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The effect of *Tulbaghia violacea* extracts on testosterone secretion by testicular cell cultures

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ABSTRACT

Aim of the study: This study aimed to determine the effect of *Tulbaghia violacea* Harv. on the male reproductive system *in vitro* by using testicular cell cultures. *Tulbaghia violacea* is a plant species indigenous to southern Africa and is used locally as a herbal remedy/medicine to treat several ailments.
Materials and methods: A 50% ethanol extract of *Tulbaghia violacea* was prepared. Three-month old male Balb/C mice were sacrificed and testicular cell cultures were prepared. Cells were then treated with varying concentrations of the *Tulbaghia violacea* ethanol extract (with/without Luteinizing hormone (LH)-treatment) and incubated for 4 h. Hormone production and cell viability were evaluated.
Results: Treatment of cells with *Tulbaghia violacea* (312.5–5000 μg ml^{-1}) significantly increased ($P < 0.05$) LH-induced testosterone production as compared to vehicle-treated control (DMSO) whereas cells without LH-treatment showed no significant change in testosterone concentrations. No significant effect on cell viability was observed at all concentrations tested.
Conclusions: The data presented shows that *Tulbaghia violacea* has androgenic properties. Further studies are warranted to determine and clarify the exact mechanisms involved.

1. Introduction

Tulbaghia violacea Harv. is a plant species belonging to the Alliaceae family, indigenous to South Africa and has been used as a herbal remedy/medicine to treat several ailments which include respiratory diseases (tuberculosis and asthma), oesophageal cancer, gastrointestinal problems as well as colds and fever (van Wyk et al., 1997). It is popularly known as wild garlic, society garlic or sweet garlic and the leaves have also been used to treat sinus headaches and as a deterrent for moles in gardens due to the strong garlic smell (Kubec et al., 2002). The Zulus, who refer to *Tulbaghia violacea* as isihaqa, have used this plant as an aphrodisiac medicine (Dyson, 1998) as well as a snake repellant (Kubec et al., 2002). Studies have shown, however, that excessive consumption of *Tulbaghia violacea* can have adverse effects which include gastroenteritis, abdominal pain and inflammation (van Wyk et al., 1997; van Wyk and Gericke, 2000).

Since *Tulbaghia violacea* has been reported to being used as an aphrodisiac medicine and as remedy for several ailments, scientific evidence is needed to determine and evaluate the potential androgenic activity of this plant. To our knowledge, no studies have been conducted to assess the effect of *Tulbaghia violacea* on the reproductive system. The aim of this pilot study is thus to determine the effect of *Tulbaghia violacea* on the male reproductive system *in vitro* by assessing testosterone and estradiol production, as well as cell viability.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals, reagents, solvents were purchased from Sigma (USA), Merck (Germany) and Roche Diagnostics (South Africa) and all other reagents were of analytical grade.

2.2. Animals

After obtaining approval from the institutional animal ethical committee, male Balb/C mice were used for this study. Mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa) and were pathogen free. The mice were then housed in a well-ventilated animal house with a light/dark cycle of 12:12. The mice had free access to normal drinking water and were fed standard mouse feed (Medical Research Council, Cape Town, South Africa).

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2.3. Cell culture

Three-month old mice were sacrificed by cervical dislocation. The testes were then removed aseptically, minced and then transferred to a tube (Greiner Bio-one) containing 10 ml serum-free medium (0.2% bovine serum albumin (BSA), 1% glutamax (Invitrogen), 1% Penicillin/Streptomycin/Fungizone mix (Sigma) and RPMI-1640 medium (Sigma)). Debris was allowed to collect at the bottom of the tube and thereafter, the supernatant (containing cells) was transferred to a new tube. Subsequent serum-free medium was added to the cells resulting in a final volume of 10 ml. The cells were then incubated at 37 °C with 5% CO_2 for 1 h. After incubation, the cells were centrifuged at 1000 × *g* for 10 min. The supernatant was then discarded and the cells were resuspended in 10 ml serum-free medium and incubated at 37 °C with 5% CO_2 for 30 min. The cells were centrifuged as before and the supernatant obtained was again discarded. The cell pellet was then resuspended in 10 ml serum-free medium to an approximate concentration of 3.4×10^6 cells/ml and then used for cell viability and hormone production determinations.

2.4. Preparation of Tulbaghia violacea ethanol extract

The voucher specimen of *Tulbaghia violacea* Harv. was identified by the University of the Western Cape Herbarium, Bellville, South Africa. Fresh plant organs (50 g) of *Tulbaghia violacea* (leaves and rhizomes) were homogenized in a Waring blender and extracted overnight in a sealed container at room temperature in ethanol (100 ml) to obtain a 50% (500 mg ml^{-1}) extract. The 50% ethanol extract was subsequently filtered through Whatman No. 4 qualitative filter paper to remove any remaining plant material and thereafter air-dried at room temperature. The extract was then reconstituted to 50% (w/v) in DMSO and stored at 4 °C until use. Dilutions of the extract were made in DMSO and these were used for subsequent assays.

2.5. Tulbaghia violacea and Luteinizing hormone (LH) treatment of cells

Cells were seeded in a 96-well tissue culture plates (Nunc, Serving Life Science, Denmark) at a volume of 50 μl per well. Varying concentrations of *Tulbaghia violacea* (5000, 2500, 1250, 625, 312.5, 156.25 and 0 μg ml^{-1}) were added to the cell cultures at a volume of 1 μl per well and the culture plates were then incubated at 37 °C with 5% CO_2 for 1 h. Cells that received dimethyl sulphoxide (DMSO) in the medium were used as a vehicle-treated control, throughout the study. The cells were thereafter incubated in the presence and absence of LH (10 mu ml^{-1}) (50 μl per well) at 37 °C with 5% CO_2 for 4 h.

2.6. Hormone production

After the 4 h incubation period, supernatant from LH-treated and non-treated cells were assayed for testosterone and estradiol concentrations using commercially available ELISA kits (DRG Instruments, GmbH, Germany) to assess the effect of *Tulbaghia violacea* on hormone production. The assays were performed as per manufacturer's instructions. The range of the testosterone and estradiol assays were between 0–16 ng ml^{-1} and 9.7–2000 pg ml^{-1}, respectively.

2.7. Cell viability

The effects of *Tulbaghia violacea* on cell viability were determined by XTT assay. In this assay, the reduction of the yellow tetrazolium salt (XTT) to an orange formazan product by viable cells, were measured. Cells were seeded in a 96-well tissue culture plate (Nunc, Apogent, Denmark) with varying concentrations of *Tulbaghia violacea* in the presence and absence of LH, as previously described. At 1 h incubation, XTT reagent mix (Roche Diagnostics GmbH, Germany) was added to the culture plate at a volume of 50 μl per well and incubated at 37 °C with 5% CO_2 for 4 h. Formazan formation was then spectrophotometrically quantified at 492 nm with a microtitre plate reader (Multiskan Ex, Thermo Electron Corporation).

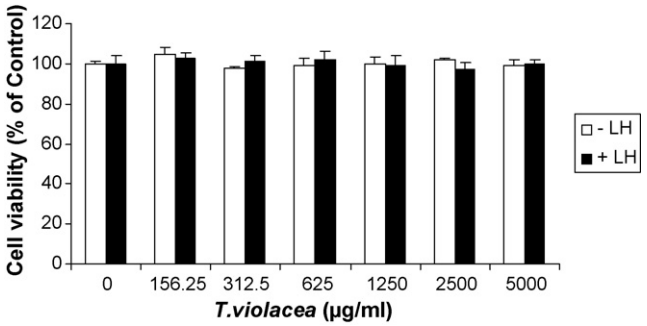

Fig. 1. Cell viability of testes cell suspension treated with varying concentrations of *Tulbaghia violacea* (with/without LH-treatment).

2.8. Statistical analysis

SigmaStat software (Systat Software Inc., USA) was used for statistical analysis. Each experiment was performed thrice in quadruplicate and data was statistically analysed via one-way ANOVA ($P < 0.001$) and regression analysis.

3. Results

3.1. Effects of Tulbaghia violacea on cell viability

Treatment of cells with varying concentrations of *Tulbaghia violacea* (with/without LH-treatment) had no significant effect on cell viability as compared to control (Fig. 1).

3.2. Hormone production

Tulbaghia violacea significantly increased ($P < 0.05$) LH-induced testosterone secretion at concentrations 312.5–5000 μg ml^{-1} as compared to control (Fig. 2). Cells incubated in the absence of LH secreted low levels of testosterone and showed no significant effect as compared to control (Fig. 2). In LH-stimulated cultures, treatment with *Tulbaghia violacea* resulted in a 30–72% increase in testosterone production as compared to control. Estradiol produc-

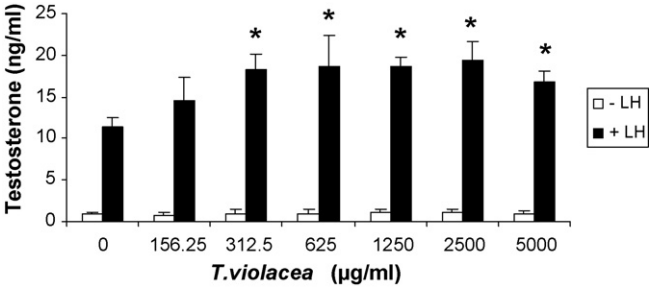

Fig. 2. Effect of *Tulbaghia violacea* on testosterone secretion (with/without LH-treatment). Cells were treated for 4 h and supernatants were screened thereafter for testosterone (*$P < 0.05$ relative to the control).

tion was undetectable at all concentrations tested (with/without LH-treatment) (data not shown).

4. Discussion

The present study investigated the effect of *Tulbaghia violacea* on the male reproductive system *in vitro* at the testicular level. To assess the potential androgenic or anti-androgenic effect of the plant extract on the reproductive system, cell viability and hormone production were investigated.

Normal functioning of the reproductive system is essential for normal sexual development, behaviour, spermatogenesis, etc. Under normal conditions, testosterone, the main sex hormone in males, is produced in the testes by the Leydig cells through steroidogenesis upon stimulation by Luteinizing hormone (LH) from the pituitary gland (Gail and Hedger, 1992; Kumar et al., 2008). Any variation in this biochemical pathway can result in an inhibition or stimulation of sex hormone synthesis, resulting in a hormonal imbalance which can cause adverse effects on the reproductive system (EPA, 2005).

A deficiency in testosterone production can have major adverse effects on reproductive health as well as other bodily functions and processes. Testosterone deficiency been associated with aging (Morley and Perry, 2000; El-Sakka and Hasobba, 2006) as well as with several diseases and disorders which include metabolic syndrome, hypogonadism, osteoporosis, obesity, diabetes mellitus type II, erectile dysfunction, Alzheimer's disease and cardiovascular disease (Jones, 2007; Schulman et al., 2009). Effects of low testosterone levels include decreased libido and low sperm count (Zitzmann, 2008), infertility, decreased muscle mass and strength, cognitive impairment and depression (Morley and Perry, 2000; Zitzmann and Nieschlag, 2000; Schulman et al., 2009). Normalization of testosterone levels is thus imperative to maintain and improve reproductive health and quality of life.

Testosterone replacement therapy has been opted as a treatment for several of the previously mentioned diseases, disorders and effects (Morley and Perry, 2003; Raynaud, 2009) and is available in oral, intramuscular, buccal, subdermal, and transdermal preparations (Schulman et al., 2009). There are several issues, however, concerning the above-mentioned available treatments such as high cost, difficulty of administration and side-effects (Bouloux, 2005; Srinivas-Shankar and Wu, 2005).

The present study shows that the ethanol extract of *Tulbaghia violacea* increased testosterone synthesis which indicates its androgenic activity. The increase in testosterone concentrations could possibly be due to an increase in steroidogenesis (increased enzymatic conversion of cholesterol to testosterone). Furthermore, the extract could be enhancing the action of LH or possibly increasing the response of Leydig cells to LH by increasing membrane-bound LH receptor expression. Unstimulated cells showed no significant effect in testosterone concentrations indicating that the extract does not stimulate testosterone production independently of LH. These findings suggest that *Tulbaghia violacea* may potentially be used as a supplement to stimulate testosterone production, thereby maintaining and improving reproductive health and quality of life.

5. Conclusion

The results obtained from this pilot *in vitro* study have demonstrated that the ethanol extract of *Tulbaghia violacea* has androgenic properties by significantly increasing LH-induced testosterone production in mouse testes with no significant change in cell viability. Further studies are warranted to determine and clarify the exact mechanisms involved.

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