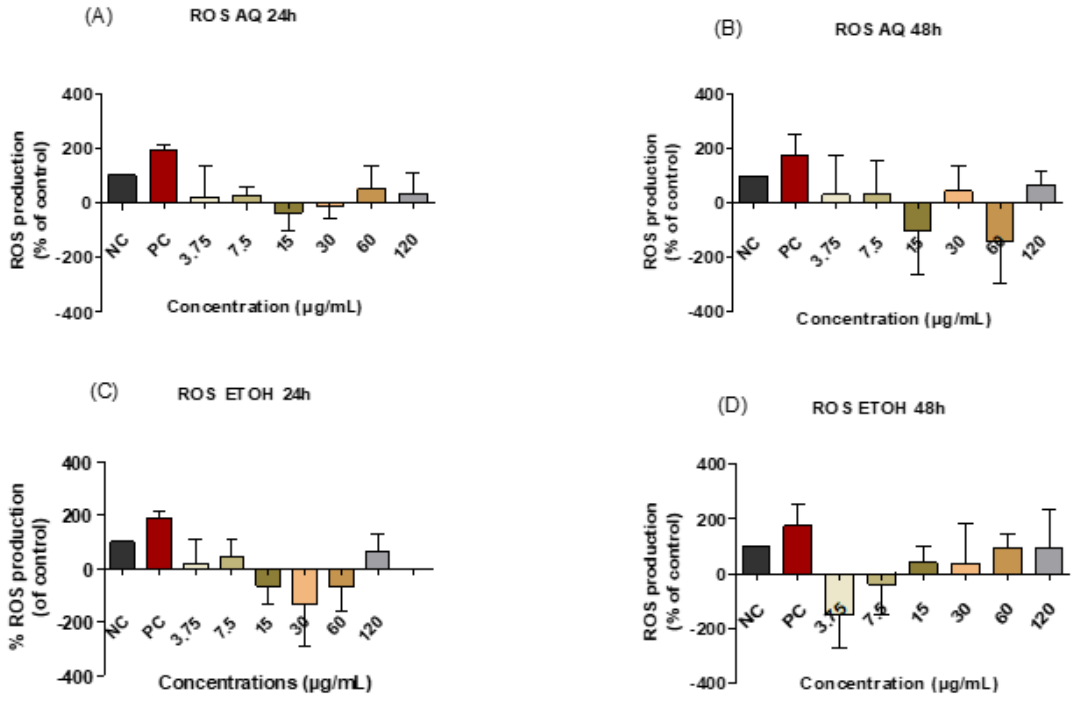

Figure 4. ROS production after 24 h and 48 h treatment of TM4 Sertoli cells with *H. cymosum* aqueous (AQ) and ethanol (ETOH) extracts. All values are represented as mean ± SEM values of three repeated assay (n = 3), level of significance ($p < 0.05$).

3.5 Total antioxidant capacity (TAC)

No significant change was noted for the total antioxidant capacity (TAC) after treatment, for both extracts and treatment times when compared to the control ($p > 0.05$). Although the TAC decreased in the PC group compared to the NC, this was insignificant ($p > 0.05$); Fig. 5A-Fig. 5D). Antioxidants are known to either scavenge the free radicals formed or intercept the radical generating process. In this study, cells treated with the AQ and ETOH extracts for 24 h and 48 h, increased TAC at lower concentrations (3.75 and 7.5 µg/mL), with no significant difference compared to the controls (Fig. 5). Overall, the observed increase in antioxidant activity (SOD, GSH and TAC) is directly proportional to a decrease in ROS production (Fig. 2-Fig. 5).

3.6 Cell morphology changes after treatment

TM4 cell morphology was observed after treatment with the AQ and ETOH extracts of *H. cymosum* for 24 h and 48 h, and the micrographs have been represented in Fig. 6. The images show normal dividing cells without any effect on cell structure integrity for both extracts at all concentrations and negative control, suggesting that the treatment were not toxic to the cells. The positive control displayed significant changes in structural integrity of the cells for both 24 h and 48 h, which was noted by cell shrinkage and round shape caused by the toxic effect of DMSO (10%) (Fig. 6).

4. Concluding remarks

The present study is the first to explore the antioxidant activities, cytotoxicity and morphological changes of AQ and ETOH extracts obtained from *H. cymosum* shoots on TM4 Sertoli cell lines. The antioxidant protective properties of *H. cymosum* against oxidative stress damage was demonstrated. Four antioxidant parameters (S OD, GSH and TAC and ROS) were analyzed which reveal better antioxidant scavenging activities with decrease ROS production at all concentrations. Additionally, extracts showed no cytotoxic effect, suggesting its ability to promote cell proliferation, which is supported by no negative effect on the morphology of the cells. Therefore, *H. cymosum* is a promising plant candidate that contains bioactive molecules that can further be explored to develop novel antioxidant drugs to manage male subfertility. However, further *in vitro*, *in vivo* and clinical research on specific compounds and mechanisms responsible for the antioxidant activities is recommended. Additionally, harnessing the antioxidant potential of *H. cymosum* extracts can unlock possibilities for innovative therapeutic strategies against oxidative stress-related diseases, propelling future research into optimizing these bioactive compounds from this plant for clinical applications.

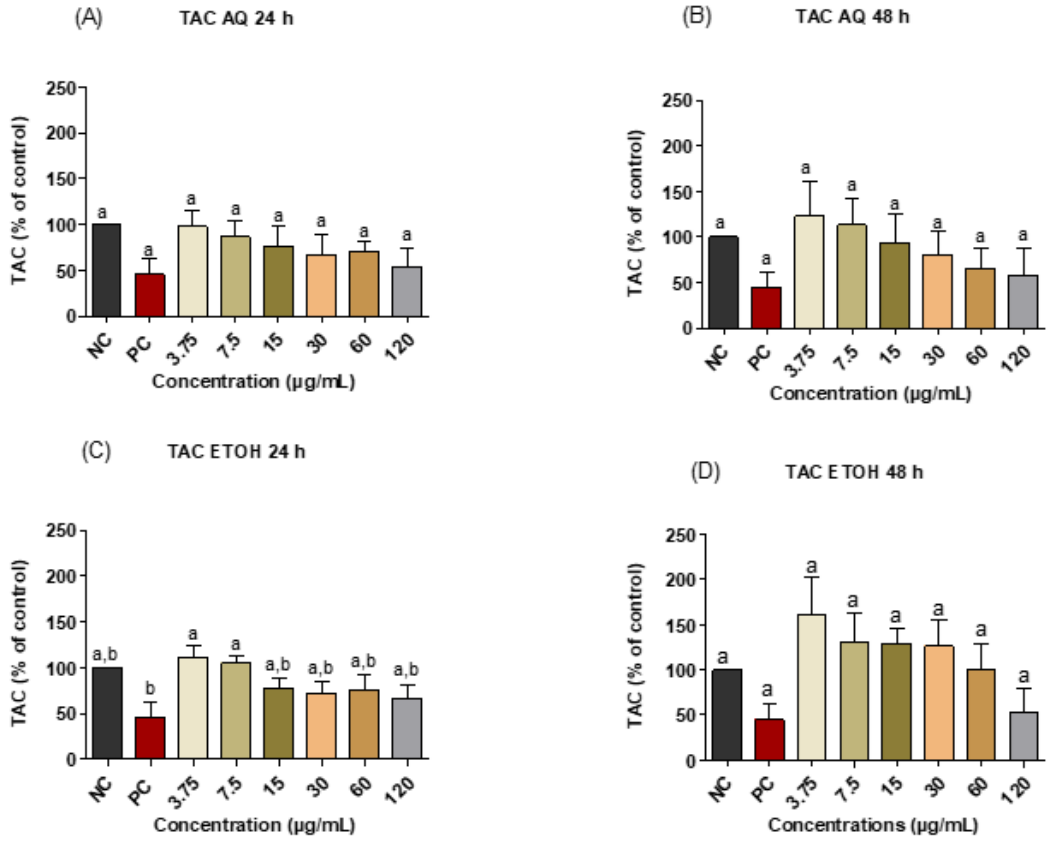

Figure 5. Total antioxidant activities of *H. cymosum* aqueous (A and B) and ethanol (C and D) extracts after 24 and 48 hours exposure to TM4 Sertoli cells. Values are represented as mean ± SEM of three repeated experiments (n = 3), 10% DMSO in DMEM was used as positive control and DMSO (0.1%) in DMEM as negative control. Bars with different letters represent significant difference ($p < 0.05$), while bars with similar letters represent no significant difference ($p > 0.05$) compared to the negative control.

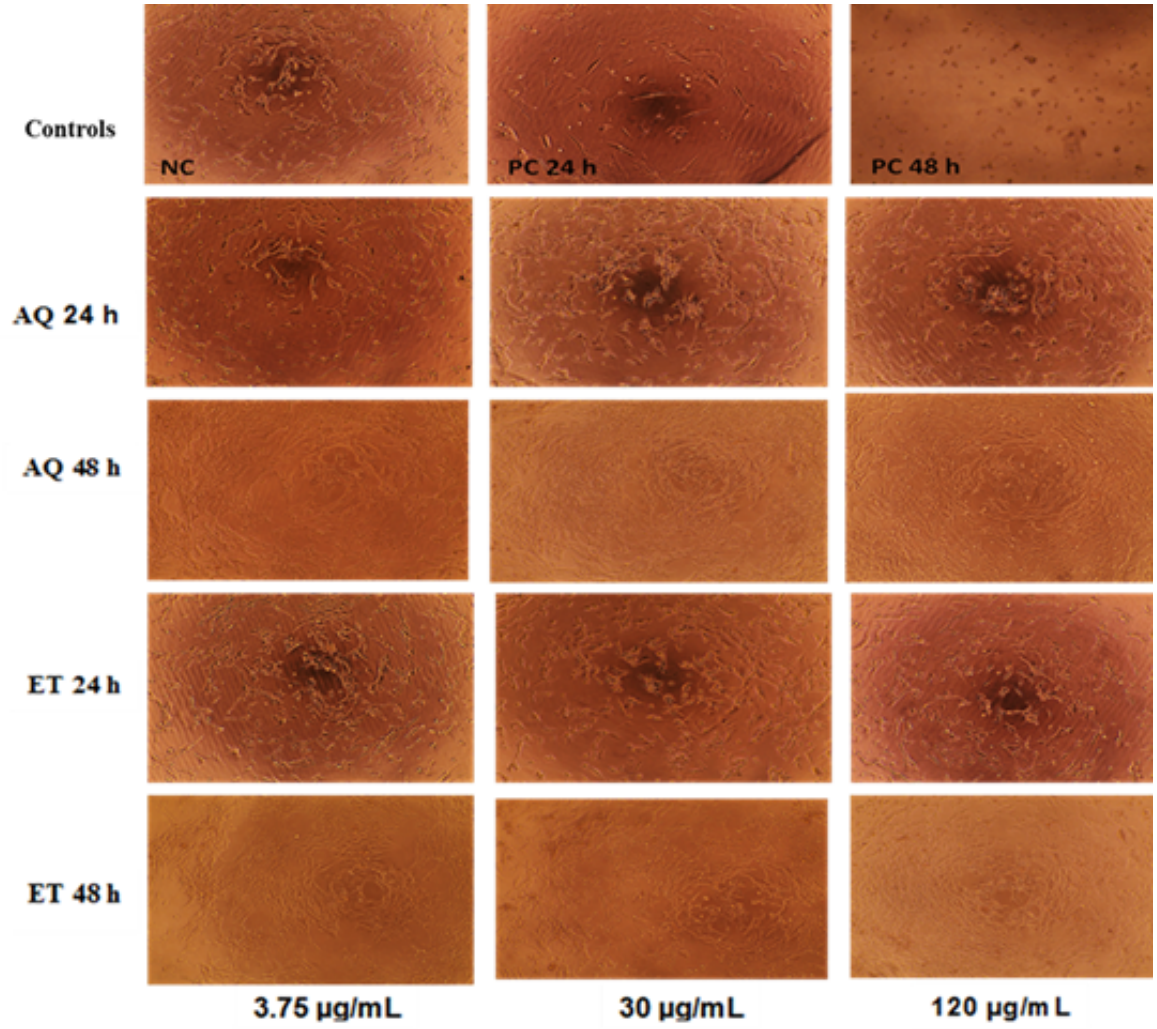

Figure 6. Micrograph images of TM4 sertoli cells captured at (10x) magnification after treatment to AQ and 70% ethanol extracts for 48 hours.

Abbreviations

ADM: Antioxidant Defense Mechanism, **ATTC**: American TYPE TISSUE CULTURE COLLECTION, **AQ**: Aqueous, **DMEM**: Dulbecco's Modified Eagle Medium, **DMSO**: Dimethyl Sulfoxide, **DNA**: Deoxyribonucleic Acid, **ETOH**: Ethanol, **FBS**: Fetal BOVINE SERUM, **FSH**: Follicle-Stimulating Hormone, **GnRH**: Gonadotropin-Releasing Hormone, **GSH**: Glutathione, **HCG**: Human Chorionic Gonadotropin, **HMG**: Human Menopausal Gonadotropin, **HPLC**: High Performance Liquid Chromatography, **LH**: Luteinizing Hormone, **NC**: Negative control, **OS**: Oxidative Stress, **PC**: Positive control, **ROS**: Reactive oxygen species, **SOD**: Superoxide Dismutase, **TAC**: Total Antioxidant Capacity, **UPLC-LCMS**: Ultra-Performance Liquid Chromatography.

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Authors contributions

Achasih Quinta Nkemzi: Conceptualization, Data curation, methodology, formal analysis, Investigation, Validation, Writing original draft, Writing review & editing Chinyerum Sylvia Opuwari: Conceptualization, methodology, review & editing. Okobi Eko Ekpo: Supervision, Conceptualization, methodology, Investigation, Validation, administration, Writing review & editing. Oluwafemi Omoniyi Oguntibeju: Funding acquisition, resources, supervision, Conceptualization, investigation, administration, validation, and review & editing.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests

The authors declare that there is no conflict of interest.

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