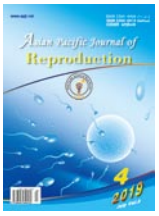

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Influence of *Glyphaea brevis* twig extract on nucleus, tight junctions and expression of inhibin-β, stem cell factor, and androgen binding protein in TM4 Sertoli cells

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ABSTRACT

Objective: To examine the influence of *Glyphaea (G.) brevis* twig extract on the mitochondrial dehydrogenase activity, integrity of the tight junctions between adjacent cells, mitochondria, apoptosis, nucleus and expression of inhibin-β, stem cell factor, and androgen binding protein in TM4 Sertoli cells.

Methods: TM4 cell line was used in this study as it exhibited properties similar to the Sertoli cells. TM4 Sertoli cells were exposed to *G. brevis* twig extract (0.1, 1.0, 10.0, 100.0, or 1 000.0μg/mL) for 24, 48 and 72 h. Parameters studied included cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay], mitochondrial membrane potential (tetra methyl rhodamine ethyl ester dye), transepithelial electrical resistance, apoptosis (Annexin V Alexa Fluor®488/propidium iodide assay) and mRNA expression (quantitative reverse transcription polymerase chain reaction).

Results: *G. brevis* twig extract had no cytotoxic impact on cell viability, thus, considerably increasing the activity of mitochondrial dehydrogenase enzyme after 24 and 72 h exposure. Transepithelial electrical resistance values revealed substantial (P<0.05) rise in treated groups, especially after 72 h of treatment. Moreover, there was a significant decrease in mitochondrial depolarization of TM4 Sertoli cells exposed to *G. brevis* twig extract when compared to controls. In addition, *G. brevis* twig extract significantly reduced necrosis and apoptosis of TM4 Sertoli cells when compared to control. Nevertheless, fluorescence microscopy revealed that the nuclei were egg-shaped and marked uniformly with consistent cell shape at the middle of the TM4 Sertoli cells. Significant stimulatory effects were observed on mRNA levels of inhibin-β, androgen binding protein and stem cell factor.

Conclusions: *G. brevis* twig extract may increase the secretory roles of TM4 Sertoli cells, cells proliferation, as well as cell-cell tight junction integrity. Thus, *G. brevis* twig may enhance spermatogenesis.

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1. Introduction

Glyphaea (G.) brevis (Spreng) Monachino belongs to the family Tiliaceae and plays a major part in conventional medicine in most parts of Africa and South America. It is employed orthodoxly to treat various illnesses for example fevers, gonorrhea, dysentery, stomach distresses, lung difficulties, parasitic infections, aphrodisiacs, insect control, *etc*[1–4]. Over the past few years, there has been a remarkable increase in the research on *G. brevis* which has led to the identification of some compounds in the plant such as ferulic, catechuic and coumaric acids[4]. Its anti-infective[5,6], antioxidant and free radical scavenging[4,7], anticonvulsant[8], anti-inflammatory[5,9], antiproliferative[10], and hepatoprotective effects[11], have led to its therapeutic importance. Moreover, male fertility problem is on the increase and major factors are involved in this process, comprising exogenous factors. Currently, natural products are used on the increase in treating fertility problems. The study by Eweoya *et al*[12] reported the probable effect of the aqueous and alcohol leaf extract of *G. brevis* on male infertility in rats.

Testis, a male reproductive organ, is the site of many reproductive agents. The primary specialized reproductive tissues in the testes are the Sertoli and Leydig cells. The Sertoli cell found in the seminiferous tubules offers a nutritive and morphogenetic guide to the germ cells[13]. However, Sertoli cells and spermatogenic cells are crucial for proliferation, differentiation, and survival of the germ cells[14]. Morphometric analyses have revealed that Sertoli cell in matured testis gives structural and dietary support to growing germ cells[15]. However, secretions produced by Sertoli cells control hormone release, thus inducing spermatogenesis further[16]. In addition, the role of Sertoli cells is to create the blood-testis barrier, which offers a specialized and enabling atmosphere for germ cell growth[17]. Therefore, any compound that can damage the viability and/or roles of these cells may intensely impact spermatogenesis[13]. Sertoli cells are a site for numerous compounds which aid as a well-documented scheme for studies in reproductive systems[18]. Although, hitherto, the impact of *G. brevis* twig exposure on the structure and function of Sertoli cells have not been reported.

Hence, the aim of this study was to investigate the influence of exposure of *G. brevis* twig on TM4 Sertoli cell mitochondria, nucleus, apoptosis, necrosis, blood-testis barrier, expression of specific proteins such as androgen-binding protein, stem cell factor and inhibin-β as well as the role of Sertoli cells.

2. Materials and methods

2.1. Collection of G. brevis twigs

G. brevis twigs (1 000 g) were obtained from Olodo village in Ogun State, Nigeria. They were identified and authenticated by Mr. Odewo at Forestry Research Institute of Nigeria and a voucher specimen number FHI 110104 was prepared.

2.2. Preparation of methanolic G. brevis twig extract

Fresh twigs of *G. brevis* were rinsed, sliced and air-dried at room temperature. The dried twigs were pulverized into powder using an electric blender (KENWOOD, Model BL490, Taiwan) and weighed. 100 g of the powder was extracted in 500 mL of methanol for 5 h *via* Soxhlet extraction and was allowed extra 5 h until solvent siphon became colorless. The mixture was concentrated using rotary evaporator (MODEL: RE-52A) to give extract used for further study.

2.3. Groupings

The groupings for the TM4 Sertoli cell lines are as follows: Cells were treated with *G. brevis* twig extract at concentrations of 0.1, 1.0, 10.0, 100.0 and 1 000.0 μg/mL for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and annexin V/ propidium iodide assay. Also, cells treated with *G. brevis* twig extract at concentrations of 10.0, 100.0 μg/mL for tetra methyl rhodamine ethyl ester (TMRE) and quantitative reverse transcriptionpolymerase chain reaction (RT-qPCR) assay, while *G. brevis* twig extract at concentrations of 100.0 and 1 000.0 μg/mL for transepithelial electrical resistance (TEER) assay. The doses were choosen from our preliminary study. The untreated cells served as normal control; the dimethylsulfoxide (DMSO) treated cells served as postive control.

2.4. Cell culture

One vial of TM4 mouse Sertoli cell line was procured from American Type Cell Culture (ATCC® CRL – 1715TM, Manassas, USA). The cells cultured in Dulbecco's modified Eagle medium were further supplied with 2.5% fetal bovine serum and 5% horse serum in a moistened environment of 5% CO_2 at 37 ℃. All cells were exposed to *G. brevis* twig extract at 70% confluence. Cells were treated with *G. brevis* twig extract at concentrations of 0.1, 1.0, 10.0, 100.0 and 1 000.0 μg/mL for MTT assay. For MTT assay, cells were grouped as 3 000 cells/well in a 96-well plate (Corning, Lowell, MA, USA) for 24 h and 1 500/well for 72 h because TM4 Sertoli cells grew and adhered to wells within this period of time. Also, we were checking for acute and chronic exposure which was 24 h and 72 h. For the TMRE, cells were placed at 1×10^5cells per well on a 12-well plate (Corning, Lowell, MA, USA). For annexin V-propidium iodide staining, the cells were placed in 24-well plates of 5×10^4 cells/well in complete culture media. For TEER, the cells were placed in 24-well plates of 3×10^5/well with complete culture media.

2.5. Cell viability studies by MTT assay

To determine the viability of the cells, 5 000 cells/mL were cultured in a 96-well plate for 24 h to allow cellular attachment. The next day, the medium was detached and the adherent cells were exposed to concentrations of 0.1, 1.0, 10.0, 100.0 and 1 000.0 μg/mL *G. brevis* twig extracts. The negative control groups were not treated while the positive control groups were treated with 10% DMSO. *G. brevis* twig extracts were detached from all the wells and cells were rinsed with phosphate buffer saline (PBS). Subsequently, 200 μL medium was added and 20 μL MTT was added and kept warm for 3 h at 37 ℃. The supernatant was removed by aspiration, 100 μL of DMSO was placed in each well and absorbance of the dye was read with an enzyme-linked immunosorbent assay reader (Thermo electron corporation, South Africa) at 560 nm with a reference wavelength of 750 nm.

2.6. TEER across TM4 Sertoli cell monolayer

The Milicell® 24-well tissue culture with 12 mm size, 0.6 cm^2 active surface area and 0.45 pore size were used in TEER assay. The filters were positioned in the wells of 24 well plates inside the laminar flow hood by the use of sterile forceps. Cells were thereafter placed at 3×10^5 per well with complete media and incubated in 37 ℃. After 24 h, the media were cast off and cells were exposed to 100.0 and 1 000.0 μg/mL and kept warm for 24, 48 and 72 h. The control group consisted of untreated cells. TEER was measured by means of a Milicell®-ERS resistance system Volt-Ohm meter (Merck, Millipore Ltd, USA). The values were expressed in Ωcm^2.

2.7. Determination of mitochondrial membrane potential using TMRE dye

TMRE is a permeable, charged, red-orange dye that detect mitochondrial potential. Loss of mitochondrial potential or inactive mitochondria is an indicator of cell death and can be detected *via* TMRE[19]. It clumped in the mitochondria of non-apoptotic cells and fluoresced bright orange or red, while in apoptotic cells it diffused throughout the cell.

This procedure was done as defined by Ricci *et al*[19] with some alterations. The cells were placed at 1×10^5 cells per well on a 12-well plate and kept warm for 24 h at 37 ℃ in a moistened CO_2 incubator. The cells were exposed to *G. brevis* twig extracts at a concentration of 10.0 and 100.0 μg/mL for 24 h. Untreated cells served as negative control, while carbonyl cyanide m-chlorophenylhydrazone and DMSO served as positive control. After the specified time, floating cells were moved into a 15 mL tube. The adherent cells were rinsed using PBS, trypsinized and combined with floating cells. The cells were centrifuged at 300 × *g* for 3 min and rinsed with PBS. This was followed by staining the cells using 300 μL TMRE dye (1 μM) for 30 min at 37 ℃. The cells were then rinsed with PBS to remove the dye and measured by flow cytometry (BD Biosciences Pharmingen, San Diego, CA, USA). Cell staining was measured at 488 nm at fluorescence channel 3 on a Becton Dickinson Accuri™ C6.

2.8. Estimation of necrotic and apoptotic events by Alexa Fluor® 488 annexin V/Dead cell apoptosis kit using flow cytometry

The principle behind the annexin V apoptotic assay was that in normal cells, phosphatidyl serine was situated on the surface of the membrane. Though, in apoptotic cells, phosphatidyl serine was translocated from the internal to the external part of the membrane. This exposed the phosphatidyl serine to the outside cellular surroundings, thereby marking the cell for recognition[20]. The cells were placed at 5×10^4 cells/well of the complete medium in a sterile 24-well plate. From our preliminary studies, we decided to use concentrations such as 1.0, 10.0, 100.0 and 1 000.0 μg/mL *G. brevis* twig extracts to estimate the proportion of apoptotic and necrotic TM4 Sertoli cells. The control group consist of 0.2% DMSO while the baseline group consist of untreated cells with media only. In brief, the culture medium was castoff, cells were rinsed with 1 mL PBS and trypsinated with 250 μL of 0.25% trypsin/ethylene diamine tetraacetic acid. Annexin V staining was achieved by following product instruction. In brief, 5 μL Alexa Fluor® 488 annexin V (Component A) and 1 μL 100 μg/mL propidium iodide (Component B) working solution was added to 100 μL of cell suspension and kept warm at room temperature for 15 min. After incubating period, 400 μL 5 × annexin-binding buffer (Component C) was added and mixed gently. All samples were kept on ice and then immediately taken for flow cytometry analysis using a fluorescence emission at 530 nm (green) for the fluorescence channel 1 and 585 nm (red) for the fluorescence channel 3. All fluorescence signals of labeled TM4 Sertoli cells were analyzed. The population of cells was separated into four quadrants: live cells, apoptotic cells, necrotic cells and dead cells *via* FlowJo software (version 10.4, Flowjo software, USA).

2.9. Cell imaging studies

TM4 Sertoli cells were developed overnight in a glass cultured dish (m-Dish, Ø 35 mm) under laboratory conditions. Then, cells were kept warm in complete medium in 5% CO_2 at 37 ℃ for 24 h. Cells were exposed to *G. brevis* twig extract at 10.0 and 100.0 μg/mL concentrations. The doses were chosen from our pilot study. These cells were rinsed twice with 1 × PBS and drained properly. 4,6-diamidino-2-phenylindole (DAPI) containing Fluoroshield histology mounting medium (Sigma-Aldrich) was dropped on each treated cells and then mounted on Zeiss fluorescence microscope and images were captured at 100× magnification.

2.10. RNA extraction and RT-qPCR

Cells were ready in 48-well plates and exposed to *G. brevis* twig extract at 10.0 and 100.0 μg/mL for 24 h. Total RNA was extracted from the cells *via* Qiagen RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). Extracted RNA was re-dissolved in 30 mL of RNase free water. Total RNA obtained from samples was kept at –80 ℃ until reverse transcription PCR (RT-qPCR) detection. Quantification of RNA was achieved by employing a Nanodrop (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). 20 ng of total RNA was reverse-transcribed *via* the SuperScript1 VILOTM cDNA Synthesis Kit (Life Technologies, Burlington, ON, Canada). Real-time PCR was achieved by means of SsoAdvancedTM Universal SYBR1 Green supermix and a CFX96 TouchTM apparatus (Bio-Rad). Gene-specific primers employed for qPCR were itemized in Table 1. To quantify relative gene expression, the threshold cycle of target gene amplification was standardized to the expression level of a housekeeping gene following the ratio, R = ECt GAPDH/ECt target, where E was the amplification efficiency for each primer pair. Experiments were done three times in duplicates.

Table 1. Sequences of primer pairs employed in real-time quantitative polymerase chain reaction.

Primer name	Primer sequences	Bases
ABP forward	5'-GCATGAGGATTGCACTAGG-3'	19
ABP reverse	5'-GGGAATGTCTTGGAGACTG-3'	19
Kitl forward	5'-GAAGAAGACACAAACTTGGA-3'	20
Kitl reverse	5'-GCGACATAGTTGAGGGTTAT-3'	20
Inhibin-β forward	5'-GCTTTGCAGAGACAGATGG-3'	19
Inhibin-β reverse	5'-CTTGGAAGTACACCTTGACC-3'	20
GAPDH forward	5'-AACTCAGGAGAGTGTTTCCTCG-3'	21
GAPDH reverse	5'-TGATGGGCTTCCCGTTGATG-3'	20

ABP: Androgen binding protein, Kitl: Stem cell factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

2.11. Data analysis

Data were analyzed as mean ± standard deviation (mean ± SD). These analyses were achieved with Graph-Pad Prism for Windows version 5.0. Values were expressed to be considerably different at $P<0.05$.

3. Results

3.1. Mitochondrial dehydrogenase enzyme activity by means of MTT assay

The potential cytotoxic effect of *G. brevis* twig extract on the viability of TM4 Sertoli cell lines was tested and the activity of the mitochondrial dehydrogenase of TM4 Sertoli cells after treatment with *G. brevis* twig extracts (0.1, 1.0, 10.0, 100.0, 1 000.0 μg/mL) for 24 h displayed substantial increase in mitochondrial dehydrogenase enzyme activity compared to the positive control group (Figure 1). A significant decrease was observed in mitochondrial dehydrogenase activity in the positive control compared to the negative group ($P<0.05$). After 72 h exposure, the enzyme activity displayed a concentration-dependent increase when compared to the positive and negative control groups.

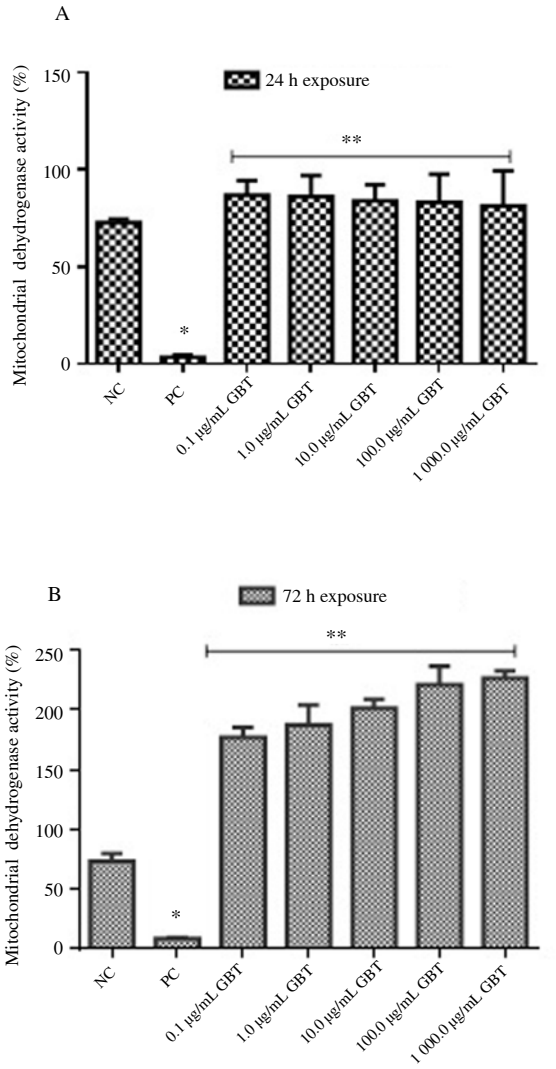

Figure 1. Mitochondrial dehydrogenase activity of basal TM4 Sertoli cell exposed to *Glyphaea brevis* twig (GBT) extract for 24 h (A) and 72 h (B). Values are expressed as mean ± SD, $n=8$. NC: negative control, PC: positive control (10% DMSO); *$P<0.05$ compare to NC group, **$P<0.05$ compared to PC group.

3.2. Effect of G. brevis twig extracts on TM4 Sertoli cells TEER study

After 24 and 72 h exposure, TM4 Sertoli cells monolayers showed a substantial increase ($P<0.05$) in TEER at 100.0 and 1 000.0 μg/mL concentrations of *G. brevis* twig extracts when compared in reference to the control group. In addition, the group which was exposed to 1 000.0 μg/mL *G. brevis* twig extracts revealed a highly substantial increase ($P<0.05$) in the resistance at 48 h (Figure 2).

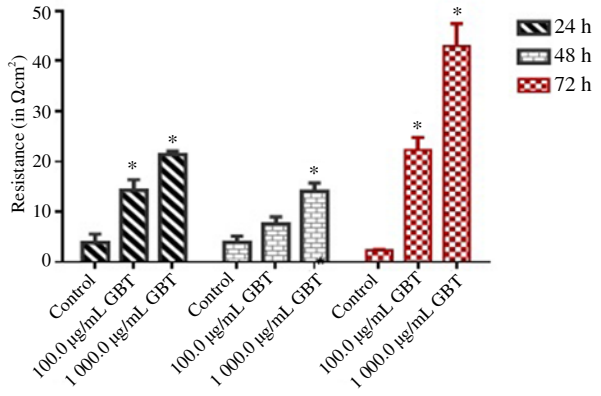

Figure 2. Effect of *Glyphaea brevis* twig (GBT) extract on transepithelial electrical resistance of TM4 Sertoli cells for 24, 48, and 72 h. Values are expressed as mean ± SD. * indicates $P<0.05$ in comparison with the control.

3.3. Effects of G. brevis twig extracts on mitochondrial depolarization in TM4 Sertoli cells

TM4 Sertoli cells exposed to *G. brevis* twig extracts showed a significant reduction in a number of cells with depolarized mitochondrial ($P<0.05$) at concentrations 10.0 and 100.0 µg/mL when compared to the positive control (Figure 3).

3.4. Impact of G. brevis twig extracts on apoptosis and necrosis in TM4 Sertoli cells

The Annexin V/propidium iodide flow cytometry assay exhibited a considerable decrease in necrotic and apoptotic cells after 24 and 72 h exposure with 1.0, 10.0, 100.0 and 1 000.0 µg/mL when compared to the control groups (Figure 4, Figure 5).

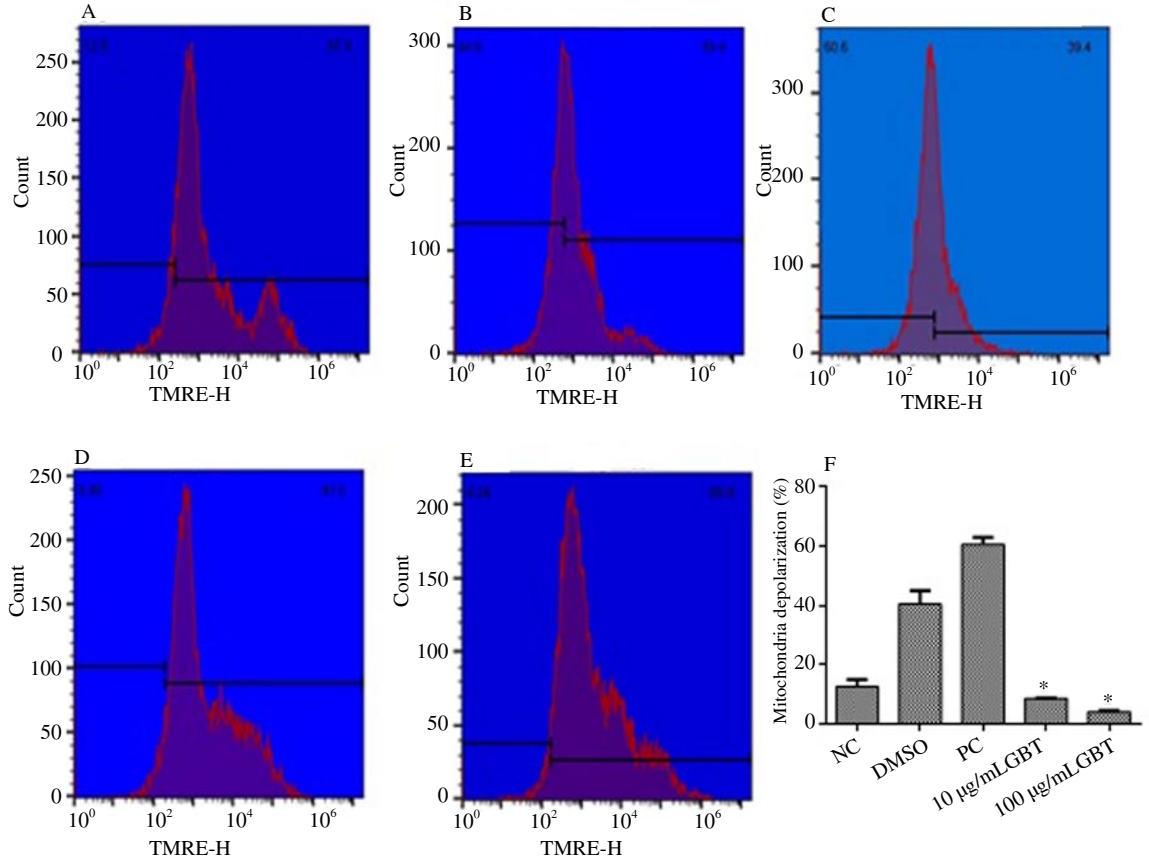

Figure 3. Flow cytometer analysis of influence of *Glyphaea brevis* (GBT) twig extracts on mitochondria depolarization in TM4 Sertoli cells (stained with TMRE dye). Histograms represent cells with depolarized mitochondria that are harvested from untreated cells (A), cells treated with DMSO (B), cells treated with treated with DMSO and carbonyl cyanide m-chlorophenylhydrazone (C), cells treated with methanolic extract of *Glyphaea brevis* twig of 10 µg/mL (D) and 100 µg/mL (E). Values are expressed as mean ± SD, $n=6$. NC: negative control (untreated), PC: positive control (treated with DMSO and carbonyl cyanide m-chlorophenylhydrazone); *$P<0.05$, compared to the negative control. DMSO: dimethylsulfoxide.

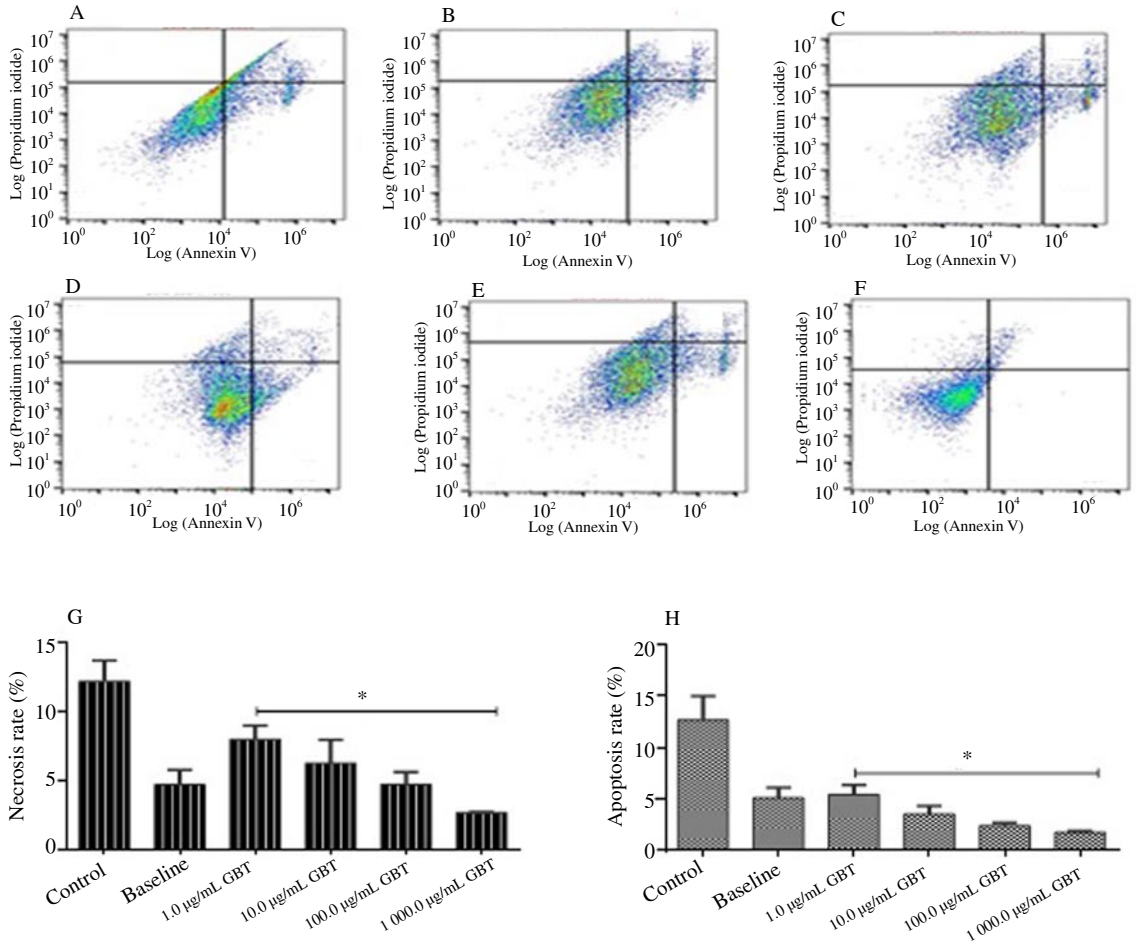

Figure 4. Effect of methanolic extract of *Glyphaea brevis* twig (GBT) on necrosis and apoptosis. Representative micrographs revealed by flow cytometry dot plot analysis of annexin-V/PI- labelled TM4 Sertoli cells after 24 h exposure with control (0.2% DMSO) (A), baseline (untreated cells with media only) (B), extract of *Glyphaea brevis* twig at different concentrations of 1.0 μg/mL (C), 10.0 μg/mL (D), 100.0 μg/mL (E) and 1000.0 μg/mL (F). Data are expressed as mean ± SD of six independent experiments performed in triplicate. **P*<0.05, when treated groups are compared with the control groups.

3.5. Effects of G. brevis twig extracts on nuclear DNA in TM4 Sertoli cells

The nuclei were marked in blue using DAPI (Figure 6). In the control group, the nuclei were rounded in shaped and marked evenly with regular cell shape in the center of the TM4 Sertoli cells. After 24 h exposure to *G. brevis* twig extracts, the nuclei were regular in shape and were rounded. The nuclei were not damaged with increasing dose. From Figure 6, *G. brevis* twig extracts were able to penetrate into the nucleus of the Sertoli cells. From the blue color of the DAPI, it showed that the extracts can penetrate and have effects in the nucleus.

3.6. Impacts of G. brevis twig extracts on mRNA levels of inhibin-β, androgen binding protein, stem cell factor in TM4 Sertoli cells

The mRNA levels of inhibin-β, androgen binding protein, stem cell factor considerably increased in groups exposure to 10 and 100 μg/mL *G. brevis* twig extracts in reference to the negative control groups (Figure 7).

4. Discussion

The main roles of the Sertoli cell is to produce the blood-testis barrier, whose roles were to moderate the route of diverse molecules inside and outside of the adluminal section of the seminiferous epithelia and to aid as an immunological barrier to establish specified and relative steady surroundings for the growth, expansion, and differentiation of germ cells[20]. Blood-testis barrier comprises of coexisting adherens and tight junctions. Interference of these junctions results in damage of spermatogenesis[21] and eventually results in sterility[22].

Mitochondrial dehydrogenase activity is frequently employed to measure TM4 Sertoli cell viability and Monsees *et al*[23] indicated that the mitochondria of the Sertoli cell may be a possible target site for toxicants. Our study, therefore, revealed that the activity

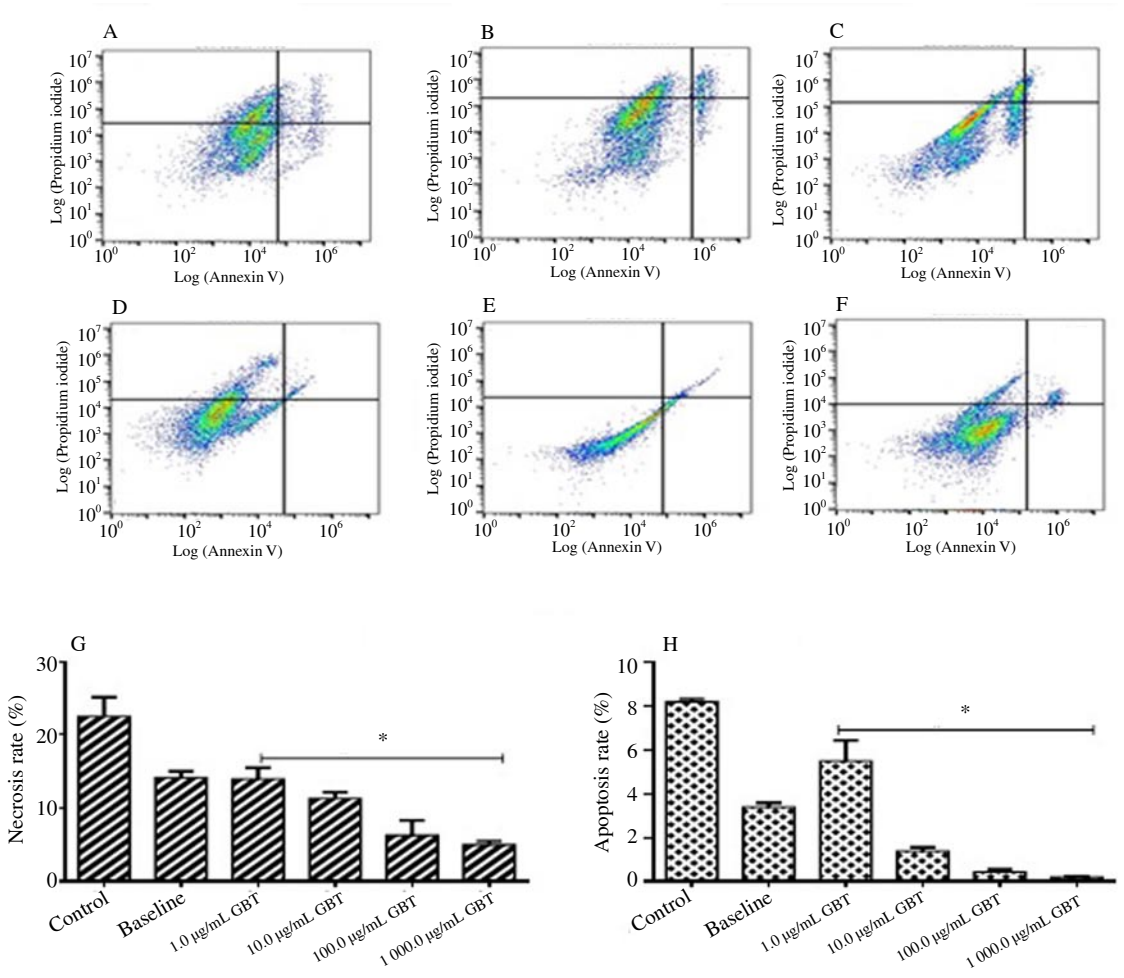

Figure 5. Effect of methanolic extract of *Glyphaea brevis* twig (GBT) on necrosis and apoptosis. Representative micrographs revealed by flow cytometry dot plot analysis of annexin-V/PI- labelled TM4 Sertoli cells after 72 h exposure with control (0.2% DMSO) (A), baseline (untreated cells with media only) (B), extract of *Glyphaea brevis* twig at different concentrations of 1.0 μg/mL (C), 10.0 μg/mL (D), 100.0 μg/mL (E) and 1000.0 μg/mL (F). Data are expressed as mean ± SD of six independent experiments performed in triplicate. **P*<0.05, when treated groups are compared with the control

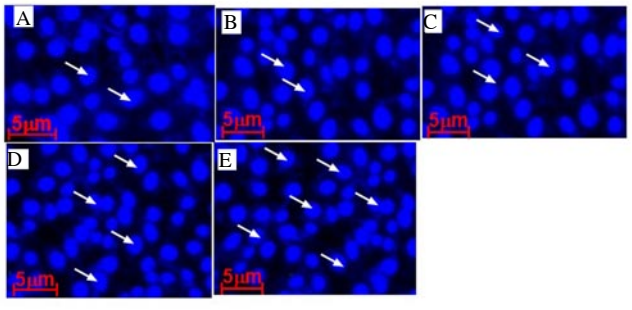

Figure 6. Effects of *Glyphaea brevis* twig (GBT) methanolic extract on nucleus structure of TM4 Sertoli cells. Nuclei of cells were stained with 4,6-diamidino-2-phenylindole. Arrowhead points to nucleus of TM4 Sertoli cells. A: the control (untreated cells), B: 1.0 μg/mL GBT, C: 10.0 μg/mL GBT, D: 100.0 μg/mL GBT, E: 1 000.0 μg/mL GBT. Scale bar: 5 μm, Magnification: 100×.

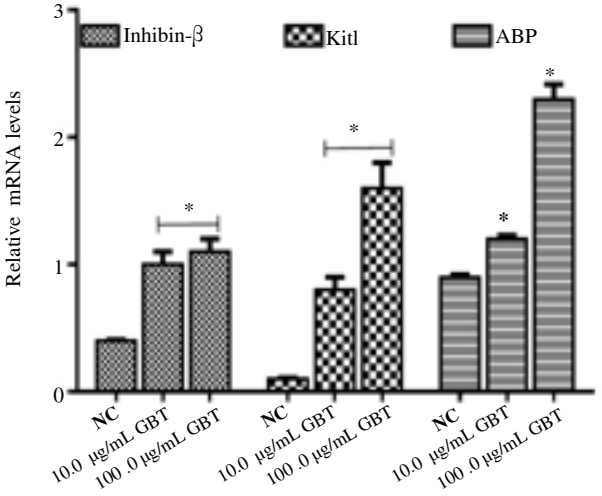

Figure 7. Quantitative RT-PCR analyses of mRNA expression levels of inhibin-β, stem cell factor (Kitl) and androgen binding protein (ABP) in control and *Glyphaea brevis* twig extract (GBT)-exposed TM4 Sertoli cells for 24 h. Gene expression levels represent mRNA expression levels relative to control levels. Values represent mean ± SD. **P*<0.05 when compared to the negative control group (NC).

of the mitochondrial dehydrogenase of TM4 Sertoli cells after exposure to *G. brevis* twig extract for 24 h displayed a significant increase in the treated groups when likened to the positive group. Furthermore, after 72 h exposure, the enzyme activity displayed a concentration-dependent increase at a concentrated test. This outcome also corroborates our previous results on the effects of *G. brevis* twig extract using TM3 Leydig cells[24]. The mitochondrion is the power house of a cell and *G. brevis* twig extract has no influence on the mitochondrial enzymes in the cells, thus it suggests that the enzyme activities in the cells may not be affected. However, as the concentration increases at 72 h exposure, there is a marked increase in the activity of the mitochondrial dehydrogenase which indicates that this *G. brevis* twig extract may target the mitochondria of the TM4 Sertoli cell.

Furthermore, the amount of TM4 Sertoli cells per cm^3 of the testis ranges from 24-41 million in rabbits and humans, respectively[25]. A significant increase in the activity of mitochondrial dehydrogenase may result in increased cell proliferation, which could increase the numbers of TM4 Sertoli cells and the growing germs cells. However, the growing germ cells in the adluminal section of the seminiferous tubule rely on the TM4 Sertoli cells for nutritional and structural supports[25]. Thus, increased interaction between TM4 Sertoli cells and the germ cells provides the required support of the TM4 Sertoli cells to the germ cells and enhances spermatogenesis. Hence, assessment of the cell viability through MTT assay showed no cytotoxic effect of *G. brevis* twig extracts on TM4 Sertoli cells.

Adjacent TM4 Sertoli cells are able to adhere through tight junctions to produce blood-testis barrier which is responsible for closing the inter-cellular space in epithelial cellular sheets and producing two different fluid compartments with the establishment of apical and basolateral membrane territories. These barriers can be affected by external stimuli as they form a dynamic equilibrium and their integrity can be quantified *in vitro* directly by measurement of TEER[26]. Hence, measurement of the resistivity of Sertoli cells monolayers by means of TEER is an estimation of the function of the blood-testis barrier[27]. Treatment of TM4 Sertoli cells with methanolic extract of *G. brevis* twig has led to a substantial increase in the electrical resistance of the epithelial cells monolayer in a concentration-dependent manner at the concentrations throughout the timeframe (24-72 h). This increase in TEER values reveals the establishment and stiffness of the tight junctions[28]. Though additional research is essential to detect the exact mechanism by which *G. brevis* twig extract has significantly raised TEER, different theories can be proposed. In this study, *G. brevis* twig extract has shown a considerable increase in the proliferation of TM4 Sertoli cells which indicates more established tight junctions and hence subsequently high TEER records. The increase in mitochondrial dehydrogenase enzyme and TEER values can be attributed to the presence of phytochemicals and major compound found in *G. brevis* twig extract[4].

Mitochondrial dysfunction is associated with the reduction of mitochondrial potential which is associated with cell death and damage to cell viability. This work centered on the activity of *G. brevis* twig extract on mitochondrial membrane potential *via* TMRE dye. Our outcomes suggest that TM4 Sertoli cells exposed to *G. brevis* twig extracts displayed a reduced amount of cells with depolarization *via* a flow cytometer. Previous reports suggested that dysfunction of the mitochondria may include depolarization and release of pro-apoptotic features related to apoptosis through the intrinsic pathway[29]. Similar studies also have highlighted that the mechanism of apoptosis can be through the mitochondrial pathway both intrinsic and extrinsic[30]. These outcomes provide us an understanding of the new probable molecular mechanism of action of *G. brevis* twig extract and its potential role in managing the disorder. Therefore, one might speculate that *G. brevis* twig extract might be a potential chemotherapeutic agent that reduces mitochondrial depolarization in TM4 Sertoli cells with non-cytotoxic effects. This current outcome corroborates the studies of Olugbodi *et al*[24].

Apoptosis is an essential biological event where the cells induce the intrinsic death cascade when exposed to different damaging effects targeting at maintaining the internal environment balance[31]. In the testis, spontaneous apoptosis is accountable for the removal of 75% of the germ cells[31]. Treatment of TM4 Sertoli cells with *G. brevis* twig extract has displayed no key modifications in apoptotic and necrosis cells proportion in comparison with untreated cells following 24 and 72 h exposure. This is concurring with the non-cytotoxic and proliferative effect that has been achieved by the cell viability assay following 24 and 72 h treatment of TM4 Sertoli cells with concentrations of *G. brevis* twig extract. This finding also corroborates with our previous results on the effects of *G. brevis* twig extract using TM3 Leydig cells[24]. Furthermore, treatment of TM4 Sertoli cells with 0.2% DMSO (control) showed a substantial increase in apoptotic and necrotic cells proportion. Thus, it was reported that DMSO induced cell death in human leukemic HL-60 cell line[31]. It was, however, indicated that the main approach by which DMSO induced cell death is comparable to methanol[31]. Hence, *G. brevis* twig extract did not cause substantial changes in apoptotic cell proportion of TM4 Sertoli cells following 24 and 72 h exposure. This backs the anti-proliferative effect identified in the microphotographs and the non-cytotoxic effects on TM4 Sertoli cells.

Apoptosis is a biological process that performs a significant function in sustaining normal growth and tissue homeostasis. A normal cell undergoing cell death is categorized by structural alterations, which comprises cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation[32]. In the testis, 75% of the germ cells are decreased by spontaneous cell death[31]. Though, extreme or insufficient apoptosis of the testicular cells will lead to irregular spermatogenesis[32]. To maintain homeostasis in the adult male body, approximately 10 billion cells are generated every day to replace the failing cells as a result of cell death[33] with the number of cells increasing significantly with elevated cell death during normal growth, aging or diseases[34]. The results obtained from the TM4 Sertoli cells exposed to *G. brevis* twig extract showed mainly a normal appearance of the nucleus with no cell membrane

blebbing, cell shrinkage, chromatin condensation or damaged nuclei. Furthermore, *G. brevis* twig extract was able to penetrate into the nucleus of the TM4 Sertoli cells, this show from the blue color of the DAPI and it shows that the extracts can penetrate and have effects in the nucleus.

Inhibin is a secretory product of TM4 Sertoli cells, is a regulating hormone comprising of inhibin-α and inhibin-β[35]. Inhibin-β is generated by Sertoli cells that moderate follicle stimulating hormone through a feedback inhibition[36]. It is regarded as a marker in toxicological studies[37]. Increased inhibin-β levels result in increased production of inhibin-β. In this study, the levels of inhibin-β increased considerably following treatment of *G. brevis* twig extract. An increase in inhibin-β levels as a result of TM4 Sertoli cells following treatment of *G. brevis* twig extract may indicate the improved function of TM4 Sertoli cells.

Androgen binding protein is a product of Sertoli cells, which binds, transports, and defends androgen from breakdown, and also manages their bioavailability, thus facilitating the growth and development of spermatogenic cells. It is recognized as a biomarker of TM4 Sertoli cell function[38]. Excessive androgen binding protein upregulates the expression of aromatase in the germ cells leading to meiotic arrest from androgen deficiency[39]. In this study, *G. brevis* twig extract increased the mRNA levels of androgen binding protein at 10.0 and 100.0 μg/mL, which indicated that the modifications in TM4 Sertoli cell function were a result of *G. brevis* twig extract exposure. Furthermore, normal biosynthesis of androgen binding protein is closely associated with sustaining *in vivo* endocrine balance and function[40].

Stem cell factor also recognized as a kit ligand is generated by Sertoli cells[41] and are essential for fertility. In the testis, there is the reaction of stem cell factor with a c-kit receptor on several phases of spermatogenesis including spermatogonia and spermatids[42]. This stem cell factor/c-kit interaction occurs mostly during testicular development and it is important in spermatogonial adhesion, survival and proliferation[41,42]. In this study, *G. brevis* twig extract increased the mRNA levels of stem cell factor at 10.0 and 100.0 μg/mL, suggesting that *G. brevis* twig extract might perform a significant function during spermatogenesis and this outcome is in line with other reported study of stem cell factor[41]. Our previous study reveals that *G. brevis* twig extract contains coumaric, catechuic and ferulic acids, and these compounds have shown diverse pharmacotherapeutic effects[4]. We can suggest that the impact of *G. brevis* twig extract on TM4 Sertoli cells might be due to the actions of these compounds on the cells.

In conclusion, *G. brevis* twig extract may have beneficial effects on TM4 Sertoli cells by regulating spermatogenesis, providing morphological support and nutrition to growing cells, and regulating the movement to and fro of nutrients and hormones into the seminiferous tubules without cytotoxic effects on TM4 Sertoli cells lines.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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