



# Phytochemical content, antioxidant activities and androgenic properties of four South African medicinal plants

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## ABSTRACT

**Introduction:** This study aimed to investigate the phytochemical contents, antioxidant activities and androgenic properties of *Peltophorum africanum* Sond, *Trichilia emetica* Vahl, *Terminalia sambesiaca*, and *Ximenia caffra*.

**Methods:** The finely powdered leaves of the selected plants were extracted using acetone, aqueous and methanol as solvents. The total phenolics and flavonoids contents were determined from gallic acid and quercetin standard curves. The antioxidant activities of these extracts were evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. The effects of plant extracts (100-1000 µg/mL) on TM3 Leydig cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Testosterone levels were measured using ELISA kit.

**Results:** The methanol extracts of *T. sambesiaca* and *X. caffra* revealed higher total phenolic and flavonoid contents (102.13 ± 2.32 mg/g gallic acid equivalent and 1.05 ± 0.04 mg/g quercetin equivalent, respectively) than other plant extracts. The acetone and methanol extracts of *P. africanum* revealed the best IC<sub>50</sub> values (12.50 ± 0.052 µg/mL) against DPPH compared to the other plant extracts and ascorbic acid. The MTT assay results showed that all concentrations of plant extracts maintained cell viability and were not cytotoxic with IC<sub>50</sub> values of greater than 20 µg/mL. The methanol extract of *T. sambesiaca* had the higher testosterone production at 500 µg/mL (0.399 ng/mL) when compared with the basal control while at the concentration of 500 µg/mL the acetone extracts of *P. africanum* and *T. sambesiaca* had significantly high testosterone production of 0.147 and 0.188 ng/mL, respectively when compared with basal control.

**Conclusion:** The results reveal that these plants possess antioxidants and androgenic properties and suggest the potential use for the treatment of male infertility.

### Implication for health policy/practice/research/medical education:

The examined medicinal plants and natural products are potent androgenic agents. Their androgenic potentials may improve sexual behaviour and performance, and also boost spermatogenesis and reproduction. The validation of these medicinal plants for their sexual behaviour and fertility may serve as a promising source for the identification of new chemicals for the management and treatment of sexual and erectile dysfunction.

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## Introduction

Medicinal plants have become the main ingredient of medications (1). Many African people, approximately 80%, rely on herbal medicine for alleviation and management of diseases (2). Preference of medicinal herbs over artificial medicine is due to their diversity, accessibility for collection, used in many cultures, self-medication (3), low costs, and minor health effects (4). Medicinal plants are rich in diversity of phytochemicals such as polyphenols

with antioxidant properties that can neutralize damaging free radicals and subsequent protecting tissues from oxidative stress (5-7).

Elevated reactive oxygen species (ROS) or free radicals such as hydroxyl radical, hydrogen peroxide and superoxide anion have been found associated with male hypogonadism and infertility (8). Male infertility has become one of the serious medical conditions globally contributing about 50% of prevalence (9). Testosterone,

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an essential sex hormone synthesised from testicles, plays a critical role in several body's physiological and biological functions. Its deficiency may result in changes that can negatively affect the body's normal function (10). Disturbances of testosterone impair spermatogenesis, male secondary sexual characteristics development, sexual performance and subsequent male infertility (11). Herbal medicines are sources of natural antioxidants used for the treatment and management of male infertility (12). In South Africa, a number of medicinal plants have been used for male reproductive healthcare (13). Some of these plants have not been scientifically evaluated for their potential activity and possible toxicity levels.

*Peltophorum africanum* Sond belongs to the Fabaceae family. It is well-known as African-wattle, Weeping wattle, Rhodesian black wattle, and African false wattle (English); “dopperkiaat, huilboom, huilbos” (Afrikaans); “Umsehla” (Ndebele); “isiKhaba-mkhombe, umSehle, or umThobo” (Zulu); “mosêtlha” (Tswana) (14). “Mosesé” (Venda) (15); and “Mosetlha” (Sepedi) (16). *P. africanum* is native to Southern Africa, geographically distributed throughout Demographic of Congo, the Northern part of the KwaZulu-Natal province of South Africa and Swaziland (17,18). The plant is also found in Kenya, Madagascar, Australia, Tanzania and the United States (18). The *P. africanum* bark and root are used for the treatment of tuberculosis, intestinal disorders and male infertility (13,19). Some researchers (20,21) have reported that the root of this plant is used to treat diarrhoea, tonsils, herpes, and as an eye drop to soothe sore eyes. Also, the bark of this multifunctional plant is used to treat erectile dysfunction, sore throat, diarrhoea, dysentery, wounds, and human immune virus/acquired immune deficiency syndrome (HIV/AIDS) (22,23). Moreover, the concoction of both *P. africanum* bark and *Elephantorrhiza elephantina* bark is also used traditionally for the alleviation of HIV/AIDS (23).

*Trichilia emetica* Vahl belongs to the Meliaceae family. This plant is well-known as “umKhuhlu” (Zulu) (24). The tree is distributed in Tropical Africa (25). The bark is used to treat stomach problems, renal problems and to relieve back pain (26,27). The leaves are used by Zulu people to treat dysentery (28). According to Shai et al (29), it is used folklorically for treatment of wounds and *Ascaris* stomach aches. *T. emetica* is also used to treat fever, asthma, eye infections, epilepsy, oedema, anthelmintic, and skin diseases. Moreover, the plant is used as aphrodisiac and diuretic (30). A ratio 1:1 of *T. emetica* bark and chopped roots of *Gymnosporia senegalensis* is used for the treatment of infertility (31).

*Terminalia sambesiaca* belongs to the Combretaceae family. *Terminalia* species are widely distributed in Africa and Asia (32). The stem bark and leaves decoctions are used as medications to treat cancer, stomach ulcers, and appendicitis (33,34). The powdered root bark is used to

treat bloody diarrhoea (34). The plant is also used to treat bilharzia, dysmenorrhoea, earache, backache, pneumonia, hookworm, and syphilis (35). *Terminalia sericea* one of *Terminalia* species is used in Limpopo Province, South Africa for treatment of male infertility and impotence (36).

*Ximenia caffra* belongs to the Olacaceae family. The plant is called large sour plum (English); and “mutshili” (Venda) (15). *X. caffra* origin spans around Kenya, Tanzania, Malawi, Mozambique, Uganda, Zambia and South Africa (14). The leaves or roots of *X. caffra* is used for constipation, leprosy, stomach pains and tuberculosis (37). *X. caffra* dried powdered leaves are applied on the skin to heal dermatophilosis, a skin disease caused by bacterium *Dermatophilus congolensis* (38), and also used for fertility and as a febrifuge (39). The leaves and/or roots are used by the Venda tribe for the treatment of sexually transmitted infections, cough, diarrhoea, and relieve headache (19). The dried powdered root of *X. caffra* is applied to the wound, and mixed with soup or added in beers to stimulate sexual desire (40). A decoction of leaves is used to treat tonsillitis and sooth inflamed eyes (40). Moreover, a boiled concoction of the *X. caffra* root and the *Tabernaemontana elegans* root is used to treat diarrhoea and gonorrhoea infections (20).

This study was therefore aimed at investigating the phytochemical contents, antioxidant activities, and androgenic properties of *P. africanum* Sond, *T. emetica* Vahl, *Terminalia sambesiaca*, and *Ximenia caffra*.

## Materials and Methods

### Collection and preparation of plant materials

The leaves of *P. africanum* Sond, *T. emetica* Vahl, *Terminalia sambesiaca* and *X. caffra* were collected in the summer season from labeled trees of Lowveld National Botanical Garden of Mpumalanga province, South Africa. The plant materials were rinsed with distilled water and air-dried. The plant materials were then ground into powder using a laboratory electric grinder, and stored in airtight containers until further use. Ethical clearance was approved by the University of South Africa (Unisa), College of the Agriculture and Environmental Sciences Ethics Committee.

### Plant material extraction

Acetone, methanol, and distilled water were used as solvents for extractions. Three grams (3 g) of powdered plant materials were weighed into 100 mL bottles and 30 mL of acetone and methanol were added. The extraction was performed as explained by Masoko et al (40) with slight modifications. The extraction was repeated three times with equal volume of extractants and each extract were merged and filtered through Whatman No 1 filter paper into weighed labeled glass vials and thereafter concentrated to dryness. The aqueous extract was derived

via concoctions and filtered through Whatman No 1. The filtrates were concentrated overnight using a freeze-drier (Ultra-Low Temperature Freezer -86°C, Vacutec). After concentration to dryness, all the crude extracts of the respective weight were determined and recorded.

#### Determination of total phenolic content

The total phenolic contents were determined using the Folin-Ciocalteu method as described by Makkar (41) and modified by Adebayo et al (42). Briefly, 25 µL of plant extracts (1 mg/mL stock solution) and gallic acid (0.02-0.1 mg/mL) was added in different test tubes and mixed with 250 µL Folin-Ciocalteu reagent for about 5 minutes. The resultant reaction was stopped with 750 µL 20% anhydrous carbonate. The solution was made up to 5 mL with 3975 µL distilled water. The solution was kept at the darkroom temperature for about 2 hours. After incubation, 300 µL of the solutions were transferred into 96-well plates and absorbance was read at 760 nm using a microplate reader (Varioska flash, AEC Amersham Pty Ltd). The experiment was performed in triplicate. Gallic acid was used for the standard curve. The phenolic content of each extract was determined from the standard curve:  $y = 0.3141x + 0.0488$ ;  $R^2 = 0.9996$  and expressed as mg/g gallic acid equivalent (GAE). The total phenolic contents of plant extracts calculated using the below formula:

$$\text{Total phenolic content} = \text{GAE} \times V/m$$

where, "GAE" is the concentration of gallic acid established from the calibration curve (mg/mL), "V" is the final volume of extract (mL), and "m" is the weight of pure plant extract (g) (43).

#### Determination of total flavonoid content

The total flavonoid content of the extracts was determined using Yadav and Agarwal (44) method and slightly modified by Adebayo et al (42). Briefly, 100 µL of plant extracts (1 mg/mL dissolved in solvents) and quercetin (0.04-2 mg/mL) was mixed with 300 µL of methanol, 20 µL of 10% aluminium chloride and 20 µL of 1 M sodium acetate. The solution was made up to 1 mL with 560 µL distilled water. The resultant solutions were allowed to stand at room temperature for about 30 minutes. After 30 minutes of incubation, the absorbance was read at 450 nm using a microplate reader (Varioska flash, AEC Amersham Pty Ltd). Experiments were performed in triplicate. The flavonoid content of each extract was determined from quercetin standard curve:  $y = 3.6518x + 0.2309$ ;  $R^2 = 0.9997$ . The values were expressed as mg/g QE. The total flavonoid contents of the plant extracts calculated using the below formula:

$$X = C \cdot V/m$$

where "X" is the total flavonoid content, mg/g is the plant extract in QE, "C" is the concentration of quercetin

established from the calibration curve (mg/mL), "V" is the final volume of extract (mL) and "m" is the weight of pure plant extract (g) (45).

#### Determination of antioxidant activity using DPPH assay

The 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging activity method as explained by Mensor et al (46), and modified by Hamzah et al (47) was used to measure the antioxidant activity of extracts. Briefly, a solution of 0.051 mM DPPH radical in methanol was prepared. One hundred microlitres of DPPH radical solution was mixed with 100 µL (40 to 500 µg/mL) each of the various plant extracts/standard drug (vitamin C) in a 96-well microplate were kept at the darkroom temperature for 30 minutes. After incubation, the absorbance was read at 517 nm using a microplate reader (Varioska flash, AEC Amersham Pty Ltd). DPPH in methanol served as negative control and methanol as blank. All samples were carried out in triplicate. The percentage of inhibition values were calculated using the below formula:

$$\% \text{ Inhibition} = 1 - (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

where,  $\text{Abs}_{\text{sample}}$  is the absorbance of the sample,  $\text{Abs}_{\text{control}}$  is the absorbance of the negative control.  $\text{IC}_{50}$  is the concentration of the sample required for 50% inhibition of DPPH free radical was determined from the linear regression plots, % Inhibition against the concentrations of tested plant extracts (mg/mL).

#### Cell culture conditions

The TM3 Leydig cell line derived from mouse testes were obtained from the University of Western Cape cell culture laboratory. The cells were cultured in the cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) added 10% fetal bovine serum (FBS) (complete medium) in the incubator (Thermo Scientific Forma Series II Water Jacket CO<sub>2</sub> Incubator) with 5% CO<sub>2</sub> and 37°C temperature (48). The cells were allowed to reach 80% confluence before being split. Cell passaging was done using Mahapatra et al (49) method, with slight modifications.

#### Cell viability and cytotoxicity assessments

To assess the effects of solvent extracts of *P. africanum*, *T. emetica*, *T. sambesiaca* and *X. caffra* on TM3 Leydig cells viability was carried out according to method of Mosmann (50) with slight modifications. The acetone and methanol crude extracts were reconstituted in acetone and aqueous in DMSO at a concentration of 100 mg/mL. TM3 Leydig cells ( $5 \times 10^3$ /100 µL/well) were exposed to 100 µL various concentrations of plant extracts (0.1 - 1 mg/mL) and incubated at 37°C with 5% CO<sub>2</sub> for 24 hours. After incubation, the medium was removed and then 25 µL MTT (5 mg/mL in PBS) was added to each well. The plates were then incubated for 2 hours at 37°C. Afterwards, the medium was removed and the converted dye (formazan blue crystals) dissolved in 100

$\mu\text{L}$  DMSO per well. Afterwards, the plates were kept in the dark place at room temperature for about 30 minutes and the absorbance was read at 570 nm and 620 nm for reference using a microplate reader (Varioska flash, AEC Amersham Pty Ltd). Non-treated cells and MTT were used as control and blank, respectively. The samples were tested in triplicate. Percentage cell viability of the sample was calculated using the below equation:

$$\% \text{ cell viability} = \frac{\text{Mean Absorbance of treated cells}}{\text{Mean Absorbance of control}} \times 100$$

The percentage inhibitions were then calculated using the following equation:

$$\% \text{ inhibition} = \frac{1 - \text{Mean Absorbance of treated cells}}{\text{Mean Absorbance of control}} \times 100$$

$\text{IC}_{50}$  results were determined from the linear regression graph, % inhibition versus test concentrations (51).

#### Determination of testosterone production

The TM3 cells were seeded at density of  $5 \times 10^3$  cells/well in 96-well plate, and treated with various concentrations (0.1, 0.25, and 0.5 mg/mL) of plant extracts in the presence of 1  $\mu\text{L}$  anti-human chorionic gonadotropin hormone (Anti-HCG) and were incubated for 24 hours at 37°C with 5%  $\text{CO}_2$ . After 24 hours, the production of testosterone was assessed using the Testosterone ELISA kit according to the manufacturer's instructions (ab108666-Testosterone ELISA Kit).

#### Statistical analysis

Statistical analysis was done using Minitab 17 statistical software, Fisher's least significant difference (LSD) and analysis of variance (ANOVA). The significant difference was  $P < 0.05$ . Data were expressed as mean  $\pm$  standard deviation.

## Results

#### Percentage yield crude extracts

The subsequent percentage yield upon the completion of extraction and concentration with various plants (*P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra*) and solvents (acetone, methanol, and water). *T. emetica* had the highest acetone extract yield (3.7%), while in the case of methanol extracts, *T. sambesiaca* had the highest yield (30.7%) and the aqueous extract of *X. caffra* had the highest yield (15.7%) (Table 1).

#### Total phenolic contents

In this study, the results of the total phenolic content are expressed in mg of gallic acid equivalent per gram (mg/g GAE). Overall, the acetone extracts of all plants had higher total phenolic content. The highest phenolic content in this study was observed in the acetone extract of *T. sambesiaca*

**Table 1.** Percentage yield of crude extracts from the selected plant species extracted using acetone, methanol, and water as solvents

Samples	Yield (%)		
	Acetone	Aqueous	Methanol
<i>P. africanum</i>	2.3 <sup>a</sup>	9.0 <sup>a</sup>	20.3 <sup>a</sup>
<i>T. emetica</i>	3.7 <sup>b</sup>	4.3 <sup>b</sup>	8.3 <sup>b</sup>
<i>T. sambesiaca</i>	3.0 <sup>c</sup>	13.7 <sup>c</sup>	30.7 <sup>c</sup>
<i>X. caffra</i>	2.3 <sup>a</sup>	15.7 <sup>d</sup>	4.0 <sup>d</sup>

Values within the same row, with different superscript are significantly different.

( $102.13 \pm 2.32$  mg/g GAE) while the lowest was observed in the methanol extract of *T. emetica* ( $2.22 \pm 0.31$  mg/g GAE). When compared with other methanol extracts, that of *X. caffra* had the highest phenolic content ( $29.7 \pm 1.45$  mg/g GAE) while the aqueous extract of *T. sambesiaca* ( $12.1 \pm 1.22$  mg/g GAE) had the highest content in among other aqueous extracts (Table 2).

#### Total flavonoid contents

The flavonoid content of these four plants and their respective crude extracts were expressed in mg of quercetin equivalent per gram (mg/g QE). The acetone extracts of all plants were determined with reference to quercetin and expressed as their equivalents (mg/g QE). The highest flavonoid content in this study was observed in the acetone extract of *X. caffra* ( $1.05 \pm 0.04$  mg/g QE) while the lowest was observed in the methanol extract of *T. sambesiaca* ( $0.02 \pm 0.00$  mg/g QE). Summarily, all three extracts of *X. caffra* had the highest flavonoid contents within each of the different solvents of extraction (Table 2).

#### Antioxidant activities

The DPPH free radical scavenging activity of the various solvent extracts of *P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra* when compared with a recognised antioxidant (Ascorbic acid) and their respective concentrations that scavenged 50% ( $\text{IC}_{50}$ ) of the radicals are presented in Table 3, respectively. From the results, all extracts showed lower  $\text{IC}_{50}$  values than that of ascorbic acid; hence the extracts were more efficient in scavenging the DPPH radical than ascorbic acid. The acetone and methanol extracts of *P. africanum* scavenged DPPH radical with  $\text{IC}_{50}$  of  $13.00 \pm 0.010$  and  $19.00 \pm 0.010$   $\mu\text{g}/\text{mL}$ , respectively. The aqueous extract of *T. emetica* had the best scavenging capacity ( $\text{IC}_{50} = 348.5 \pm 0.077$   $\mu\text{g}/\text{mL}$ ) when compared to other aqueous extracts.

#### Cell viability

The TM3 Leydig cells were exposed to increasing concentrations of plant extracts (0.1, 0.25, 0.5, 0.75, and 1 mg/mL) for 24 hours using MTT assay and percentage cell viabilities are illustrated in Figure 1. *T. sambesiaca* and *X. caffra* showed higher percentages of viable cells at

**Table 2.** Total phenolic and flavonoid contents of four selected plant species extracted using acetone, aqueous and methanol

Samples	Total phenolic contents (mg/g GAE)			Total flavonoid contents (mg/g QE)		
	Acetone	Aqueous	Methanol	Acetone	Aqueous	Methanol
<i>P. africanum</i>	76.07 ± 2.99 <sup>a</sup>	7.34 ± 0.45 <sup>a</sup>	2.26 ± 0.29 <sup>a</sup>	0.84 ± 0.17 <sup>a</sup>	0.007 ± 0.00 <sup>a</sup>	0.06 ± 0.05 <sup>a</sup>
<i>T. emetica</i>	7.1 ± 0.73 <sup>b</sup>	12.1 ± 1.22 <sup>b</sup>	2.22 ± 0.31 <sup>b</sup>	0.44 ± 0.01 <sup>a</sup>	0.03 ± 0.01 <sup>b</sup>	0.10 ± 0.02 <sup>b</sup>
<i>T. sambesiaca</i>	102.13 ± 2.32 <sup>c</sup>	12.1 ± 0.35 <sup>c</sup>	3.25 ± 0.49 <sup>c</sup>	0.19 ± 0.01 <sup>b</sup>	0.02 ± 0.00 <sup>b</sup>	0.02 ± 0.01 <sup>a</sup>
<i>X. caffra</i>	85.5 ± 9.27 <sup>d</sup>	6.86 ± 0.26 <sup>d</sup>	29.7 ± 1.45 <sup>d</sup>	1.05 ± 0.04 <sup>c</sup>	0.10 ± 0.00 <sup>c</sup>	0.12 ± 0.01 <sup>c</sup>

Abbreviations: mg/g GAE, milligram gallic acid equivalent per gram of extract; mg/g QE, milligram quercetin equivalent per gram of extract. Values are expressed as mean ± standard deviation of 3 replicates. All the superscripts indicate significant difference ( $P < 0.005$ ) between the means. Values within the same column, with different superscript are significantly different.

**Table 3.** IC<sub>50</sub> values of selected plant species on DPPH radical and TM3 Leydig cells using MTT assay

Samples	DPPH IC <sub>50</sub> (µg/mL)			MTT IC <sub>50</sub> (µg/mL)		
	Acetone	Aqueous	Methanol	Acetone	Aqueous	Methanol
<i>P. africanum</i>	12.50±0.052 <sup>a</sup>	830.0±0.513 <sup>a</sup>	19.00±0.010 <sup>a</sup>	850.0±0.257 <sup>a</sup>	946.0±0.043 <sup>a</sup>	68.00±0.001 <sup>a</sup>
<i>T. emetica</i>	109.0±0.055 <sup>a</sup>	348.5±0.077 <sup>b</sup>	24.00±0.003 <sup>a</sup>	65.00±0.023 <sup>b</sup>	214.0±0.002 <sup>b</sup>	222.0±0.048 <sup>b</sup>
<i>T. sambesiaca</i>	685.5±0.108 <sup>a</sup>	536.5±0.077 <sup>ab</sup>	500.5±0.076 <sup>b</sup>	451.0±0.018 <sup>c</sup>	501.0±0.008 <sup>c</sup>	905.0±0.025 <sup>c</sup>
<i>X. caffra</i>	521.5±0.209 <sup>a</sup>	472.0±0.102 <sup>ab</sup>	174.0±0.014 <sup>c</sup>	246.0±0.197 <sup>d</sup>	749.0±0.138 <sup>d</sup>	244.0±0.004 <sup>d</sup>
<b>Ascorbic acid</b>	<b>1473.3±1.335</b>					

The IC<sub>50</sub> value represents the concentration of the extract required for 50% inhibition of cells. Values are expressed as mean ± standard deviation of 3 replicates. All the superscripts indicate significant difference ( $P < 0.005$ ) between the means. Values within the same column, with different superscript are significantly different.

1 mg/mL (78%, and 106%, respectively) (Figure 1A). *P. africanum* and *T. emetica* showed higher percentages of viable cells at 0.75 mg/mL and 0.5 mg/mL (72% and 83%, respectively) (Figure 1A). *P. africanum* and *T. sambesiaca* showed significantly high percentages of viable cells at 0.25 mg/mL (94% and 116%, respectively). *T. emetica* and *X. caffra* showed higher percentages of viable cells at 0.1 mg/mL (91% and 103%, respectively) (Figure 1B). *P. africanum* and *T. emetica* showed significantly high percentage of viable cells at 0.75 mg/mL and 1 mg/mL (110% and 109%, respectively). *T. sambesiaca* and *X. caffra* both showed high percentages of viable cells at 0.1 mg/mL (115% and 87%, respectively) (Figure 1C).

### Cytotoxicity

Table 3 shows the results of the cytotoxic effects of the extracts. The cytotoxic effects were expressed as IC<sub>50</sub> values for the percentage inhibition graph (data not shown). The higher IC<sub>50</sub> values indicate the lower cytotoxicity. According to the findings, acetone and aqueous extracts of *P. africanum* IC<sub>50</sub> values (850.0 ± 0.257 and 946.0 ± 0.043 µg/mL) respectively, the methanol extract of *T. sambesiaca* IC<sub>50</sub> value (905.0 ± 0.025 µg/mL) while the aqueous extract of *X. caffra* IC<sub>50</sub> value (749.0 ± 0.138 µg/mL). The above extracts showed the lowest cytotoxicity against TM3 Leydig cells (Table 3).

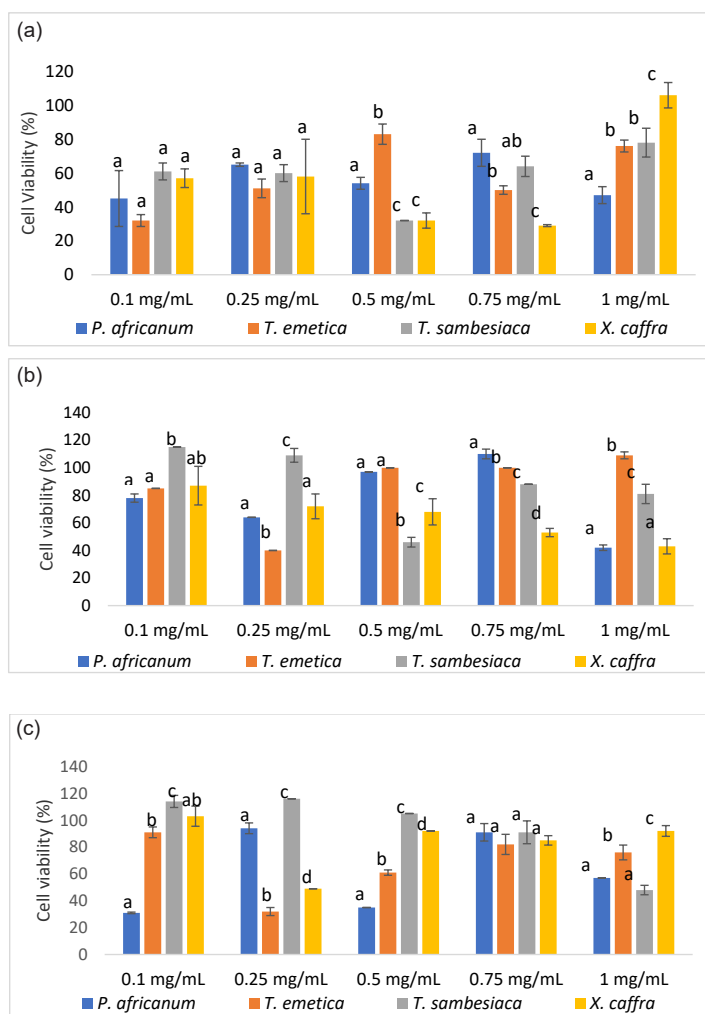
### Testosterone production

Table 4 shows the results of testosterone production in TM3 Leydig cells exposed to selected concentrations of *P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra* solvent

extracts treated with anti-human chorionic gonadotropin hormone (anti-hCG). As soon as the plant extracts were introduced, there was an increase in testosterone production levels in non-stimulated TM3 Leydig cells in comparison to control. It is noteworthy to state that at 500 µg/mL, the methanol extract of *T. emetica* (0.399 ng/mL) had the highest testosterone production on TM3 Leydig cells in comparison to control. Also, three extracts (acetone, methanol and aqueous extracts) of *P. africanum* at 500 µg/mL showed increased testosterone production on TM3 Leydig cells. In addition, 250 µg/mL of acetone extract, 100 and 250 µg/mL of the methanol extract, and 100 µg/mL of the aqueous extract of *P. africanum* caused significant ( $P < 0.05$ ) decrease in testosterone levels when compared with the control. At the different concentrations tested, *T. emetica* solvent extract had better testosterone production potential when compared to control. Meanwhile, in *T. sambesiaca* only at 100 µg/mL of the acetone extract had significantly ( $P < 0.05$ ) lower levels in testosterone when compared with control. Finally, the acetone and methanol extracts of *X. caffra* caused significant ( $P < 0.05$ ) decrease in testosterone levels when compared with control while the aqueous extract at 250 µg/mL showed an increase in the testosterone production on the TM3 Leydig cells in comparison with control (Table 4).

### Discussion

Phytochemical screening of medicinal plants is mainly depended on the solvent selected for extraction (6,40). The structures of compounds determine their solubility in the solvent polarity (52). Therefore, it is very important



**Figure 1:** Percentage cell viability of TM3 Leydig cells exposed to different concentrations (0.1, 0.25, 0.5, 0.75 and 1 mg/mL) of 4 selected plant species for 24 h using MTT assay. (A) Acetone extracts (B) methanol extracts and (C) aqueous extracts. The values in the bar chart are expressed as mean (cell viability)  $\pm$  SD (n = 3). Mean separation was done by LSD ( $P < 0.05$ ). Sets of bars (the same concentration) with different alphabets are significantly different;  $P < 0.05$ .

to isolate non-polar compounds with nonpolar solvents and polar compounds with polar solvents (53). In the current study, it was observed that methanol was the best extractant in comparison to other solvents (Table 1). Our results are in support with the study of Masoko et al (54) reported the highest quantity of crude extract obtained with methanol as solvent of extraction.

Phenolic compounds are major secondary metabolites found abundantly in medicinal plants (55). They possess the antioxidant properties that protect the damaging effect of free radical scavenging activity (56). They are believed to account for the antioxidant activity of most plant species (57). From our findings, the acetone extracts of *P. africanum*, *T. sambesiaca*, and *X. caffra* had significantly higher total phenolic and flavonoid contents in comparison with other solvent extracts evaluated in this study (Table 2). The study by Mulaudzi et al (38) revealed the total phenolic content of 50% methanol leaf extract of *X. caffra*  $11.87 \pm 0.08$  mg/g GAE lower than

that of our study where 100% methanol was used. The presence of higher phenolic content may be responsible for the antioxidant activity of selected plant species. The results showed that acetone could be regarded as a good solvent for isolating phenolic compounds. Our findings are in line with Unuofin et al (6). They reported that the acetone extract of *V. mespilifolia* had the ability to extract more phytochemical content than other extractants used. Also, the findings are in line with Masuku and Lebelo (58) which obtained higher total phenolic and flavonoid contents from acetone extract of *Kigelia africana* leaves.

Over the years, the impacts of extractants on medicinal plants and natural products/remedies have been reviewed. In order to have a greater yield of desired phytochemicals during extraction procedures, the polarity index of the solvent of extraction must be carefully addressed (6,59,60). In recent times, researchers have correlated the abundance of polyphenolic contents and other bioactive compounds in natural products to their radical scavenging potential

**Table 4.** Testosterone production in TM3 Leydig cells exposed to selected varying concentrations of the extracts for 24 h in the presence of anti-human chorionic gonadotropin hormone (hCG)

Samples	Testosterone (ng/mL)									
	Acetone			Methanol			Water			Negative control
	100 µg/mL	250 µg/mL	500 µg/mL	100 µg/mL	250 µg/mL	500 µg/mL	100 µg/mL	250 µg/mL	500 µg/mL	
<i>P. africanum</i>	0.063±0.003*	0.011±0.001	0.147±0.007*	0.002±0.000	0.008±0.000	0.084±0.004*	0.017±0.001	0.053±0.003*	0.064±0.003*	
<i>T. emetica</i>	-	0.065±0.003*	0.042±0.031	0.050±0.002*	-	0.042±0.002	0.092±0.004	0.054±0.003*	0.046±0.002*	
<i>T. sambesiaca</i>	0.008±0.000	-	0.118±0.006*	-	0.042±0.002	0.399±0.019*	-	0.105±0.005*	0.067±0.003*	
<i>X. caffra</i>	-	0.008±0.000	0.015±0.001	0.004±0.000	0.023±0.001	0.019±0.001	0.031±0.001	0.073±0.003*	0.038±0.002	
<b>Negative control</b>	0.038±0.002									

Values represent mean ± SD of three determinations.

\* $P < 0.05$  when anti-hCG-stimulated treated groups were compared with the negative control. (-) indicates that the concentration was not measured.

(61-64). Phytochemicals in the form of phenols and flavonoids possess different capacities such as electron donation, metal chelating and oxygen quenchers potential (65,66). All these abilities aid in combating/reducing the accumulation of free radicals in living organisms.

Numerous assays have been developed to measure the free radical scavenging activity of antioxidants in food, beverages, and plant extracts. Good examples are the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethyl-benzothiazoline-sulfonic acid (ABTS) radical scavenging assays. These two assays are regarded as the most popular spectrophotometric methods used to evaluate the antioxidant activity of samples (67). These assays are simple to use and rapid to assess the antioxidant activity, but they are very sensitive to light (68). In the current study, DPPH radical assay was used to determine the radical scavenging potentials of *P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra* extracts. The DPPH radical is a purple colour compound, upon reaction with antioxidant it decolorizes to light yellow diphenyl-2-picryl hydrazine by accepting hydrogen atom from antioxidants (69). The degree of colour change at the end of the reaction is proportional to the free radical scavenging activity of antioxidants (70).

Our findings revealed that all plant extracts effectively reduced the DPPH radical than ascorbic acid. The presence of lower  $IC_{50}$  value indicates stronger antioxidant activity against damaging free radicals (71). The acetone and methanol extract of *P. africanum* had the best scavenging potential against DPPH radical with  $IC_{50}$  of  $12.5 \pm 0.052$  µg/mL and  $19.00 \pm 0.010$  µg/mL respectively when compared with other plant extracts and standard (ascorbic acid). The methanol extracts of *T. emetica*, *T. sambesiaca*, and *X. caffra* had the best scavenging power with  $IC_{50}$   $24.00 \pm 0.003$  µg/mL,  $500.5 \pm 0.076$  µg/mL and  $174.0 \pm 0.014$  µg/mL, respectively when compared to other solvent extracts of these plants. The aqueous extract of *T. emetica* had the best scavenging strength ( $IC_{50}$   $348.5 \pm 0.077$  µg/mL) when compared with the other aqueous

extract tested against DPPH radical (Table 3). The study by Bizimenyera et al (72) measured the DPPH radical scavenging activity of acetone extract of *P. africanum* (leaf, root, and bark) with  $IC_{50}$  values of  $6.54 \pm 0.49$ ,  $3.82 \pm 0.58$  and  $4.37 \pm 0.41$  µg/mL. Adebayo et al (42) determined the antioxidant activity of 70% acetone leaves extract of *P. africanum* using DPPH assay, in which the results showed  $IC_{50}$  of  $7.71 \pm 0.36$  µg/mL. The review article by Komane et al (73) reported the DPPH radical reducing activity of methanol and aqueous leaf extracts of *T. emetica* showed  $IC_{50}$  values of 17.9 and 43.9 µg/mL. Tshikalange et al (74) demonstrated the DPPH radical inhibition activity of ethanol extracts of *X. caffra* bulbs, roots, and seeds, in which the results showed  $IC_{50}$  values of 11.77, 10.41 and 9.21 µg/mL. On the contrary to our study, the antioxidant activity of acetone, hexane, dichloromethane, methanol, and ethyl acetate extracts of *T. sambesiaca* leaves was observed on the thin layer chromatography using DPPH methanol solution spray as an indicator. Acetone, ethyl acetone and methanol exhibited inhibition activity on the TLC plates (75,76). Inconsistent with the findings reported by Bizimenyera et al (72) and Adebayo et al (42), the  $IC_{50}$  values of *P. africanum* and *T. emetica* leaves extracts showed weak inhibition activities against DPPH radical in comparison with other studies. It could be as a result of variation in season of harvest of plant materials and geographical location (77). The  $IC_{50}$  values of ethanolic extracts of *X. caffra* bulbs, roots, and seeds were lower in comparison with acetone, aqueous, and methanol leaves extracts of *X. caffra* of our study. In terms of *T. sambesiaca*, to our knowledge, this is the first study to determine the  $IC_{50}$  value of these plant crude extracts. Several variables may influence the levels of bioactive compounds of the plant and antioxidant capacities such as type or polarity of solvents used, extraction technique, duration of extraction, temperature, the origin of the plant, and harvest seasons (77,78). These reasons may explain the contrasting result gotten from different studies.

Apart from phenolic compounds, it was reported that

the antioxidant activity could be due to other bioactive compounds (alkaloids and saponin) in the extracts (62). Catechin, gallotannin, berginin, proanthocyanidins, coumarins, benzenoids, condensed flavonoids, lactone, amino acid, and terpenoids are detected from *P. africanum* extract (79-81). Kurubasch aldehyde, kurubasch aldehyde benzoate, trichilin A, dregeana 4, trichilia substance (Tr-A, Tr-B, and Tr-C), nymania 1, *seco*-A protolimonoid, sendanin, and rohituka-3, rohituka-4, and rohituka-7 have been isolated from *T. emetica* extract (73,82). The presence of the saponin-like compound has been identified on the TLC plate loaded *T. sambesiaca* crude extract (32) and beta-sitosterol was isolated from *T. sambesiaca* extract by nuclear magnetic resonance (74). In the study of Zhen et al (83), the phytochemical analysis of *X. caffra* leaf extract using liquid chromatography-mass spectrometry (LC-MS) showed the presence of gallic acid, quercetin, kaempferol, and catechin. In addition, the antioxidant capacity of the plants is most times dependent on the chemical structure of the antioxidant and its interaction with other plant constituents (84,85). Therefore, other bioactive compounds that contribute to the therapeutic effect of medicinal plants need to be considered as their mechanisms of action (59).

3-(4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay is a popular method used to evaluate the effects of toxic substances on the cell viability and cytotoxicity (86). MTT is a yellow tetrazolium salt soluble in water. When cells are exposed to MTT solution, it changes to purple formazan product by viable cells. The MTT formazan product cannot pass through the cell membrane of dead cells. It is produced when the tetrazolium ring split by mitochondria succinate dehydrogenase enzyme of viable cells. For this reason, when the number of viable cells increases, the intensity of the purple colour of MTT formazan increases (51). The TM3 Leydig cells were used in this present study. They were gotten from mouse testis. The cells are sited within the interstitial space where almost 95% of androgenesis takes place (87-89). The study revealed the indices of cytotoxicity via cell mortality using MTT assay in TM3 Leydig cell line after 24 h incubation period with the different plant extracts. The results from this study revealed that the concentrations of 100-1000 µg/mL of all different solvent plant extracts did not alter the viability of TM3 Leydig cells upon 24 h treatment. This was also observed by Opuwari and Monsees (88), who reported the concentration-dependent increased percentage cell viability from 250-1000 µg/mL when TM3 Leydig cells were exposed to *Camellia sinensis* (unfermented and fermented rooibos) and *Aspalathus linearis* (green tea and black) but reduced at 5000 µg/mL. The inhibition of growth of the normal TM3 Leydig cells by different plant extracts ranged between 65 and 946 µg/mL. Different researchers have stated that the National Cancer Institute residing in the United State of America

put up a threshold of an IC<sub>50</sub> less than 20 µg/mL for crude extracts as cytotoxic level on cell lines with an exposure period of 24 hours (61,90). From the above assertion, we can say that our different extracts were not cytotoxic.

Human chorionic gonadotropin (hCG) purified from the urine of pregnant women is one the hormones used in therapy to stimulate or improve spermatogenesis in men with low or no spermatogenesis caused by a deficiency in gonadotropin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Human chorionic gonadotropin has a similar effect as LH, hence it has been used to replace LH. It stimulates testosterone production by Leydig cells (91). The TM3 Leydig cells and anti-hCG were used in this study to examine the effects of varying concentrations of *P. africanum*, *T. emetica*, *T. sambesiaca* and *X. caffra* in testosterone production. We determined the testosterone levels via ELISA kit by using cell supernatant obtained after treatment with varying concentrations of *P. africanum*, *T. emetica*, *T. sambesiaca* and *X. caffra* with anti-hCG for 24 hours. The testosterone levels with the treatment of 100, 250 and 500 µg/mL of the various solvent extracts of the plants increased significantly. Remarkably, the testosterone levels were highest with 500 µg/mL of the methanol extract of *T. sambesiaca*, 0.399 ng/mL which were significantly greater ( $P < 0.05$ ) than the control group (0.038 ng/mL). The acetone extracts of *P. africanum* and *T. sambesiaca* at 500 µg/mL also recorded significantly better testosterone production (0.147 and 0.118 ng/mL, respectively) in comparison with control. Also, the methanol extract of *P. africanum* had a greater testosterone level (0.084 ng/mL) when compared with the basal control (0.038 ng/mL). In addition, the aqueous extract of *T. sambesiaca* at 250 µg/mL (0.105 ng/mL) and *T. emetica* at 100 µg/mL (0.092 ng/mL) showed a significantly high testosterone production level when compared with basal control. It is noteworthy that only the aqueous extract of *X. caffra* at 250 µg/mL (0.073 ng/mL) was able to stimulate testosterone when compared to the control and other solvent extracts of the plant. Generally, sperm cells are defenseless against ROS; hence the enhanced or the increased testosterone product exhibited by the different plant extracts most especially by the solvent extracts of *P. africanum* and *T. sambesiaca* could be due to their potent radical scavenging activity. Steroid producing cells are also prone to attack by ROS and they also produce free radicals from the electron transport chain in the mitochondria by way of cytochrome P450 pathway enzymes (88,92,93). Naturally, Leydig cells possess a number of antioxidant enzymes that help mop up free radicals; however, elevated levels of ROS would suppress testosterone production (93). Owing to promising antioxidant activity against free radicals, androgenic property and noncytotoxic, the selected medicinal plants could be used to boost the body's antioxidant defensive system and protect male reproduction functions from oxidative damage. Basically,



herbal medicines are traditionally prepared using water as a solvent for remedies. Therefore, traditional healers and the African population as a whole could be encouraged to further use water as an extractant to prepare the concoction of *P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra* for treatment of diseases associated to elevated free radicals, infertility, and aphrodisiacs.

### Conclusion

According to the results of this study, different solvent extracts of *P. africanum*, *T. emetica*, *T. sambesiaca*, and *X. caffra* showed a significant high content of flavonoids and phenolics, potent antioxidant activity. They were nontoxic on TM3 Leydig cells and enhanced testosterone production. The study suggests that the selected plant species might be used as natural antioxidants to replace the synthetic antioxidants and may be introduced in drug discovery for the development of male fertility drugs with fewer side effects. However, further study is needed to examine LC-MS analysis and high-performance liquid chromatography/photodiode array detection (HPLC-PDA). Also, *in vivo* studies to find out the possible mechanisms of action still need to be done.

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### Authors' contributions

NPM, JOU and SLL conceived and designed the study. NPM performed the experiments, NPM and JOU prepared all figures and tables in the manuscript, while NPM wrote the draft manuscript. JOU and SLL coordinated and helped to revise the manuscript. NPM, JOU and SLL read and approved the final manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest

### Ethical considerations

The study was approved by the University of South Africa (Unisa), College of the Agriculture and Environmental Sciences Ethics Committee with protocol number 2016/CAES/037.

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