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Original Article

The toxicogenic effect of *Terminalia phanerophlebia* Engl. & Diels leaf extract on oxidative stress parameters in an *in vitro* Hek293 model

[Efecto toxicogénico de extracto de hoja de *Terminalia phanerophlebia* Engl. & Diels sobre parámetros de estrés oxidativo en un modelo *in vitro* con Hek293]

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Abstract

Context: Medicinal plants are a highly sought-after alternative to current pharmaceutical drugs because they can be locally cultivated, inexpensive and possess minimal adverse effects. Given that *Terminalia phanerophlebia* (*TP*) possesses many useful properties and plays a role in modulating lethal diseases, the cytotoxic effect should be evaluated before its application for therapeutic use.

Aims: To investigate the oxidative effect and molecular mechanisms of *TP* on human embryonic kidney (HEK293) cells.

Methods: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and adenosine triphosphate (ATP) assays were used to determine the cell viability whilst the thiobarbituric acid reactive species (TBARS) assay was used to detect lipid peroxidation. Endogenous antioxidants, catalase, superoxide dismutase, glutathione peroxidase, heat shock protein 70 and nuclear factor erythroid 2-related factor 2 (Nrf2), were used as oxidative stress markers and were detected via western blotting.

Results: A decrease in cell viability with an IC_{50} of 1.36 mg/mL and ATP were noted. The concentration of malondialdehyde (MDA) increased significantly ($p<0.005$). Superoxide dismutase, Nrf2 and heat shock protein concentrations were increased. However, glutathione, glutathione peroxidase and catalase were depleted.

Conclusions: The results obtained suggest that *Terminalia phanerophlebia* extract is toxicogenic and induced oxidative stress in HEK293 cells.

Keywords: antioxidants; chronic kidney diseases; cytotoxicity; Hek293 cells; lipid peroxidation; oxidative stress; *Terminalia phanerophlebia*.

Resumen

Contexto: Las plantas medicinales son una alternativa muy buscada a los medicamentos farmacéuticos actuales porque pueden cultivarse localmente, son económicas y tienen efectos adversos mínimos. Dado que *Terminalia phanerophlebia* (*TP*) posee muchas propiedades útiles y juega un papel en la modulación de enfermedades letales, el efecto citotóxico debe evaluarse antes de su aplicación para uso terapéutico.

Objetivos: Investigar el efecto oxidativo y los mecanismos moleculares de *TP* en células de riñón embrionario humano (HEK293).

Métodos: Se utilizaron ensayos de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) y trifosfato de adenosina (ATP) para determinar la viabilidad celular mientras que el ensayo de especies reactivas con ácido tiobarbitúrico (TBARS) utilizado para detectar la peroxidación de lípidos. Se utilizaron antioxidantes endógenos, catalasa, superóxido dismutasa, glutatión peroxidasa, proteína 70 de choque térmico y factor 2 relacionado con el factor nuclear eritroide 2 (Nrf2), como marcadores de estrés oxidativo y se detectaron mediante transferencia Western.

Resultados: Se observó una disminución de la viabilidad celular con una CI_{50} de 1,36 mg/mL y ATP. La concentración de malondialdehído (MDA) aumentó significativamente ($p<0,005$). Se incrementaron las concentraciones de superóxido dismutasa, Nrf2 y proteína de choque térmico. Sin embargo, se agotaron glutatión, glutatión peroxidasa y catalasa.

Conclusiones: Los resultados obtenidos sugieren que el extracto de *Terminalia phanerophlebia* es toxicogénico e induce estrés oxidativo en células HEK293.

Palabras Clave: antioxidantes; células Hek293; citotoxicidad; enfermedades renales crónicas; estrés oxidativo; peroxidación lipídica; *Terminalia phanerophlebia*.

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INTRODUCTION

Medicinal plants have been used for centuries to treat a wide variety of ailments including cardiovascular diseases (CVD), diabetes, bacterial infections, cancer and sexually transmitted infections (STIs) (Anand et al., 2019). Additionally, they have been used to treat diarrheal symptoms, headaches, inflammation and for wound healing (Petrovska, 2012; Jamshidi-Kia et al., 2018). Medicinal plants are a highly sought-after alternative to current pharmaceutical drugs because they can be locally cultivated, inexpensive and possess minimal adverse effects (Petrovska, 2012; Jamshidi-Kia et al., 2018).

The genus *Terminalia* is one of the most popularly used medicinal plants due to its many traditional medical applications. Of the 11 *Terminalia* species spread over the southern African region, *Terminalia phanerophlebia* Engl. & Diels *(TP),* family *Combretaceae*, is endemic to the northern KwaZulu-Natal and Mpumalanga regions (Madikizela et al., 2014). In previous studies, *TP* has been identified to possess antioxidant, antidiabetic, anti-inflammatory (Nair et al., 2012), antifungal and antibacterial properties (Shai et al., 2008). These properties are due to the phytochemicals like flavonoids (Adebayo et al., 2015), β-sitosterol (Nair et al., 2012), tannins, saponins and terpenoids, which are present in *TP* extracts (Akhalwaya et al., 2018). Phenolic compounds are strong antioxidants whose mechanism of action is to interact with receptors and enzymes involved in signal transduction in order to protect cell constituents against oxidative damage by free radicals and therefore avert their deleterious effects on nucleic acids, proteins and lipids in cells.

Chronic kidney diseases (CKDs) have become a global challenge. Progressive CKD leads to end-stage renal failure (ESRF) and mortality. Some of the risk factors leading to CKD and cardiovascular diseases are oxidative stress, hypertension, diabetes and smoking. During renal re-modelling, cells rely on chemokines, growth factors and cytokines for interaction (Daenen et al., 2019). Hepatocyte and vascular endothelial growth factors as well as osteogenic protein 1 protect the kidney from renal damage by activating intracellular signal transduction pathways (Gao et al., 2020). The overproduction of ROS stimulates activation of the MAPKs signalling pathway, which facilitates the regulation of inflammatory and immune responses (Signorini et al., 2017). MAPKs function in a broad range of processes such as renal cellular responses to stimulating growth factor production by interacting with DNA-binding sites and activating protein-1 (AP-1) triggering regulation on DNA synthesis, fibrogenesis and cellular proliferation (Cassidy et al., 2012). The kidney is involved in the detoxification of the blood and requires vast quantities of energy to carry out their function efficiently. For this reason, the kidney contains many mitochondria to provide energy. This means that oxidative stress in the kidney cells due to an increase in ROS or depletion of antioxidants results in CKDs. Progression of this state may result in atherosclerosis, anemia, hypertension, inflammation, water retention and in some cases death (Daenen et al., 2019). Based on studies done in 2015, 10% of mortality and morbidity cases in the world are from CKD with most of the cases coming from the African continent (Kaze et al., 2018). Current treatments available depending on severity of CKD are dialysis, medication such as diuretics and surgery. However, these methods are invasive in the event of surgery, time-consuming in cases of dialysis and overall expensive. Given the high rate of poverty in Africa and limited facilities in rural regions, an alternative natural treatment is needed to combat the symptoms and effects of CKD. To date, there is a literature gap investigating the toxic effects of *TP* on the kidney. Therefore, the purpose of this study is to investigate the oxidative effect and molecular mechanisms of *TP* on human embryonic kidney (HEK293) cells.

MATERIAL AND METHODS

Materials

All tissue culture reagents and apparatus were obtained from Whitehead Scientific (Johannesburg, South Africa). The bicinchoninic acid (BCA) assay kit, β-actin and methylthiazol tetrazolium (MTT) salt were purchased from Sigma. Promega luminometry kits and Cell Signalling Technology (CST) antibodies were procured from Anatech (Johannesburg, South Africa), while protease and phospha-

tase inhibitors were obtained from Roche Diagnostics (Johannesburg, South Africa). Western blot reagents were purchased from Bio-Rad (Hercules, CA, USA) and all other reagents were obtained from Merck (Johannesburg, South Africa), unless specified otherwise.

Tissue culture

A vial of cryopreserved HEK293 cells received from the Discipline of Medical Biochemistry, Howard College, University of KwaZulu-Natal, Durban was thawed at 37°C and reconstituted in complete culture media [(CCM: Dulbecco's Modified Eagle's Medium (DMEM), 10% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin-streptomycin-fungizone and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) (HEPES) buffer)]. The HEK293 cells were incubated at 37°C with 5% carbon dioxide supply for 4 h. The media was changed to remove residual dimethyl sulfoxide (DMSO). Thereafter the cells were maintained by changing the media as appropriate every 24-48 h. Once confluence was reached the media was discarded, the cells were resuspended in CCM, counted using the trypan blue method (150 µL CCM; 50 µL trypan blue and 50 µL cells) and utilized for various assays.

Plant material

Terminalia phanerophlebia Engl. & Diels (*TP*) (Assession no: 18267) leaves were collected in September 2018 from Sherwood, Durban, South Africa (29° 49′ 48.5″: 30° 58′ 38.5″). The tree was identified by Dr S. Ramdhani, authenticated by Mr EN Khathi and deposited into the botanical herbarium at University of Kwa-Zulu Natal, Westville campus Durban, South Africa.

TP leaf extract was obtained from the Department of Medical Biochemistry, Howard College, University of KwaZulu-Natal, Durban (voucher specimen 5544000 and accession No.18267). A 10 mg/mL aqueous stock solution of the extract was prepared, and the solution was filtered (0.45 µm) and used to prepare the concentrations of *TP* crude extract required for the study.

The leaves were separated from the stalks and dried at room temperature (RT) for 5 d or until completely dry. The air-dried leaves were weighed and ground into a fine powder using a Sunbeam standard household mechanical blender (Australia) and 500 mL dH_2O was added and left for 24 h at RT while continuously stirring. The mixture was subjected to centrifugation (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 2000 ×g for 10 min at RT and the supernatant was harvested and lyophilised for 2 d using the Vis Tis sp Scientific freeze dryer (Warminster, Pennsylvania, USA) (-46°C, 79 mT,). The final weight of the extracts was obtained, and the percentage yield of the extracts was determined. The extracts were stored in the dark at 4°C until further use (Wang et al., 2015). The percentage yield obtained was 23.36% (initial yield = 18.791 g and final yield = 4.390 g).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was used to determine the half maximum inhibitory concentration (IC_{50}) (Bahuguna et al., 2017). A confluent flask of HEK293 cells was washed thrice using phosphate buffer solution (PBS) each time. The cells were dislodged by agitation and resuspended in (CCM). Cells were counted, and 20 000 cells (200 µL) were seeded per well in triplicate for each treatment that was used in the MTT assay. Cells were allowed to adhere for 24 h after which the treatment medium (*TP*) was added to the relevant wells from 0-5 mg/mL. After 24 h the treatment medium was removed and replaced with a solution containing 4 mg MTT salt, 800 µL PBS and 4000 µL warm CCM. The solution was left for 4 h and replaced with DMSO for 1 h (to dissolve the purple formazan). The absorbance was then read at 570 nm with a reference wavelength of 690 nm using a BioTek µQuant spectrophotometer (USA) (Perumal et al., 2019). The absorbance values were used to calculate the cell viability according to the equation [1] (Vijayarathna and Sasidharan, 2012).

$$\text{Cell viability (\%)} = \left(\frac{absorbance\ of\ treated\ cells}{absorbance\ of\ control\ cells}\right) \times 100 \quad [1]$$

The log concentration and cell viability were analysed using GraphPad Prism (V) to produce the regression curve (Fig. 1) from which the IC_{50} (i.e., maximum inhibition where 50% of the cells were inhibited) was determined. The IC_{25} was calculated as 50% of the IC_{50}. For each subsequent assay three 25 cm^3 flasks with confluent cells were treated with different concentrations of *TP* (Control; IC_{25} = 0.7 mg/mL and IC_{50} = 1.4 mg/mL). All assays were conducted in triplicate to obtain comparable results.

Thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay was used to test for lipid peroxidation, which results from oxidative stress (Bartsch and Nair, 2004). Treated cells (100 000 cells) as well as the treatment medium were used to determine the levels of lipid peroxidation. A positive control [containing 1 µL of malondialdehyde (MDA)] and the negative control (a blank without MDA), samples (untreated control, IC_{25} and IC_{50}) were also used. The cells were resuspended in 200 µL CCM and homogenized by passing through a needle. To each test tube representing each sample, 200 µL of 7% H_3PO_4 (4.1 mL in 45.9 mL distilled water) was added, after which 400 µL of TBA/BHT solution (0.1 g NaOH; 0.5 g TBA; 250 µL from 20 mM stock (440.8 mg in 100 mL ethanol) all dissolved and made up to 50 mL using distilled water) was added to each test tube excluding the blank (negative control). To the blank 400 µL of 3 mM of HCl (30 µL from 1 M stock in 9.97 mL distilled water) was added. To each sample 200 µL of 1 M HCl (4.92 mL from 32% HCl topped to 50 mL) was added and all test tubes were vortexed before being placed in a water bath at 100°C for 15 min and then cooled to room temperature. Butanol was then added to each test tube (1500 µL each) and each test tube was vortexed. The samples were allowed to settle until two distinct phases were visible. To respective Eppendorf's, the upper phase was pipetted and 100 µL of each sample was plated in duplicates on a 96-well microtitre plate (Satyo et al., 2020). The absorbance at 532 nm with a reference wavelength of 600 nm was measured using a BioTek µQuant (USA) spectrophotometer. The equation [2] was used to convert the absorbance values to MDA concentration (Basak et al. 2001).

$$\text{MDA} = \left(\frac{absorbance\ of\ Samples - absorbance\ of\ blanks}{156\ mM}\right) \times 100 \quad [2]$$

Nitric oxide synthase (NOS) activity assay

The NOS assay was used to test and quantify the reactive nitrogen species (Vishwakarma et al., 2019). Cells (50 000 per well) were homogenized in 50 µL PBS. The treatment media (50 µL) was used to measure reactive nitrogen species present in the CCM. From a 1000 µM stock solution, 6 serial dilutions (0-200 µM) were prepared and 50 µL of each standard was added to a 96-well microtitre plate in triplicate. The sample (50 µL of control, IC_{25} and IC_{50} for both the medium and cells) were plated in duplicate. To each well 50 µL of vanadium chloride, 25 µL of sulfanilamide and 50 µL of N-(1-naphthyl) ethylenediamine dihydrochloride were added to each well. The plate was incubated for 45 min at 37°C before reading the absorbance at a wavelength of 540 nm and a reference wavelength of 690 nm (Tsotetsi et al., 2020). A standard curve was prepared, and sample nitric oxide concentrations were extrapolated from the standard curve.

Luminometry

GSH and ATP assay

The GSH assay was used to detect mitochondrial stress by quantifying ATP and GSH concentration (Rahman et al., 2006; Birket et al., 2011). For both assays, 20 000 cells/well were plated in duplicate into an opaque 96-well white plate. The ATP and GSH assay reagents were prepared according to the manufacturer`s protocol and 25 µL each was added to the respective wells. The plate was left overnight to adhere. The culture medium was removed, and the treatment (*TP*) was added (control, IC_{25} = 0.7 mg/mL and IC_{50} = 1.4 mg/mL) to respective wells for 24 h. The treatment medium was removed and 50 µL of prepared 2× GSH-GloTM or Cell Titer-Glo reagents were added to each well. The plate was mixed briefly on a shaker and then incubated at room temperature for 30 min after which the ATP plate was read. For GSH, reconstituted luciferin detection reagent (50 µL) was added to each well. The

plate was mixed briefly on a shaker before incubating it for 15 min. The luminescence was then measured.

Western blot

The western blot was used to quantify the proteins/antioxidants produced due to oxidative stress, which were SOD (#13141), catalase (#12980), GPx (#3286), HSP70 (#4872) and Nrf2 (#12721) (Yang et al., 2014).

Protein isolation and standardization

Flasks with confluent cells were treated with *TP* (Control, IC_{25} = 0.7 mg/mL, and IC_{50} = 1.4 mg/mL) and were incubated for 24 h at 37°C with 5% CO_2 supply. The media was discarded, and cells were washed twice with PBS. Cytobuster containing protease and phosphatase inhibitors (300 µL) was added to each flask. The cells were incubated on ice for 15 min. Cells were scrapped, transferred to an Eppendorf and centrifuged (2000×g; 4°C, 5 min). The supernatant was collected, and protein quantified using the BCA assay (25 µL sample/standard solution + 200 µL BCA working solution) and incubated in the dark for 30 min at 37°C before reading the absorbance at 562 nm on a BioTek µQuant spectrophotometer (USA). The absorbance was used to extrapolate crude protein concentration, which was used to standardise the protein to 1 mg/mL. Sample/Laemmli buffer (5× dilution) was prepared and used to dilute the standardized protein (4 parts crude protein: 1-part buffer). Samples were boiled for 5 min to denature the proteins then cooled to room temperature (Tsotetsi et al., 2020).

Protein separation

The mini-PROTEAN 3 SDS-PAGE apparatus were assembled according to the manufacturer`s guidelines. A 10% resolving gel was prepared [dH_2O, acrylamide/Bis, 1.5 M Tris (pH 8.8), 10% w/v SDS, 10% APS and TEMED] and 4% stacking gel [dH_2O, 0.5 M Tris (pH 6.8), 10% SDS, Bis/acrylamide, 10% APS and TEMED]. 1× electrode buffer [dH_2O, Tris, glycine, SDS pH (8.3)] was added to the tank and 25 µL of samples and 5 µL of molecular weight markers were loaded to respective wells. Running buffer was added and electrophoresis was carried out (150 V for 90 min) using a Bio-Rad compact power supplier until the tracker dye reached bottom of the gel (Mhlanga et al., 2019).

Protein transfer

Transfer buffer [25mM Tris (pH 7.4), 192 mM glycine, 20% v/v methanol; pH 8.3] was used to equilibrate the gel and nitrocellulose membrane for 10 min. A gel sandwich was prepared in a transblot plate and a constant current of 2.5 mA (25 V) was applied for 30 min. When the transfer was completed the membrane was placed in blocking solution [5% BSA in TTBS, NaCl, KCl, Tris (pH 7.4)] for 2 h. Thereafter primary antibodies 5% BSA in TTBS (1:1000 dilution) were added. The membranes were placed in on a shaker for 1 h before being left overnight at 4°C. Membranes were then allowed to return to room temperature before being washed five times with Tris buffered saline (TTBS) (10 mL) and probed with matched secondary antibodies (anti-mouse or anti-rabbit IgG) in 5% BSA in TTBS (1:2500) for 2 h at room temperature on a shaker. Membranes were then washed with TTBS (10 mL) 5 times and rinsed with deionized water. The membrane was covered with chemiluminescence reagent (mixed luminol/enhancer and peroxide buffer in 1:1 ratio, each 500 µL) the proteins of interest were viewed using Molecular Image ®Chemidoc TMXRS and Bio-Rad imaging system. The bands were then analyzed by Image Lab software (6.0.1) by Bio-Rad. The membranes were then prepared for probing for the housekeeping protein. The membrane was washed with 10 mL water for 1 min. The water was discarded and, 5 mL of H_2O_2 was added and incubated at 37°C for 30 min. The H_2O_2 was then discarded after incubation and the membrane was washed with 10 mL of water, then 10 mL of TTBS for 1 min each. Thereafter, the buffer was discarded followed by blocking the membrane with 5% BSA for 2 h. HRP-conjugated house-keeping antibody β-actin (abd1214) (1:5000 dilution in 5% BSA/TTBS 1 h) was then added. After successive washes in TTBS, the membrane was viewed as described previously (Madide et al., 2020).

Ethical approval

Ethical approval was obtained from the Biomedical Research Ethics Administration under the Reference number: BE368/19.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software version 5.0. Bars in the graphs are mean ± standard deviation. Significant difference was determined using One-way Analysis of Variance (1-way ANOVA) with Tukey's post-test and Students *t*-test with Welch's correction. The 95% confidence interval was set at $p<0.05$.

RESULTS

Cell viability

The MTT assay was used to determine cell viability from which an IC_{50} was derived. Fig. 1 shows that cell viability decreased with increased concentrations. However, a threshold-point was reached at the lowest cell viability (0.30103 mg/mL), and then it started to increase again from 0.39794 mg/mL. An IC_{50} value was calculated using GraphPad Prism 5.0 and was determined to be 1.4 mg/mL for *TP* in HEK293 cells.

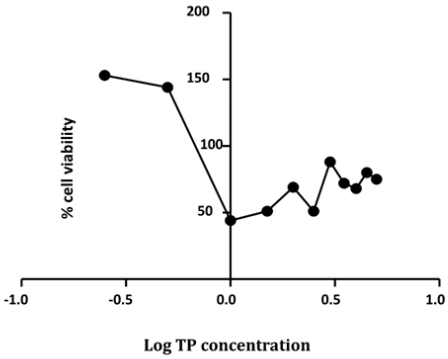

Figure 1. The effects of increased *TP* treatment concentration on the cell viability.

Overall *TP* decreased the cell viability below that of control cells.

Effect of *Terminalia phanerophlebia* on ATP level

The enzyme luciferase cleaves luciferin, thus producing light, which can be measured by luminometer. The light is directly proportional to the amount of or activity of a molecule of interest; therefore, luminometry can be used to quantify the activity of various molecules such as ATP. A non-significant depletion in ATP occurred following treatment with *TP* (Fig. 2).

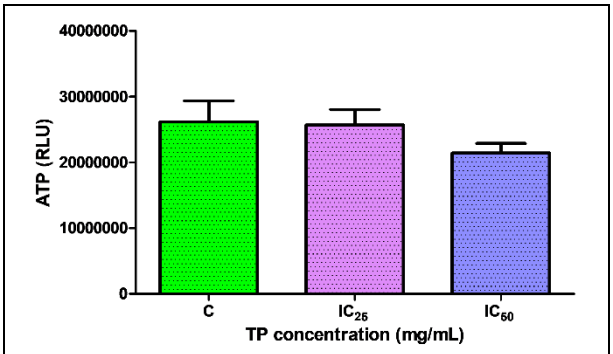

Figure 2. ATP activity *vs.* treatment concentration.

Decreased ATP concentration is greater when the IC_{50} is compared to the control. (RLU- Relative light units).

TBARS assay

Malondialdehyde (MDA) concentration was measured using the TBARS assay in both the cells and the supernatant treatment medium. The concentration of MDA, a by-product of lipid peroxidation, increased significantly in the treatment medium ($p<0.0043$, 1-way ANOVA with Tukey's post-test), but the slight increase in MDA in cells following exposure to *TP* was not significant (Fig. 3). For the media, the levels of MDA were much higher than the ones observed in the cells.

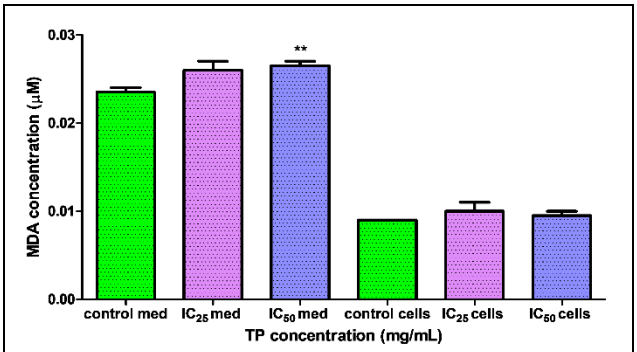

Figure 3. Treatment concentration vs. MDA concentration.

A 0.04 ± 0.01 increase was observed for the IC_{50} treatment media. **p=0.0018, Students *t*-test with Welch's correction.

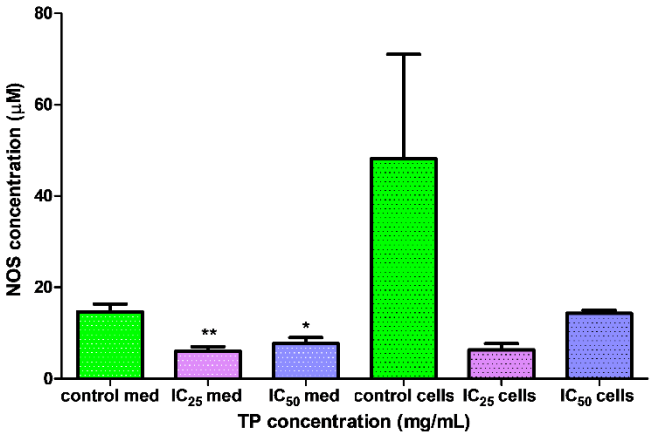

Figure 4. The effect of increased treatment concentration on NO concentration.

RNS were significantly decreased for all treatments compared to the control. *p=0.0108 and ** p=0.0045 when compared to the control. Students *t*-test with Welch's correction.

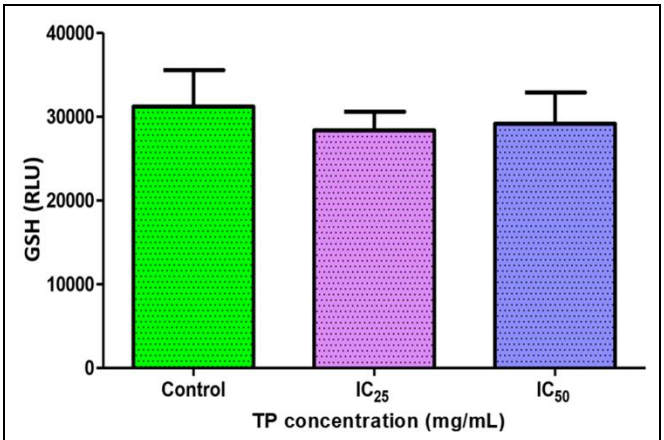

Figure 5. The effect of increased treatment concentration on glutathione concentration.

A 2-3% decrease in GSH concentration is noted following treatment with *TP*.

NOS assay

RNS were indirectly determined using the NOS assay. The NOS concentration was decreased in both the cells ($p<0.0158$, 1-way ANOVA with Tukey's post-test) and the treatment media ($p<0.0005$, 1-way ANOVA with Tukey's post-test) (Fig. 4) compared to the controls. A 50-55% decrease was noted for the treatment media and 60-80% decrease for the cells.

GSH

A decrease in GSH concentration is a marker of oxidative stress. A number of physiological substances inactivate GPx such as nitric oxides and carbonyl compounds. The slight decreases in GSH concentration were not significant when compared to the control (Fig. 5).

Western blot

Western blotting was used to quantify and determine the presence of proteins/antioxidants produced in order to validate if oxidative stress was present. Fig. 6 depicts the relative changes in the protein expression for SOD, catalase, GPx, Nrf2 and HSP70. Both *TP* concentrations increased SOD (Fig. 6A, $p=0.0002$ using 1-way ANOVA with Tukey's posttest), Nrf2 (Fig. 6D, $p=0.0218$ using 1-way ANOVA with Tukey's post-test) and HSP70 (Fig. 6E, $p=0.002$ using 1-way ANOVA with Tukey's post-test) relative to the control. GPx was not significantly decreased for both concentrations (Fig. 6C), but catalase was only decreased for the IC_{50} treatment (Fig. 6B, $p=0.0112$ using 1-way ANOVA with Tukey's post-test, $p=0.0112$ using 1-way ANOVA with Tukey's post-test, $p=0.0112$ using 1-way ANOVA with Tukey's post-test).

DISCUSSION

Given the high rate of poverty in Africa and limited facilities in rural regions, alternative natural treatment is sort to combat the symptoms and effects of many illness. *Terminalia phanerophlebia* (*TP*) extracts have been reported for their beneficial effects against various ailments. However, to date, there is a literature gap investigating the toxic effects of *TP* on the kidney. Therefore, the purpose of this study is to investigate the oxidative effect and molecular mechanisms of *TP* on human embryonic kidney (HEK293) cells.

The IC_{50} was determined to be 1.4 mg/mL through the MTT assay, which was used to quantitatively assess mitochondrial activity. The yellow MTT salt enters the cells and then the mitochondria where it is reduced by mitochondrial dehydroge-

nase to formazan (purple insoluble salt) (van Meerloo et al., 2011). Reduction can only be measured in metabolically active cells. Fig. 1 indicates that *TP* has a hormesis/"U-shaped" dose–response effect indicating that it may impart beneficial or stimulatory effects at low doses but adverse effects at higher doses (Calabrese, 2019).

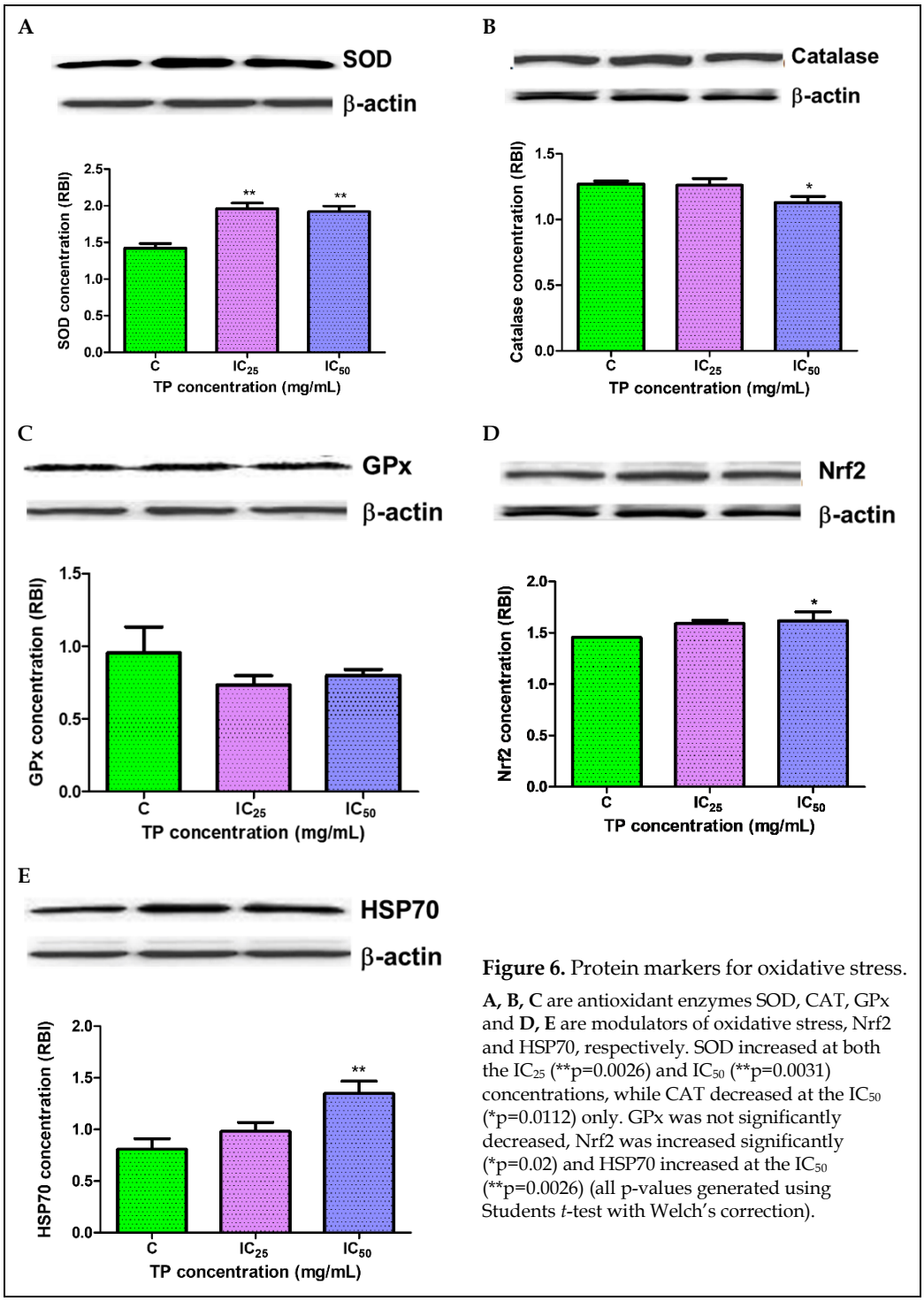

Figure 6. Protein markers for oxidative stress.

A, B, C are antioxidant enzymes SOD, CAT, GPx and **D, E** are modulators of oxidative stress, Nrf2 and HSP70, respectively. SOD increased at both the IC_{25} (**p=0.0026) and IC_{50} (**p=0.0031) concentrations, while CAT decreased at the IC_{50} (*p=0.0112) only. GPx was not significantly decreased, Nrf2 was increased significantly (*p=0.02) and HSP70 increased at the IC_{50} (**p=0.0026) (all p-values generated using Students *t*-test with Welch's correction).

It is presumed that *TP* caused uncoupling of oxidative phosphorylation, a process whereby NADH transfers electrons to O_2 through a series of electron carriers to produce ATP (Stier et al., 2014). The uncoupling inhibits ATP synthesis resulting in the decrease in ATP (Fig. 2) and increased ROS production. Major ROS production occurs in the mitochondria where the oxygen molecule is reduced to oxidants such as O_2^- (Dan Dunn et al., 2015). MnSOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, thus an increase in O_2^- up-regulates MnSOD production (Schott et al., 2017). The increase in MnSOD protein concentration (Fig. 6A) suggests that there was increased O_2^- produced that required detoxification to H_2O_2. The results also suggest that SOD was successful in competing with NO for O_2^- inhibiting further production of RNS such as $ONOO^-$ (Fig. 4) (Phaniendra et al., 2015). The next possible fate for H_2O_2 was production of OH. by Fenton-type reactions; $OH^\cdot$ is a potent initiator of lipid peroxidation. It does this by abstracting hydrogen from polyunsaturated fatty acids, thus increasing the production of aldehydes such as MDA (Ayala et al., 2014). In the present study, MDA concentration increased (Fig. 3), which agrees with previous studies done on *Terminalia* species *in vivo* on rats (Mahesh et al., 2009). Alternatively, *Terminalia* species contain methyl gallate (MG), a compound that has membrane-damaging activities, which could have interfered with the membrane integrity and numerous cellular functions resulting in oxidative stress (Acharyya et al., 2015). Previous studies have confirmed the presence of MG in *Terminalia chebula Retz, Terminalia macroptera, Terminalia myriocapa, Terminalia calamansanai* (Acharyya et al., 2015; Madikizela et al., 2014).

Reduced GSH is crucial in the cellular defense against free radicals and lipid hydroperoxides (Liu et al., 2015), and therefore prevents lipid peroxidation by producing stable lipid alcohols. The depletion of GSH in this study (Fig. 5) suggests that it was employed to minimize peroxidation of lipids (Fig. 3). H_2O_2 can also be decomposed to water by either GSH (through GPx at lower concentrations) or CAT at higher concentrations (Kurutas, 2015). The depletion of these intracellular antioxidants (Fig. 6C and 6B, respectively) is an indication of increased free radicals requiring oxidation and may result in the onset of oxidative stress. Decrease in ATP production is also associated with decrease in protein synthesis, which could be the reason for decreased antioxidant enzymes, GPx, CAT and GSH (Kurutas, 2015). The 2GSH: GSSG ratio is critical and if in imbalance results in oxidative stress because GSSG is toxic and should be reduced to GSH by GSH reductase in the presence of NAD(P)H. Since GSH, GPx and CAT concentrations decreased in trying to combat the increasing concentration of oxidants, lipid peroxidation was therefore prolonged and led to the disruption of the lipid membranes in the mitochondria and in turn destroyed the integrity of ATP as displayed by Fig. 2 resulting in oxidative stress.

HSP70, an endogenous chaperone protein increases in response to stimuli, stress or damage (Fig. 6) and its production was triggered by oxidative stress (Martine et al., 2019). HSP70 protects the cell from stress by regulating signaling pathways, most of which are related to cell death (Radons, 2016; Shrestha and Young, 2016). Another modulator of oxidative stress is Nrf2, which detected the oxidative stress environment and was up-regulated (Fig. 6) to cause an increase in the transcription of proteins and ultimately decrease oxidative stress (Ma, 2013). Important intracellular antioxidants like SOD, GPx and CAT are modulated by Nrf2 at the transcriptional level. This could explain the minimal depletion of the antioxidants in *TP*-treated Hek293 cells.

CONCLUSIONS

Medicinal plants have a range of phytochemical properties believed to combat a variety of disease ailments. However, this study has shown that aqueous *TP* leaf extracts induced the production of free radicals in HEK293 cells. The antioxidant response was insufficient to scavenge the ROS generated and resulted in lipid peroxidation. This study highlights the importance of studies that investigate the effects of potential extracts before administration of medicinal plants, to avoid detrimental side effects.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Nyahada MR	Amoako DG	Somboro AM	Arhin I	Khumalo HM	Khan RB
Concepts or ideas	x	x	x			x
Design	x	x			x	x
Definition of intellectual content	x	x	x	x	x	x
Literature search	x	x		x		x
Experimental studies	x	x	x			x
Data acquisition	x	x	x	x	x	x
Data analysis	x	x	x	x	x	x
Statistical analysis	x	x		x		x
Manuscript preparation	x	x		x		x
Manuscript editing		x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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