

Original Research

The Effect of Aqueous *Lessertia frutescens* Extract on TM3 Leydig Cells Exposed to TNF- α *in vitro*

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Abstract

Background: Extractions of *Lessertia frutescens* (*Lf*) are shown to have immune modulation, anti-inflammatory and antioxidant properties. However, *Lf* is also cytotoxic, antiproliferative, and pro-apoptotic *in vitro*. Furthermore, *Lf* extractions may influence steroidogenesis. Nevertheless, the impact on Leydig cell function has not previously been investigated. As tumor necrosis factor-alpha (TNF- α) is known to cause Leydig cell dysfunction under inflammatory conditions, it is further proposed that *Lf* extracts may protect against the negative impact of TNF- α on Leydig cells. The aim of the study was to investigate the effect of an aqueous *Lessertia frutescens* extract (*LFE*) on Leydig cells exposed to TNF- α *in vitro*. **Methods:** Human chorionic gonadotrophin-stimulated TM3 Leydig cells were exposed for 24 h to (a) TNF- α (0.1, 1, 10, 100 ng/mL), (b) *LFE* (0.01, 0.1, 1, 10, 100 ng/mL), and (c) co-exposure to 10 ng/mL TNF- α and *LFE* (0.01, 0.1, 1, 10, 100 ng/mL). We analyzed cell viability, cytotoxicity, caspase 3/7 activation, testosterone concentration, and intracellular superoxide. **Results:** TNF- α exposure decreased cell viability, increased cytotoxicity, and caspase 3/7, with no significant effect on intracellular superoxide in TM3 Leydig cells. When *LFE* concentrations of 0.01–10 ng/mL were tested, we observed improved vitality and reduced levels of caspase 3/7. At 100 ng/mL, *LFE* decreased viability and increased cytotoxicity and caspase 3/7. However, *LFE* did not affect intracellular superoxide. Furthermore, *LFE* protected against 10 ng/mL TNF- α -induced cytotoxicity and apoptosis, except at the highest concentration. *LFE* alone and in co-culture with 10 ng/mL TNF- α increased testosterone at high concentrations. **Conclusions:** In our TM3 Leydig cell model, *LFE* protected against TNF- α -induced cytotoxicity and early apoptosis, except at the highest experimental concentrations, where it was cytotoxic. These effects were not mediated through a change in intracellular superoxide. Although further investigations are warranted, aqueous *LFE* may protect against TNF- α -induced Leydig cell dysfunction.

Keywords: *Lessertia frutescens*; Sutherlandia; tumor necrosis factor-alpha; inflammation; Leydig cells; apoptosis

1. Introduction

Cytokines influence the development and function of the adrenals, testes, and ovaries, and further modulate steroidogenesis [1,2]. Inflammatory cytokines are elevated in the male reproductive tract during both acute and chronic inflammatory diseases, contributing to the development of male hypogonadism [3–5]. Several cytokines, including the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF- α), are secreted locally by leukocytes (such as macrophages and lymphocytes) in the male reproductive tract [3]. Sertoli cells and Leydig cells also synthesize and secrete various cytokines, including TNF- α [4].

Originally discovered as a protein that mediates tumor necrosis with a role in cancer cachexia [6], TNF- α is now considered a pleiotropic cytokine with a central role in acute and chronic inflammatory responses [7,8]. In murine and human testes, TNF Receptor-1 (TNFR1) is the main TNF receptor expressed by Leydig cells, as well as in interstitial

macrophages and lymphocytes. TNF Receptor-2 (TNFR2) has been identified in rat Sertoli cells and human peritubular cells [3]. High levels of TNF- α inhibit Leydig cell steroidogenesis, resulting in reduced testosterone, impaired spermatogenesis, and reduced steroidogenic enzyme gene expression [9,10]. In healthy human males, acute administration of TNF- α transiently increases serum luteinizing hormone (LH) and decreases testosterone, with no change in follicle-stimulating hormone (FSH) and sex hormone binding globulin (SHBG) [11]. Yet, the mechanisms by which these changes occur remain unclear. However, the induction of oxidative stress and apoptosis may play a role as potential mechanisms for TNF- α induced toxic effects on Leydig cells [12].

Several clinically approved protein-based TNF- α antagonists are used in the treatment of inflammatory diseases, such as etanercept, infliximab, adalimumab, recombinant human TNF receptor, Fc-fusion protein, and golimumab [13]. Although effective, these drugs have a series



of systemic side effects as they have been shown to increase the risk of lymphoma and infections, cause cardiovascular and neurologic problems, and induce auto-antibodies and lupus-like syndromes [14–16]. Therefore, some focus has been redirected to herbal extracts containing different chemical classes (polyphenols, alkaloids, steroids, terpenoids, fatty acids, macrolides) which can act as TNF- α antagonists or potentially protect against mechanisms of TNF- α induced cytotoxicity [13,16,17]. Natural extracts can surpass synthetic compounds as they are more likely to be safer, less toxic, and cost-effective [17]. In male hypogonadism associated with inflammation, natural products and plant extracts that are immune regulating and act as antioxidants may be useful in Leydig cell dysfunction [18].

Lessertia frutescens (Linnaeus (L.) Goldblatt and Manning) (abbreviated as *Lf* in this manuscript), indigenous to Southern Africa, is traditionally considered an important medicinal herb [19,20]. Common local names for *Lf* are Sutherlandia or Cancer bush (English), Kankerbos (Afrikaans), Umnwele (isiXhosa), Insiswa (isiZulu), and Phetola (Sotho) [19]. Numerous constituents of *Lf* extractions from leaves have been identified, including free amino acids (asparagine, proline, and L-arginine), non-protein amino acids (L-canavanine and γ -aminobutyric acid), pinitol, cycloartane glycosides (sutherlandiosides A, B, C and D), flavonol glycosides (sutherlandins A, B, C and D), hexadecanoic acid, γ -sitosterol. Furthermore, various polysaccharides make up to 35% of the dry weight of the aqueous extract [21–24].

Lf is traditionally used for blood purification (bitter tonic), as an anti-stress remedy, and in the prevention and treatment of cancer [19,25,26]. Using *in vitro* and *in vivo* experimental models, extractions from *Lf* have been shown to be hypoglycaemic and antidiabetic [27–32], immune modulating [31,33–37], antioxidant [38–40], analgesic [31], anti-bacterial [38] and anti-human immunodeficiency virus [41]. *Lf* has also been classified as an adaptogen, promoting adaptability and resilience to environmental stressors [42] and promoting an anti-stress response [43–45]. Furthermore, *Lf* is antiproliferative and pro-apoptotic in cancer cell lines [21,46–53].

It is currently unknown what effect *Lf* has on Leydig cell function. It is further plausible that *Lf* may protect against inflammation-mediated Leydig cell dysfunction. However, no investigation has been done in this direction. As the impact of *Lf* on Leydig cell function and dysfunction remains unclear and poorly investigated, the aim of this study was to investigate the potential protective effect of *Lf* extraction on Leydig cells exposed to TNF- α *in vitro*.

2. Materials and Methods

2.1 Study Design

In this study, an *in vitro* human chorionic gonadotrophin (hCG)-stimulated TM3 Leydig cell culture

model was used [54]. TM3 cells are an immortalized Leydig progenitor cell line with epithelial morphology and adherence culture properties that are derived from 11–13 d mouse testes [55]. The TM3 Leydig cells for laboratory use were purchased without Mycoplasma contamination from the American Type Culture Collection (Manassas, VA, USA) under the catalog number CRL-1714 and were cultured according to supplier recommendations as described below. The cell model was exposed to three different conditions in 25 mIU/mL hCG-enriched cell culture medium for 24 h: (a) increasing concentrations of TNF- α (0.1, 1, 10, 100 ng/mL), (b) increasing concentrations of *Lessertia frutescens* extract (*LFE*) (0.01, 0.1, 1, 10, 100 ng/mL), and (c) co-exposure to 10 ng/mL TNF- α with increasing concentrations *LFE* (0.01, 0.1, 1, 10, 100 ng/mL). The study outcomes for analysis were cell viability, cytotoxicity, caspase 3/7 activation, testosterone concentration, and intracellular superoxide (Fig. 1). As an *in vitro* study, ethical clearance was not required. The study design is outlined in Fig. 1.

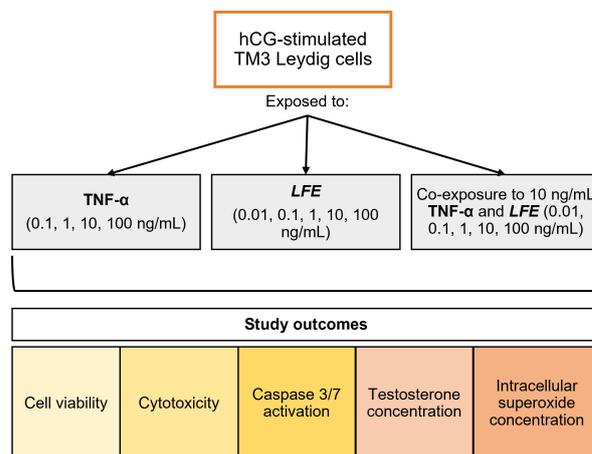


Fig. 1. Study design workflow. The figure illustrates the culture model, experimental conditions, and study outcomes analyzed. *LFE*, *Lessertia frutescens* extract; TNF- α : tumor necrosis factor- α .

2.2 Preparation of TNF- α

Recombinant TNF- α , expressed in *Escherichia coli*, was obtained as a lyophilized powder from Sigma-Aldrich (St Louis, Missouri, USA). In brief, according to the manufacturer’s instructions, the lyophilized powder was reconstituted in distilled and sterilized water to produce a 100 μ g/mL solution. Subsequently, this solution was diluted at a ratio of 1:10 with cell culture medium to produce a 10 μ g/mL stock solution, which was aliquoted and stored at -20 $^{\circ}$ C until preparation for experimental use. For the experiments, based on previous *in vitro* studies [54,56–58], concentrations of 0.1, 1, 10, and 100 ng/mL of TNF- α were prepared in 25 mIU/mL hCG-enriched medium.

2.3 Preparation of LFE

The plant name has been checked with <http://www.thaplantlist.org> (date of access December 13, 2022). *Lessertia frutescens* plants were grown by Parceval Pharmaceuticals (Wellington, South Africa) as a non-commercial product on the Ezibusweni farm without artificial fertilizers, pesticides, or herbicides and watered 3 times per week. Following harvest, a sample of the fresh plant material was authenticated as *Lf* by a botanist, Mr Frans Weitz (Curator of the Herbarium at the University of the Western Cape, Bellville, South Africa). The samples were subsequently archived at the Herbarium under the voucher number UWC6971. Following authentication, the harvested fresh leaves were dried in a ventilated oven for 3 days (Mommert, Schwabach, Germany) at 35 °C and milled into finely ground dried leaf material. Extraction of *Lf* leaves with hot water has higher total phenolic content, flavonoid content, and total reducing power ($\mu\text{g}/\text{mL}$ dried leaf) compared to cold water, methanol, ethanol, and acetone [40], with aqueous extractions previously reported [27,28,44,59]. For this study, *Lf* underwent extraction using a glass soxhlet extractor, where 10 g of dried milled leaves were extracted in 200 mL distilled water (dH_2O) at a regulated temperature of 70 °C for 4.5 h with a total of 4 refluxes. Following this, the extraction was filtered 3 times using a suction filter with Watman 1 filter paper. The resulting aqueous extract was freeze-dried over 3 days using a Mitsubishi electric GOT2000 freeze dryer (Chiyoda City, Tokyo, Japan) to yield the water-soluble extract. This process was repeated 3 times to produce an average 1.2% yield of a brown, powdery crude aqueous LFE. Subsequently, 10 mg of the LFE were dissolved in 1 mL medium (Dulbecco's Modified Eagle Medium F-12 - DMEM/F-12; Thermo Fisher, Waltham, MA, USA) to obtain a final concentration of 10 mg/mL, which was stored at -20 °C until use. For experimental exposure, concentrations of 0.01, 0.1, 1, 10, and 100 ng/mL of LFE were prepared in 25 mIU/mL hCG-enriched medium. As there have been no previous studies investigating LFE on Leydig cells, this range of concentrations was based on previous cell culture experiments using Chang liver (PC3) and human prostate cancer (LNCaP) cell lines [28,47].

2.4 TM3 Cells Experimental Exposure

TM3 Leydig cells were cultured using medium that consisted of DMEM/F-12 with 10% fetal bovine serum (Thermo Fisher) and 1% of 50 mg/mL penicillin-streptomycin (Sigma-Aldrich) [56] in 75 mL flasks. The cells were incubated in an atmosphere of 5% CO_2 at 37 °C under sterile conditions. Detachment of cells for experiments was done using 0.25% Trypsin/EDTA (Thermo Fisher) from 80–90% confluent 75 mL cell culture flasks.

For each experiment and control, the cell culture medium was supplemented with 25 mIU/mL hCG (Sigma-Aldrich) [54]. Following confluency and detachment, 4.5×10^3 TM3 Leydig cells were seeded with 300 μL hCG-

enriched medium per well in sterile 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and allowed to acclimatize and attach over 24 h. Following this, the medium was removed, and the cells were then cultured in 300 μL hCG-enriched medium with experimental exposures and co-exposure of LFE and TNF- α for a further 24 h. Working dilutions were prepared for experimental concentrations of TNF- α (0.1, 1, 10, 100 ng/mL) with hCG-enriched medium from stock solutions. Similarly, dilutions were prepared for experimental concentrations of LFE (0.01, 0.1, 1, 10, 100 ng/mL) with hCG-enriched medium from stock solutions. Based on previous studies [56,58] and the results of the experiments exposing TM3 cells to TNF- α , TM3 cells were then co-exposed to 10 ng/mL of TNF- α with the range of concentrations used for LFE (0.01, 0.1, 1, 10, 100 ng/mL). For all experiments, the control was TM3-cell exposure to hCG-enriched medium only. When cells were exposed to 10 ng/mL TNF- α and co-cultured with various LFE concentrations (co-culture experiments), these were further controlled with 10 ng/mL TNF- α only in hCG-enriched medium as a positive control. The experiments were all terminated after 24 h of exposure to the experimental conditions.

2.5 Assessment of Cell Viability, Cytotoxicity, and Caspase 3/7 Activation

Cell viability, cytotoxicity, and caspase 3/7 activation were determined using the ApoTox-Glo™ Triplex assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The ApoTox-Glo™ Triplex assays were done in white opaque bottom 96-well plates. Following cell incubation with TNF- α and LFE according to the study design, 20 μL of viability/cytotoxicity reagents (glycylphenylalanylaminofluorocoumarin, GF-AFC and bis-alanyl-alanyl-phenylalanyl-rhodamine 110, bis-AAF-R110 substrates, respectively) were added to the wells and incubated at 37 °C for 1 h. Fluorescence was measured at different wavelengths based on excitation (Ex) and emission (Em) spectra of GF-AFC (400 and 505 nm, respectively) and bis-AAF-R110 (485 and 520 nm, respectively). To determine caspase 3/7 activation, 100 μL of Caspase-Glo® 3/7 reagent was added to each well and incubated in the dark for 1 h. Fluorescence and luminescence were measured using the GloMax™ multi-scan plate reader (Promega Corporation, Madison, USA) at wavelength 580–640_{Ex}/525_{Em} nm. Data for cell viability, cytotoxicity, and caspase 3/7 was then converted into percentages compared to the control.

2.6 Assessment of Intracellular Superoxide

Intracellular superoxide was determined using the dihydroethidium (DHE) fluorescence stain (Thermo Fisher) in white opaque bottom 96-well plates. After the termination of the incubation, according to the study design, cells were washed with phosphate-buffered saline (PBS) (Oxoid,

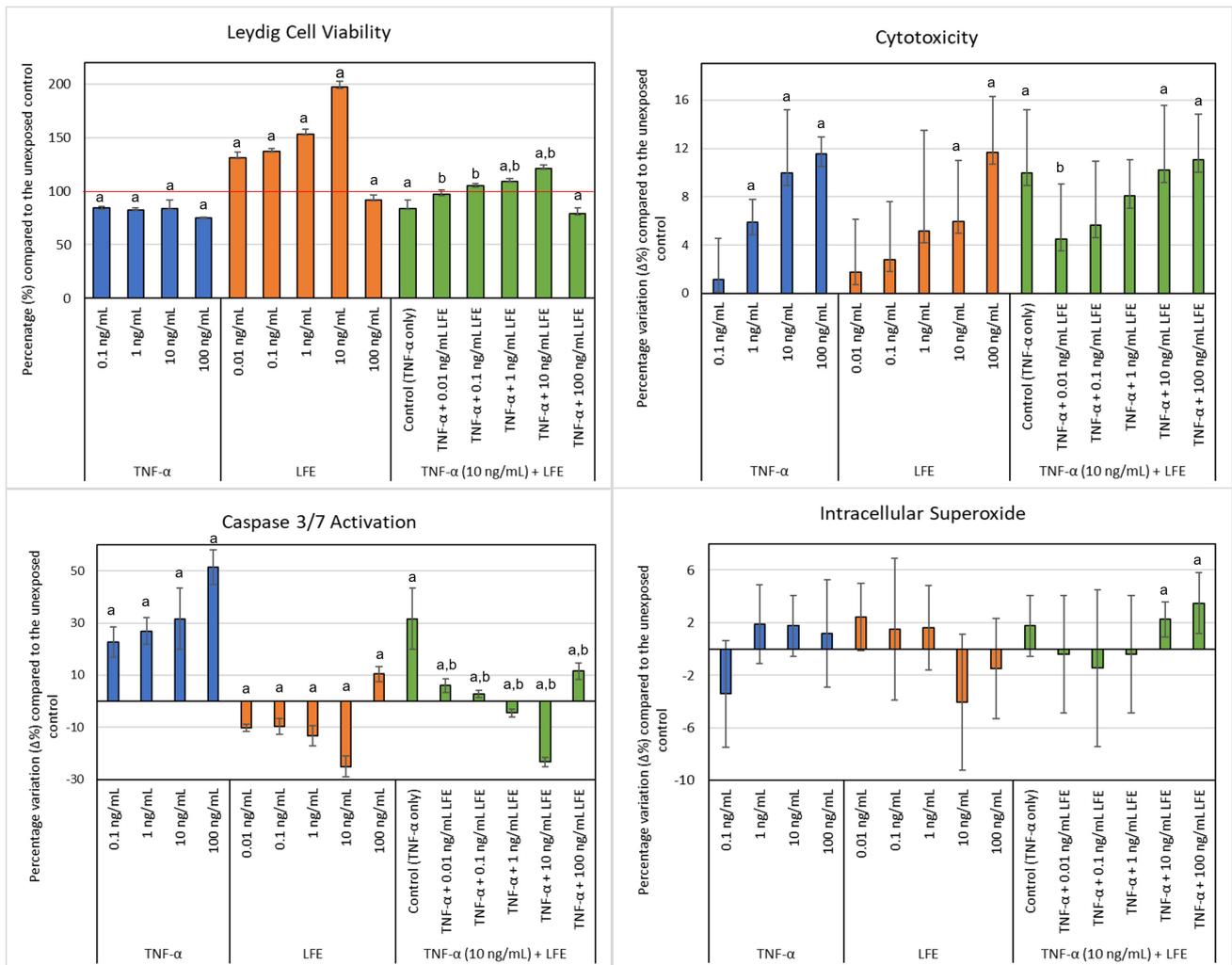


Fig. 2. TM3 Leydig cell viability, cytotoxicity levels, caspase 3/7 activation, and intracellular superoxide after exposure to various concentrations of TNF-α and LFE. Results for cell viability are reported as a percentage compared to the unexposed control cells (100%, indicated by the red line). Results for cytotoxicity levels, caspase 3/7 activation, and intracellular superoxide are reported as percentage variation (Δ%) compared to the unexposed control cells. On the bars, the following letters indicate (a) $p < 0.05$ compared to cells exposed only to hCG media and (b) $p < 0.05$ compared to cells exposed to hCG media with 10 ng/mL TNF-α. The error bars indicate the standard deviation.

Basingstoke, Hampshire, UK). Subsequently, the cells were incubated with 10 μM DHE fluorescence stain in a cell culture medium with 5% CO₂ at 37 °C for 1 h. Following the incubation period, the fluorescence was measured at excitation and emission wavelengths of 518 and 606 nm, respectively, using a GloMax Multi Detection System plate reader (Promega Corporation). Data for superoxide was then converted into percentages compared to the control.

2.7 Assessment of Testosterone Concentration

The testosterone concentration in the culture medium was determined using the Testosterone ELISA kit (DRG International, Springfield, NJ, USA) according to the manufacturer's instructions. Following the termination of each experiment, 300 μL of the medium was pipetted into a centrifuge tube and centrifuged at 1500 rpm for 10 min-

utes to remove excess cells. This supernatant was immediately stored at -20 °C until assayed. In brief, 25 μL of cell supernatant and 200 μL enzyme conjugate were mixed in the antibody-coated microplate and incubated for 1 h at room temperature, along with the standards provided in the kit. The mixture was then removed, and the wells were washed with 400 μL of diluted wash solution 3 times. Subsequently, 200 μL of the substrate solution was added to each well for 5 minutes at room temperature, and the process terminated by adding 100 μL of stop solution. The absorbance was then determined at 450 nm with an ELISA plate reader (Labtech, Heathfield, East Sussex, UK). The final concentration was quantified based on the standard curve determined by the supplied standard concentrations for the assay and represented as ng/mL. Each set of experiments was done with the corresponding control group. As

these experiments were done over time, with minor different subsets of cell line passages from frozen storage, absolute values of controls can vary, resulting in a minor variation in the 3 control groups.

2.8 Statistical Analysis

In our study, we included 3 biological replicates, which were analyzed in triplicates for a total of 9 measurements in each experiment. The analysis was conducted by using MedCalc® statistical software (version 20.109, Ostend, Belgium). To analyze whether or not the data was normally distributed, the Shapiro-Wilk test was performed, followed by repeated measures ANOVA or Friedman test, depending on data distribution. Data was expressed as mean \pm standard deviation (SD) or percentage (%), and a p -value of <0.05 was considered statistically significant.

3. Results

3.1 Cell Viability

A decrease in cell viability was observed after exposing the TM3 cells to increasing concentrations of TNF- α ($p < 0.00001$) compared to unexposed control cells (hCG media only). Cell viability was increased in cells exposed to concentrations of 0.01–10 ng/mL of *LFE* extract compared to unexposed control cells ($p < 0.0001$). However, at the highest concentration *LFE* (100 ng/mL), viability significantly decreased ($p = 0.02$) compared to unexposed control cells. This is summarised in Fig. 2 and **Supplementary Table 1**. When TM3 cells were co-exposed to TNF- α (10 ng/mL) and various concentrations of *LFE* for 24 h, a significant increase in cell viability was observed for concentrations of *LFE* between 0.01–10 ng/mL ($p < 0.0001$) compared to TNF- α (10 ng/mL) control cells. However, viability significantly decreased ($p < 0.0001$) at the highest *LFE* concentration (100 ng/mL) compared to unexposed control cells. There was also a significant decrease in vitality for cells exposed to TNF- α only compared to unexposed control cells ($p < 0.0001$) (Fig. 2, **Supplementary Table 1**).

3.2 Cytotoxicity

Cytotoxicity did not significantly change with exposure to 0.1 ng/mL TNF- α ($p = 0.4198$). However, it significantly increased after cell exposure to higher concentrations of 1 ng/mL, 10 ng/mL, and 100 ng/mL TNF- α ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively) compared to unexposed control cells. Exposure to *LFE* between 0.01 and 1 ng/mL did not significantly increase cytotoxicity. However, cells exposed to 10 and 100 ng/mL *LFE* showed significantly higher cytotoxicity when compared to unexposed control cells ($p = 0.0027$ and $p = 0.0005$, respectively) (Fig. 2, **Supplementary Table 1**). A significant decrease in cytotoxicity was observed for 0.01 ng/mL *LFE* concentration compared to TNF- α (10 ng/mL) control cells ($p = 0.0140$). On the contrary, the level of cytotoxicity significantly increased after cell co-exposure to TNF- α and

high concentrations of 10 and 100 ng/mL *LFE* ($p = 0.0004$ and $p < 0.0001$, respectively) when compared to unexposed control cells. There was also a significant increase in cytotoxicity for cells exposed to TNF- α only compared to unexposed control cells ($p < 0.0001$) (Fig. 2, **Supplementary Table 1**).

3.3 Early Apoptosis Measured by Caspase 3/7 Activation

Exposure to TNF- α significantly increased early apoptosis levels measured by caspase 3/7 activation for all concentrations ($p < 0.0001$) compared to unexposed control cells. Exposure to *LFE* resulted in a significant reduction of caspase 3/7 activation ($p < 0.001$) for 0.01 ng/mL ($p = 0.0005$), 0.1 ng/mL ($p = 0.0027$), 1 ng/mL ($p = 0.0006$), 10 ng/mL ($p < 0.0001$), with a significant increase for caspase 3/7 activation at 100 ng/mL ($p = 0.0015$) *LFE*, when compared to unexposed control cells (Fig. 2, **Supplementary Table 1**). There was a significant increase in caspase 3/7 activation after co-exposure to TNF- α (10 ng/mL) for the lowest (0.01 ng/mL and 0.1 ng/mL) and highest (100 ng/mL) *LFE* concentrations ($p = 0.0001$, $p = 0.0049$, and $p < 0.0001$, respectively) compared to unexposed control cells. Conversely, caspase 3/7 activation significantly decreased after co-exposure to TNF- α (10 ng/mL) with 1 and 10 ng/mL *LFE* ($p = 0.0001$ and $p < 0.0001$, respectively) compared to unexposed control cells. When compared to TNF- α (10 ng/mL) control cells, there was a significant decrease in caspase 3/7 activation for concentrations of *LFE* (0.01, 0.1, 1, 10, and 100 ng/mL) ($p < 0.0001$, $p < 0.0001$, $p < 0.001$, $p < 0.0001$, $p < 0.0001$, respectively) (Fig. 2, **Supplementary Table 1**). There was also a significant increase in caspase 3/7 for cells exposed to TNF- α only compared to unexposed control cells ($p < 0.0001$).

3.4 Intracellular Superoxide

Intracellular superoxide staining did not significantly change after exposure to TNF- α compared to unexposed control cells. The DHE stain did not result in any change regarding intracellular superoxide after exposing cells to different concentrations of *LFE* compared to unexposed control cells. Similarly, after co-exposure to TNF- α (10 ng/mL) and *LFE*, intracellular superoxide showed no significant change compared to TNF- α (10 ng/mL) control cells (Fig. 2, **Supplementary Table 1**). However, a significant increase in intracellular superoxide concentration was observed after co-exposing cells to TNF- α (10 ng/mL) and 10–100 ng/mL *LFE* ($p < 0.0001$ and $p < 0.0001$, respectively) compared to unexposed control cells.

3.5 Testosterone

Testosterone significantly decreased after exposing cells to the lowest concentration of TNF- α (0.1 ng/mL, $p = 0.0165$) compared to unexposed control cells. A non-significant decrease in testosterone was observed for increasing concentrations of TNF- α (1, 10, and 100 ng/mL).

Table 1. Testosterone concentration after exposure to various concentrations of TNF- α and LFE.

Exposure	Tested groups	Testosterone (Mean \pm SD)
TNF- α	Control (hCG media only)	0.29 \pm 0.03
	0.1 ng/mL	0.23 \pm 0.01 ^a
	1 ng/mL	0.25 \pm 0.02
	10 ng/mL	0.25 \pm 0.04
	100 ng/mL	0.24 \pm 0.02
LFE	Control (hCG media only)	0.24 \pm 0.02
	0.01 ng/mL	0.22 \pm 0.03
	0.1 ng/mL	0.20 \pm 0.02
	1 ng/mL	0.22 \pm 0.02
	10 ng/mL	0.22 \pm 0.01
TNF- α (10 ng/mL) + LFE	Control (hCG media + TNF- α)	0.26 \pm 0.02
	TNF- α + 0.01 ng/mL LFE	0.23 \pm 0.003 ^b
	TNF- α + 0.1 ng/mL LFE	0.26 \pm 0.01
	TNF- α + 1 ng/mL LFE	0.29 \pm 0.05 ^b
	TNF- α + 10 ng/mL LFE	0.27 \pm 0.02
	TNF- α + 100 ng/mL LFE	0.39 \pm 0.04 ^b

Results are reported as ng/mL. Mean \pm SD values are reported. ^a $p < 0.05$ compared to cells exposed to hCG media only; ^b $p < 0.05$ compared to cells exposed to 10 ng/mL TNF- α .

Testosterone did not change after exposing cells individually to LFE, except for the highest LFE concentration, showing a significant increase in testosterone compared to unexposed control cells ($p = 0.0072$) (Table 1). Following TM3 co-exposure to TNF- α (10 ng/mL) and 0.01 ng/mL of LFE, testosterone was significantly decreased ($p < 0.05$) compared to TNF- α control cells. However, testosterone was significantly increased ($p < 0.05$) with LFE 1 ng/mL and 100 ng/mL, and non-significantly increased for LFE 10 ng/mL, compared to TNF- α (10 ng/mL) control cells (Table 1).

4. Discussion

Since *Lf* is reported to have modulating effects *in vivo* and *in vitro* on immune function [31,33–37], oxidative stress [38–40], steroid hormone synthesis [43–45], and apoptosis [21,46–53], we investigated the effect of aqueous LFE in protecting Leydig cells against the damage induced by TNF- α . The results of this study found that TNF- α dose-dependently decreased cell viability across all concentrations tested. This is consistent with a previous study showing that TM3 Leydig cell viability was significantly reduced *in vitro* over 48 h at similar TNF- α concentrations of 0.1, 1, 10, and 100 ng/mL, although the effect on viability was much more dramatic at 10 and 100 ng/mL compared to the results of this study [54].

The effects of LFE on Leydig cell viability and cytotoxicity have not been previously reported. This study found that LFE increased cell viability at the lower concentrations but decreased viability at the highest one, with a corresponding increase in cytotoxicity. This suggests that the LFE may stimulate Leydig cells at lower concentrations

but exerts a cytotoxic effect at higher concentrations used in this study. There are only a few studies on cell viability for LFE *in vitro*, all of which use higher LFE concentrations compared to this study. SH-SY5Y neuroblastoma cells treated with 20–60 μ g *Lf* for 24 h showed increased cell viability, contrary to reduced viability at a much lower concentration of 100 ng/mL LFE in our study [60]. However, the extraction method is described only as 1 Kg of dried leaves in an unknown volume of boiling water before freeze-drying, and the dosage is provided as an absolute number (20–60 μ g) only [60]. In cultured renal LLC-PK1 and MDBK epithelial cells, exposure to an aqueous extraction over 48 h resulted in no significant change in cell viability at concentrations of 0.3–6 mg/mL compared to the reduced viability for LFE at 100 ng/mL, although there was a significant reduction at 12–24 mg/mL [61]. The variation in differences in viability at different dosages across these studies is, however, not clear and may be a response to different cell lines used. This different response of cell lines is further reported, where exposure of normal human lymphocytes to 2.5 mg/mL of ethanolic and aqueous LFE showed an increase in cytotoxicity and decrease in cell viability after 24 h [62], and no effect was observed on LLC-PK1 and MDBK cells at similar dosages [61].

LFE cytotoxicity is more widely reported in cancer cell lines. In prostate cancer *in vitro* and *in vivo*, methanolic LFE inhibits cell proliferation [47], similar to what was observed for ethanolic LFE in breast carcinoma and leukemia cells *in vitro* [21]. MCF-7 breast adenocarcinoma cells have been shown to be more susceptible to cytotoxic effects of 1 mg/mL aqueous LFE (48 h exposure) than non-tumorigenic MCF-12A cells [46]. Similarly, at 1.5 mg/mL, antiprolif-

erative effects and morphological signs of apoptosis were observed in MCF-7 cells after 24 h of exposure [48]. Furthermore, *Lf* has been shown to cause cytotoxicity in the *Salmonella typhimurium* strain TA97a, even if this was not evident in strains TA98, TA100, and TA102 [63]. Although our results showed reduced viability and increased toxicity at 100 ng/mL, hot water extracts of *Lf* leaves did not affect cell viability in Chinese Hamster Ovary (CHO) or Lung Carcinoma Epithelial (A549) cells exposed for 24 h at *Lf* concentrations up to 500 µg/mL, even if reduced cell viability in HepaRG cells was apparent at concentrations higher than 100 µg/mL [40]. However, it is difficult to compare the results of these different *LFE* and concentrations on different physiological and pathological cell lines that are currently available in the literature.

Our results suggest that *LFE* protects cells against TNF- α induced cytotoxicity at lower concentrations, but it may be cytotoxic as well at higher concentrations. Furthermore, the results of this study show that although cytotoxicity was increased for TNF- α concentration of 10 ng/mL, this was not observed for the lower concentrations of *LFE* when co-cultured with TNF- α , thus supporting the protective effect of lower *LFE* concentrations on Leydig cell toxicity. In a study using 1-methyl-4-phenylpyridinium (MPP+) to reduce viability in SH-SY5Y neuroblastoma cells, pre-treatment with *LFE* (20 µg) prevented this decrease [60]. Similarly, *LFE* concentrations between 10 µg/mL and 500 µg/mL protected against reduced cell viability induced by 50 µM t-BHP over a period of 24 h in CHO, A549, and HepaRG human liver cells, which was not apparent for 1000 µg/mL *LFE* [40].

TNF- α is known to regulate apoptosis in many cell types through TNFR1 [9]. Following receptor binding, intracellular mediators of TNF- α -induced apoptosis include activation of caspase-8, Bid (a Bcl-2 family protein), Bax-Bak, and cytochrome c, which results in a protein complex consisting of apoptotic protease activating factor-1, cytochrome c, and caspase-9, that then activates caspase-3 as a major execution caspase that initiates cell death [9]. With exposure to toxicants, the nuclear factor of activated T-cells promotes FasL transcription in Leydig cells [64]. Although numerous toxins may induce oxidative stress and apoptosis in Leydig cells *in vitro*, including di(2-ethylhexyl)phthalate [65], hydrogen peroxide [66–69], triptolide [70], it is not known whether TNF- α can induce Leydig cell apoptosis. The results of this study found that TNF- α increases caspase 3/7 activation across all concentrations of TNF- α tested. In similar studies where Leydig cells were exposed to toxins, caspase-3 activation was required for ethane dimethanesulfonate-induced apoptosis [71]. Corticosterone also induced apoptosis in isolated rat Leydig cells [72], mediated through activation of Fas/FasL and caspase-3, alongside reduced mitochondrial membrane potential and increased reactive oxygen species (ROS) generation [73]. Di(2-ethylhexyl)phthalate induced oxidative

stress and apoptosis in TM3 Leydig cells, thus increasing caspase-3 and -9 alongside Bax/Bcl2 ratio [65]. Further, hydrogen peroxide-induced oxidative stress in TM3 Leydig cells via increased ROS, Bax, and caspase-3 and reduced Bcl-2, PI3K, and p-AKT [68]. Similarly, activation of caspase-3 in Leydig cell apoptosis is reported *in vitro* for lead acetate [74] and alcohol [75].

Previously, it has been shown that high concentrations of *LFE* can be toxic. In cultured renal LLC-PK1 and MDBK epithelial cells, exposure for 48 h to an aqueous *LFE* significantly increased caspase-3 and -7 activity [61]. In human lymphocytes, 24 h exposure to 2.5 mg/mL of ethanolic and aqueous *LFE* each induced necrosis, reduced ATP, increased DNA fragmentation, and inhibited caspase 3/7 [62]. However, the apoptotic effect has been more widely reported in cancer cell lines. *LFE* (2.5 and 5 mg/mL) induced apoptosis in an oesophageal cancer cell line, with increased Annexin V/propidium iodide and caspase-3 and -7 [50]. In SiHa cervix carcinoma cells, a methanol *LFE* (50 µg/mL–200 µg/mL) reduced cell proliferation and increased caspase 3/7 activity after exposure for 4–24 h [76]. Ethanolic *LFE* extracts reduced proliferation and induced apoptosis in CaCo2 colon cancer cells, with down-regulation of PI3-K and Akt phosphorylation pathways and decreased forkhead transcription factor (FKHR) phosphorylation [53]. However, this increase in caspase 3/7 with *LFE* exposure is not always consistent. Ethanolic *LFE* (0.15–2.5 mg/mL) inducing apoptosis at higher dosages in various melanoma cell lines showed no activation of caspase 3/7, -8, and -9 over a period of 24 h, although this increased after 48 and 72 h of exposure [52]. However, the use of a pan-caspase inhibitor suggested that apoptosis may also occur independently of caspase activation with *LFE* exposure [52].

In our study, it appears that *LFE* is protective against TNF- α induced early apoptosis in Leydig cells. In SH-SY5Y neuroblastoma cells exposed to MPP+, pre-treatment with *LFE* reverted an increase in caspase 3/7 and -9 activity [60]. Additionally, different herbal extracts have been shown to improve toxin-induced apoptosis in Leydig cells, including taurine [65], astaxanthin [66], melatonin [77], resveratrol [67], rutin [68], quercetin [70] and extracts from *Morinda officinalis* [69].

The apoptotic effect of TNF- α on Leydig cells can also be mediated by intracellular ROS production, predominantly from the mitochondria. ROS may promote cytochrome-C release, leading to caspase-3 cleavage and apoptosis [9]. However, this was not observed in our study. Based on our results, it appears that the protective effect of *LFE* on Leydig cell cytotoxicity and early apoptosis may not be mediated through antioxidant inhibition of superoxide over a period of 24 h. *LFE* has, however, been shown to both reduce and increase cellular ROS in different experimental models. This includes protection against toxin-induced ROS production in CHO, A549, and HepaRG cells

[40], primary rat cortical neurons and microglial cells [2], neutrophils [39], and macrophages [21,37] and neuroblastoma cells [60]. However, ROS has been increased by *LFE* in neuroblastoma cells [51] and macrophages [36].

TNF- α has been suggested to negatively affect the hypothalamus-pituitary-testis (HPT) axis, where *in vivo* (including humans) administration is associated with a decrease in serum testosterone [1,11]. However, although the results of this study found that TNF- α decreased testosterone across all concentrations, this was significant only for 0.1 ng/mL TNF- α . Testosterone and progesterone synthesis were previously decreased in TM3 Leydig cells in a dose-dependent manner after incubating for 48 h with 0.1–100 ng/mL TNF- α , suggesting a downregulation of steroidogenesis cascades [54]. However, this is not always consistent as it has also been reported that TNF- α increases testosterone concentrations in adult rat Leydig cell cultures over periods between 7 and 72 h [78]. Although the mechanism for the downregulation of testosterone by TNF- α appears to be through the downregulation of steroidogenic enzymes, it still remains unclear. In rats, intratesticular injection of TNF- α inhibited basal or hCG-stimulated steroidogenesis, mediated through a downregulation of steroidogenic acute regulatory (StAR) protein [79]. This mechanism is supported in cultured porcine Leydig cells, where TNF- α 20 ng/mL over a period of 72 h inhibited LH/hCG-steroidogenesis through decreased StAR [80]. The same concentration tested in this study for co-exposure with *LFE* concentrations (TNF- α 10 ng/mL) was previously shown to upregulate Leydig cell IL-6 production and nitric oxide production, upregulate COX-2 mRNA expression, and downregulate the expression of Sirt1 mRNA (Sirt1 is known to promote steroidogenesis through upregulation of SF-1) [56]. Similarly, in R2C Leydig cells *in vitro*, this same concentration of 10 ng/mL TNF- α over 12 h downregulated steroidogenesis through activation of NF- κ B and subsequent inhibition of Nur77 and SF-1 [58]. In mouse Leydig cells, TNF- α at concentrations of 0.1 and 10 ng/mL inhibited cytochrome P450 side chain cleavage (CYP11A1) and 17 α -hydroxylase/17,20 lyase (CYP17A1) mRNA [57] and downregulated 3 β -hydroxysteroid dehydrogenase (3 β -HSD) expression [81]. LC-540 Leydig cells treated with TNF- α (10 ng/mL) for 12 h, 24 h, and 48 h showed suppressed histone H3 acetylation and methylation, with increased total histone deacetylases activity and upregulation of HDAC7 [82]. In a similar model, TNF- α upregulated DAX-1 mRNA and protein through the JNK/ERK MAP kinase pathway [83].

There are no previous studies in the literature investigating the effects of *LFE* on testosterone synthesis in Leydig cells. However, our results showed an increase in testosterone at high concentrations of aqueous *LFE* (4 mg/mL) orally administered in healthy Wistar rats significantly increased corticosterone, with no effect on testosterone after 28 days [44]. Studies in adrenal

steroidogenesis, however, suggested mechanisms for modulating corticosterone and potentially testosterone in these cells. *LFE* and sutherlandioside B (10 and 30 μ M) inhibited steroidogenic enzymes and modulated adrenal hormone biosynthesis in COS-1 and adrenal H295R cells [45]. *LFE* reduced total steroid production, including cortisol, deoxycortisol, and mineralocorticoid, and inhibited progesterone conversion by CYP17A1 and cytochrome P450 21 α -hydroxylase (CYP21A2). Sutherlandioside B decreased cortisol and androgen precursors, inhibiting CYP17A1 and 3 β -HSD but not CYP21A2 [45]. In ovine adrenocortical microsomes and mitochondria, aqueous extracts of *LFE* inhibited substrate binding to cytochrome P450 21-hydroxylase (CYP21) and cytochrome P450 11 β -hydroxylase (CYP11B1), limiting the conversion of progesterone and pregnenolone [43]. Furthermore, chloroform and methanol *LFE* extractions showed inhibitory effects on deoxycorticosterone binding to CYP11B1 and on progesterone binding to CYP21 [43]. However, it is unclear how *LFE* may have increased testosterone production in the TM3 Leydig cells. We could speculate that this effect may be due to the short (24 h) exposure in the experiments and other unknown effects on testosterone metabolizing enzymes, including 5 α -reductase and aromatase.

According to our results, *LFE* may protect against a decline in testosterone at higher concentrations. However, there are very few studies in the literature investigating the effect of *LFE* on testosterone production. There was no change in testosterone concentration in rats undergoing stress immobilization, although there was an increase in corticosterone in the stressed rats. The administration of aqueous *LFE* (4 mg/mL) had no significant effect on testosterone concentrations but did reduce corticosterone levels significantly [44]. Although animal studies suggest *LFE* may inhibit progesterone synthesis from pregnenolone through down-regulated CYP17A1 in adrenal cells [45], the effects on testosterone synthesis in Leydig cells may not be affected. Further investigations into the effect of *LFE* on testosterone synthesis within the broader context of steroidogenesis in different cell types are required.

Due to the exploratory design of the study, there was a limitation in the number of variables investigated. Only one concentration of TNF- α was selected for co-exposure experiments with *LFE*, and the experiments were limited to 24 h. The interpretation of the results within the existing literature should be done with caution, as there are no similar studies available. Furthermore, the current literature on both TNF- α and *LFE* is relatively poor and varies widely in experimental models, study design, and outcomes. There is also a lack of information about which specific compounds from *LFE* might play a more critical role in the regulation of Leydig cell function. Several phytochemicals have been identified in *LFE*, including amino acids (alanine, asparagine, proline, gamma amino butyric acid), flavonoids, pinitol, and triterpenoids, whose anti-

cancer, antiproliferative, antidiabetic, and antioxidant activities have been demonstrated in different models [84]. Despite the above-mentioned limitations, our study represents the first attempt to investigate the *LFE* potential of protecting against inflammation-mediated Leydig cell dysfunction, which represents one of the possible contributing factors to male infertility. In fact, considering the physiological role of the androgens, Leydig cell dysfunction may result in inadequate masculinization and spermatogenic failure [85]. This is also related to older men, where increased inflammation and androgen decline are observed [86]. Hence, it is important to investigate the possible use of natural extracts, which are more likely to be safer, less toxic, and cost-effective than synthetic compounds, through their action as immune regulators and antioxidants. In the future, it would be interesting to further investigate our findings through comparison with an additional treatment group consisting of TNF- α inhibitors, which are currently used in clinical practice, and perform immunocytochemistry experiments to further investigate Leydig cell-specific markers and observe their changes under experimental conditions.

5. Conclusions

This study reports that TNF- α decreases Leydig cell viability and increases cytotoxicity with early apoptosis and downregulation of testosterone. *LFE* protected against TNF- α -induced cytotoxicity and apoptosis, except at the highest experimental concentrations, where it was cytotoxic. Although TNF- α did not significantly decrease testosterone in most concentrations, co-culture with *LFE* significantly increased testosterone at higher concentrations. These effects were not mediated through a change in intracellular superoxide. Therefore, our results suggest that aqueous *LFE* may be useful in the protection of inflammation-induced Leydig cell dysfunction demonstrated by increased cytotoxicity and apoptosis. Further studies are recommended to understand the most optimal *LFE* concentration and the role of specific *LFE* isolates that may modulate Leydig cell function.

Abbreviations

Lf, *Lessertia frutescens*; *LFE*, *Lessertia frutescens* extract; TNF- α , tumour necrosis factor-alpha; hCG, human chorionic gonadotrophin.

Availability of Data and Materials

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

Author Contributions

All authors meet the requirements of ICMJE for authorship. KL designed the research study. KL, RH, RF, and CZB performed the research. RF and CZB analyzed

the data. KL, RF, and CZB wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

As an *in vitro* study, ethical clearance was not required and informed consent is not applicable.

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Conflict of Interest

The authors declare no conflict of interest. The National Research Foundation (NRF) did not influence the collection, analysis, and interpretation of data. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2809213>.

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