

## ORIGINAL ARTICLE

**Effect of *Cissampelos capensis* rhizome extract on human spermatozoa *in vitro***S. M. Shalaweh<sup>1</sup>, N. Erasmus<sup>1</sup>, F. Weitz<sup>2</sup> & R. R. Henkel<sup>1,3</sup>

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**Summary**

*Cissampelos capensis* is commonly known by the Afrikaans name 'dawidjies' or 'dawidjieswortel'. *C. capensis* is the most important and best-known medicinal plant of the family Menispermaceae used by the Khoisan and other rural people in the western regions of South Africa. Among numerous other ailments, it is traditionally taken to treat male fertility problems. Yet, no studies have investigated the effects of this plant or its extracts on human spermatozoa. The aim of study was to investigate the effects of *C. capensis* extracts on sperm function. A total of 77 semen samples were collected. Spermatozoa were washed with HTF-BSA medium and incubated with different concentrations of *C. capensis* (0, 0.05, 0.5, 5, 50, 200  $\mu\text{g ml}^{-1}$ ) for 1 h at 37 °C. Sperm motility, vitality, acrosome reaction, reactive oxygen species (ROS), capacitation, Annexin V binding, DNA fragmentation and mitochondrial membrane potential ( $\Delta\psi_m$ ) were determined. While viability, Annexin V positivity and  $\Delta\psi_m$  were not affected, the percentages of ROS-positive, TUNEL-positive, capacitated and hyperactivated spermatozoa increased significantly and dose-dependently. It is concluded that the alkaloids present in the extract of *C. capensis* rhizomes triggered sperm intrinsic superoxide production leading to sperm capacitation and DNA fragmentation.

**Introduction**

Due to rapid growing populations and socio-economic factors, herbal medicines/remedies are becoming popular as alternate treatment and are being used by many people as a form of therapy worldwide (Calixto, 2000). Modern, Western medicines are expensive, and most people living in Third World countries, especially in Africa, South America or Asia, are forced to rely only on the use of herbal medicine as treatment because of its affordability and accessibility. Amongst these, various plants of Asia, Europe and South America have been reported to influence male reproductive functions such as *Tribulus terrestris* (devil's weed), used for centuries to increase sexual desire and enhance erection (Adimoelja, 2000); *Panax ginseng* (Asian ginseng), for the treatment for impotency and improving sexual stamina (Nocerino *et al.*, 2000); *Eurycoma longifolia* (Tongkat Ali), *Astragalus membranaceus* (yellow leader) and *Acanthopanax senticosi* (Siberian ginseng), applied as an aphrodisiac to enhance testosterone levels, treat erectile dysfunction and to increase

human sperm motility *in vitro* (Liu *et al.*, 2004; Tambi & Imran, 2010); and *Lepidum meyenii* (Maca) is used to improve male fertility (Gonzales *et al.*, 2003).

Whereas in Asia or South America traditional remedies are more investigated, in Africa effectiveness and more importantly the safety of using herbal medicine is scientifically rather unknown and its efficacy can only be verified by the knowledge of Izinyangas or Sangoma's (traditional healers) passed down from generation to generation. This has slowly changed as the interest in employing herbs to treat male fertility/infertility is fast growing. Consequently, in West Africa, a study on plant extracts of *Hibiscus macranthus* and *Basella alba* (Malabar spinach) showed that these extracts enhance testosterone production (Moundipa *et al.*, 2006). The bark of the Yohimbe tree (*Pausinystalia yohimbe*) contains an alkaloid called yohimbine that is used to treat sexual dysfunction and enhances libido (Carey & Johnson, 1996). An *in vitro* study on *Mondia whitei*, which is used as an aphrodisiac in Ghana, has shown that an aqueous administration *in vitro* enhanced total motility and progressive motility

(Lampiao *et al.*, 2008). Additionally, the African medicinal plants, such as *Securidaca longipedunculata* (Polygalaceae) and *Fadogia agrestis* (Rubiaceae), have also been shown to treat erectile dysfunction (Ho & Tan, 2011).

South Africa has an exceptional variety of more than 30 000 higher plants, of which about 3000 are used as traditional remedies (van Wyk *et al.*, 2009). However, only very little is known about the action and use of these plants, their extracts and their phytochemicals, particularly for male reproductive health problems. One of the plants that are traditionally used, but have not been scientifically investigated on its effects on male fertility/infertility, is *Cissampelos capensis* of the family Menispermaceae. This plant is endemic to the Western Cape, South Africa.

In Khoisan ethnomedicine, *C. capensis* is of special significance (van Wyk & Gericke, 2000; van Wyk *et al.*, 2002; de Wet & van Wyk, 2008) as the rhizomes are used as a blood purifier to treat ailments such as fever, diabetes, stomach and skin cancer, cholera, and syphilis (van Wyk & Gericke, 2000; Von Koenen, 2001). In general, it is assumed that the medicinal therapeutic activity of the rhizomes is due to alkaloid content of the bisbenzyltetrahydroisoquinoline group with cissacapine, 12-O-methylcurine and cycleanine as main alkaloids (de Wet *et al.*, 2011). Bisbenzyltetrahydroisoquinolines are known to have anti-inflammatory effects, muscle relaxant and anticarcogenic activities (van Wyk *et al.*, 2002, 2009).

However, although this plant is reportedly used as traditional remedy to treat male fertility problems, no studies are available describing the effects *C. capensis* on male fertilising function. Therefore, this study aimed at investigating the effect of an aqueous *C. capensis* extract on the functional parameters of male spermatozoa in humans.

## Materials and methods

### Chemicals

Unless otherwise mentioned, all chemicals were obtained from Sigma (St. Louis, MO, USA).

### Herbal extract

*Cissampelos capensis* rhizomes were collected in the Cape Nature Reserve, Bellville, South Africa, cleaned from adhering soil and chopped into small segments of about 1–2 cm of length. These pieces were then placed into a mill to form a powdery substance that was infused with hot (about 70 °C) distilled water and filtered with a Whatman 1 filter paper (Whatman, Madestone, UK) to remove particles. Thereafter, this concoction was allowed to cool down and frozen at –20 °C. Subsequently, the

frozen extract was freeze-dried in a Virtis freeze drier (Virtis, Warminster, PA, USA), and the dried extract was stored at 4 °C until use.

The concentrations of the plants extract used in this study were deduced by the assumption that an average male weighs 80 kg. The traditional healers 'prescribe' the use of a handful of rhizomes to be used for making the concoction. Based on this information, three handful of rhizomes were weighed to obtain the averages (36 g per handful). Aqueous extraction resulted in 9.47 g extract per 100 g powdered rhizome. Subsequent calculation resulted in an amount of 3.41 g extract per man per day. From there, a standard 'normal' concentration of 50 µg ml<sup>-1</sup> *C. capensis* rhizome extract (CRE) was calculated. A stock solution containing 2000 µg ml<sup>-1</sup> CRE in HTF-BSA was prepared and mixed with sperm samples in HTF-BSA to obtain final concentrations of 0.05, 0.5, 5, 50, 200 µg ml<sup>-1</sup> CRE, with which the samples were incubated for 1 h at 37 °C. HTF-BSA without the extract served as a control. After the incubation, motility, viability, acrosome reaction, capacitation production of reactive oxygen species (ROS), DNA fragmentation, mitochondrial membrane potential ( $\Delta\psi_m$ ) and Annexin V binding were evaluated.

### Sperm sample collection and preparation

This study was ethically approved by the local Institutional Review Board, and patients and sperm donors gave informed consent. A total of 77 semen samples were collected after 3- to 5-day abstinence from patients ( $n = 45$ ) attending the infertility clinic of Tygerberg Hospital, Tygerberg, South Africa, and Vincent Palotti Hospital, Pine-lands, South Africa, respectively, as well as fertile sperm donors ( $n = 35$ ).

After liquefaction at room temperature, semen samples were diluted 1 : 5 with human tubular fluid medium (Quinn *et al.*, 1985), supplemented with 1% bovine serum albumin (HTF-BSA) (280 mOsmol kg<sup>-1</sup>) and centrifuged for 10 min at 500 g. The supernatant was discarded and the pellet resuspended in fresh HTF-BSA. Subsequently, sperm suspensions were incubated with CRE at the different concentrations for 1 h at 37 °C.

### Determination of sperm motility and vitality

Sperm motility was measured with the Motility/Concentration module of the Sperm Class Analyzer version 4.1.0.1 (Microptic S.L., Barcelona, Spain). After 1 h of incubation at 37 °C with CRE, an aliquot of 10 µl of the sperm suspension was put on a slide and the motility of at least 100 spermatozoa was analysed according to WHO criteria set by the SCA system with a Zeiss Photomicroscope III and a 100× oil-immersion objective. The

different kinematic parameters that were analysed are as follows: total motility (%), progressive motility (%), beat cross-frequency (BCF; Hz), linearity (LIN; %), straightness (STR; %), average path velocity (VAP;  $\mu\text{m s}^{-1}$ ), curvilinear velocity (VCL;  $\mu\text{m s}^{-1}$ ), straight line velocity (VSL;  $\mu\text{m s}^{-1}$ ) and hyperactivation (%).

Vitality was determined using the Nigrosin–Eosin Y staining technique (WHO, 2010). In brief, 50  $\mu\text{l}$  of Nigrosin–Eosin Y was mixed with 50  $\mu\text{l}$  of the semen sample, and a smear of 50  $\mu\text{l}$  was made on a slide. Slides were then air-dried and viewed with a 100 $\times$  oil-immersion objective in the bright field using a light microscope (Zeiss, Oberkochen, Germany). The percentage of live sperm was calculated.

#### Determination of acrosome reaction

Acrosome reaction was determined in 40 semen samples using the triple-staining technique as described by Henkel *et al.* (1993). In brief, semen samples were diluted 1 : 5 with HTF-BSA and centrifuged for 10 min at 500 g. The supernatant was discarded, the pellet resuspended with fresh HTF-BSA and samples were then incubated for 2 h at 37 °C. After incubation, 100  $\mu\text{l}$  of 2% Trypan blue in HTF was added and incubated for 15 min at 37 °C. Thereafter, samples were washed with 1 ml HTF (without BSA) and the pellet was fixed in 200  $\mu\text{l}$  of 3% glutaraldehyde for 20 min at 37 °C. Smears of 10  $\mu\text{l}$  were made and air-dried. Subsequently, slides were stained with 0.8% Bismarck Brown for 5 min at 40 °C, washed with deionised water and counter-stained with 0.8% Rosé Bengal for 1 h at room temperature. Slides were then washed with tap water, dehydrated in 100% ethanol and viewed with a light microscope at a 1000 times magnification. The percentage of live acrosome-reacted spermatozoa was evaluated in at least 200 spermatozoa.

#### Determination of capacitation

The capacitation state of the spermatozoa was assessed in a different set of experiments ( $n = 37$ ) using the chlorotetracyclin (CTC) fluorescence assay method as described previously (Green *et al.*, 1996). In brief, a 100 mg  $\text{ml}^{-1}$  stock solution of Hoechst 33258 was made up in distilled water and stored at 4 °C for up to 1 month. Before use, this stock solution was diluted 1 : 1000 in HTF and then further 1 : 100 with sperm suspension in HTF-BSA. Thereafter, the samples were incubated at room temperature for 2 min before being washed by centrifugation through 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF at 900 g for 5 min.

The CTC staining solution was prepared on the day of use and contained 750  $\mu\text{M}$  CTC in a buffer of 130 mM

NaCl, 5 mM cysteine and 20 mM Tris-HCl. The pH was adjusted to pH 7.8. This solution was kept wrapped in foil at 4 °C until use. Hoechst-treated spermatozoa (45  $\mu\text{l}$ ) were mixed with an equal volume of the CTC solution, and 8  $\mu\text{l}$  of 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4) was added. Subsequently, 10  $\mu\text{l}$  of the this suspension was placed on a slide and one drop of 0.22 M 1,4-diazabicyclo(2.2.2)octane (DABCO) dissolved in glycerol:PBS (9 : 1) was mixed in carefully to retard fading of the fluorescence. Slides were viewed with a 100 $\times$  oil-immersion objective using a fluorescence microscope. In each sample, 200 live (Hoechst-negative) cells were assessed for CTC staining patterns, they are as follows: uniform fluorescence over the entire head (characteristic of noncapacitated, acrosome-intact cells); fluorescence-free band in the post-acrosomal region (characteristic of capacitated, acrosome-intact cells); and dull or absent fluorescence over the sperm head (characteristic of capacitated, acrosome-reacted cells).

#### Determination of reactive oxygen species producing sperm

Sperm ROS production was determined according to Henkel *et al.* (2005). In brief, a stock solution of 20  $\mu\text{M}$  dihydroethidine (DHE; Molecular Probes, Eugene, OR, USA) in PBS, pH 7.4, (Oxoid, Basingstoke, Hampshire, UK) was prepared. After incubation of sperm samples with different concentrations of CRE for 1 h at 37 °C, aliquots of 100  $\mu\text{l}$  were centrifuged for 10 min at 500 g, resuspended in 100  $\mu\text{l}$  PBS and 20  $\mu\text{l}$  of DHE stock was added. Then, the samples were incubated for 15 min at 37 °C. Thereafter, 10  $\mu\text{l}$  of each sample was taken for evaluation at a 630 times magnification using an epifluorescence microscope with 488 nm excitation and 590 emission filters (Zeiss). The percentage of red fluorescing (ROS-positive) sperm was calculated.

#### Determination of DNA fragmentation

Sperm DNA damage in terms of nuclear DNA fragmentation was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay (Detection System fluorescein; Promega, Mannheim, Germany) according to the protocol of Henkel *et al.* (2004). After incubation of sperm samples with different concentrations of CRE, samples were centrifuged for 10 min at 300 g and the supernatant was discarded. The pellet was resuspended with PBS, and a smear was made on Superfrost slides (Knittel Gläser, Braunschweig, Germany). Slides were then fixed with freshly prepared 4% formaldehyde (KIMIX Chemicals, Epping, South Africa) in PBS for 25 min at 4 °C and washed in PBS for

5 min at room temperature. Subsequently, spermatozoa were permeabilised with 0.2% triton X-100 in PBS for 5 min and rinsed twice with PBS at room temperature. After removing excess liquid, samples were equilibrated for 5–10 min in 100  $\mu$ l equilibration buffer. Thereafter, 20  $\mu$ l TdT buffer was added, the sample was covered with a cover slip and incubated for 1 h at 37 °C. Eventually, slides were immersed in 2 $\times$  SSC for 15 min, washed thrice in distilled water and evaluated in an epifluorescence microscope at 630 times magnification. The percentage of green-fluorescing spermatozoa (TUNEL-positive) was calculated.

#### Determination of the mitochondrial membrane potential

The percentage of spermatozoa with intact mitochondrial membrane potential ( $\Delta\psi_m$ ) was determined using the DePsipher kit (TREVIGEN, Gaithersburg, MD, USA) according to Henkel *et al.* (2012). In brief, the reaction buffer was diluted with distilled water 1 : 10, and 20  $\mu$ l stabiliser was added per milliliter buffer. A volume of 1  $\mu$ l of this solution was added to 500  $\mu$ l prepared reaction buffer, vortexed thoroughly and finally centrifuged for 1 min at 10 000 g. Fifty microlitre of this supernatant was then used to resuspend the pellet of 100  $\mu$ l washed spermatozoa and incubated for 20 min at 37 °C. After incubation, the sperm suspension was centrifuged and the supernatant was discarded. Sperm was resuspended in 100  $\mu$ l of pre-warmed 1 $\times$  reaction buffer. Smears were prepared using 50  $\mu$ l of the samples, viewed with a fluorescence microscope (Zeiss). While spermatozoa displaying a green fluorescence in the mid-piece of the flagella were regarded as having a disrupted  $\Delta\psi_m$ , spermatozoa displaying a red fluorescence in the mid-piece were regarded as having an intact  $\Delta\psi_m$ .

#### Determination of apoptosis (Annexin V)

For the identification of apoptotic spermatozoa, externalised phosphatidylserin (PS) to the outer leaflet of the plasma membrane as an early step in the apoptotic process, the Annexin V-FITC Apoptosis Detection Kit was used. Annexin V is a calcium-dependent phospholipid-binding protein with a very high affinity for PS. To differentiate apoptotic from necrotic spermatozoa, the sperm nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Because this fluorescent probe does not enter live cells, fluorescing cells are regarded as dead, thus indicating necrosis of the sperm cells.

The procedure was conducted according to the protocol recommended by the manufacturer. In brief, after incubation of sperm sample with different concentrations of CRE, samples were centrifuged for 10 min at 300 g and the

supernatant was discarded. The pellet was resuspended with binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). Subsequently, 5  $\mu$ l Annexin V was added to 195  $\mu$ l sperm suspension, mixed and incubated for 10 min at room temperature. After incubation, 10  $\mu$ l of DAPI (5  $\mu$ g ml<sup>-1</sup>) was added. The sample was then centrifuged for 5 min at 500 g and resuspended in 190  $\mu$ l binding buffer. Thereafter, smears were prepared on slides and one drop of 0.22 M DABCO dissolved in glycerol:PBS (9 : 1) was mixed in carefully to retard fading of the fluorescence. Slides were viewed with a 100 $\times$  oil-immersion objective using fluorescence microscopy. A total of 200 spermatozoa were randomly assessed per slide in five fields and identified as follows: normal, nonapoptotic sperm: Annexin V(-)/DAPI(-), sperm in early apoptosis: Annexin V(+)/DAPI(-), sperm in late apoptosis: Annexin V(+)/DAPI(+) or necrotic sperm: Annexin V(-)/DAPI(+).

#### Statistical analysis

All statistical calculations were performed using the MED-CALC Statistical Software (version 12.3.0; Medcalc Software, Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov–Smirnov test, appropriate tests (Spearman's rank correlation, ANOVA, repeated measures analysis and independent *t* test) were used for further analysis. A *p* value  $P < 0.05$  was regarded as significant.

#### Results

Summary results of the motility parameters analysed are shown in Table 1. Although total and progressive motility were not affected by increasing concentrations of CRE, significant dose-dependant effects on various motion parameters were obvious. While the values for VAP, VSL and linearity decreased with increasing concentrations of CRE, the percentage of hyperactivated sperm and the beat cross-frequency increased significantly (Table 1). The VCL of the sperm cells and the straightness of the motion path remained unchanged. In addition, the treatment for human spermatozoa with increasing concentrations of CRE did not have an effect on the percentage of normal, nonapoptotic spermatozoa (Annexin V(-)/DAPI(-), the percentage of spermatozoa with intact mitochondrial membrane potential ( $\Delta\psi_m$ ) as well as on sperm viability (Table 2).

However, incubation of the germ cells with increasing concentrations of the extract caused a significant (ANOVA:  $P < 0.0001$ ), dose-dependent increase in the percentage of ROS-positive spermatozoa (Fig. 1). This increase goes along with significant (ANOVA:  $P < 0.0001$ ), also dose-dependent increases, in sperm DNA damage (Fig. 2) and the percentage of capacitated spermatozoa (Fig. 3). In

**Table 1** Summary statistics of sperm motility parameters. While total and progressive motility remained unchanged, parameters that are associated with sperm hyperactivation changed significantly

	Control	0.05 µg ml <sup>-1</sup>	0.5 µg ml <sup>-1</sup>	5 µg ml <sup>-1</sup>	50 µg ml <sup>-1</sup>	200 µg ml <sup>-1</sup>	ANOVA ( <i>P</i> value)
Total motility (%)	52.33 ± 19.13	47.47 ± 17.38	51.95 ± 18.51	54.86 ± 20.45	50.14 ± 17.59	51.90 ± 18.68	0.296
Progressive motility (%)	21.39 ± 12.45	19.53 ± 9.84	19.39 ± 8.89	20.62 ± 9.80	19.98 ± 9.89	18.97 ± 8.70	0.736
VAP (µm s <sup>-1</sup> )	62.71 ± 20.30	59.56 ± 19.70	58.87 ± 19.06	58.39 ± 17.95	56.03 ± 19.11	50.56 ± 14.27	0.005
VCL (µm s <sup>-1</sup> )	89.38 ± 21.19	88.13 ± 20.91	87.12 ± 22.48	88.91 ± 21.58	88.72 ± 23.01	85.83 ± 21.15	0.935
VSL (µm s <sup>-1</sup> )	52.07 ± 20.15	48.13 ± 18.36	47.71 ± 17.70	47.69 ± 19.02	44.90 ± 17.96	41.35 ± 16.05	0.02
Hyperactivation	1.54 ± 0.71	1.60 ± 0.76	1.64 ± 0.75	1.68 ± 0.98	1.81 ± 0.78	2.04 ± 0.85	0.004
BCF (Hz)	19.27 ± 2.36	20.10 ± 4.02	19.81 ± 3.46	20.38 ± 2.93	20.62 ± 3.06	22.00 ± 3.12	<0.001
LIN	55.60 ± 10.20	54.25 ± 11.83	53.80 ± 11.59	53.18 ± 11.71	50.36 ± 11.83	46.61 ± 8.90	<0.001
STR	76.52 ± 8.56	76.11 ± 9.66	75.26 ± 11.20	76.97 ± 8.37	75.84 ± 8.60	74.42 ± 8.35	0.629

VAP, velocity average path; VCL, velocity curvilinear; VSL, velocity straight line; BCF, beat cross-frequency; LIN, linearity; STR, straightness.

**Table 2** Summary statistics of the functional sperm parameters of human spermatozoa after incubation with increasing concentrations of *Cissampelos capensis* extract. No effect on Annexin V binding,  $\Delta\Psi_m$  and sperm viability can be seen

	Control	0.05 µg ml <sup>-1</sup>	0.5 µg ml <sup>-1</sup>	5 µg ml <sup>-1</sup>	50 µg ml <sup>-1</sup>	200 µg ml <sup>-1</sup>	ANOVA ( <i>P</i> value)
Normal, nonapoptotic sperm (Annexin V (-)/DAPI (-)) (%)	53.81 ± 8.17	53.47 ± 7.99	53.67 ± 6.22	52.58 ± 6.94	53.53 ± 7.29	53.83 ± 6.89	0.981
Sperm with intact $\Delta\Psi_m$ (%)	57.97 ± 12.05	56.80 ± 13.78	56.05 ± 13.03	56.87 ± 13.31	56.60 ± 13.36	59.57 ± 13.156	0.864
Viability (%)	62.49 ± 12.57	61.94 ± 13.13	63.29 ± 14.45	64.66 ± 13.39	65.19 ± 12.81	64.24 ± 13.17	0.664

$\Delta\Psi_m$ , mitochondrial membrane potential.

addition, the percentage of ROS-positive sperm correlates significantly positively with sperm DNA damage ( $r = 0.232$ ;  $P = 0.0017$ ) as well as with the percentage of capacitated spermatozoa ( $r = 0.176$ ;  $P = 0.0087$ ).

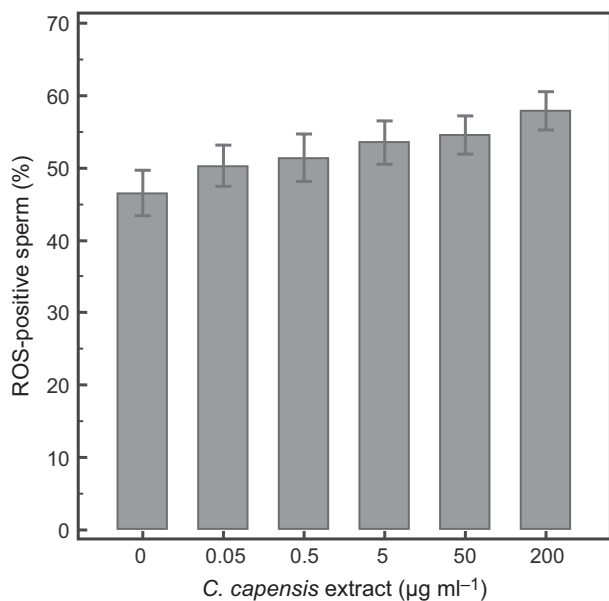
In contrast, acrosome reaction of capacitated spermatozoa as determined by the CTC test (Fig. 4) appeared to reveal only a marginal increase. While the difference between the control and the highest concentration (200 µg ml<sup>-1</sup>) of the extract used is only marginally significant (ANOVA:  $P = 0.0718$ ), the result obtained after ANOVA is not significant ( $P = 0.229$ ). Yet, an ANOVA trend analysis after repeated measures reveals a significant trend ( $P = 0.003$ ). On the other hand, acrosome reaction as determined by means of the Triple stain showed, although there is a marginal ( $P = 0.082$ ) decline after incubation with 5 µg ml<sup>-1</sup> of the extract as compared with the control, no effect of the extract is obvious (ANOVA:  $P = 0.850$ ) (Fig. 5). There was also no difference between the control and the highest concentration used ( $P = 0.9846$ ).

## Discussion

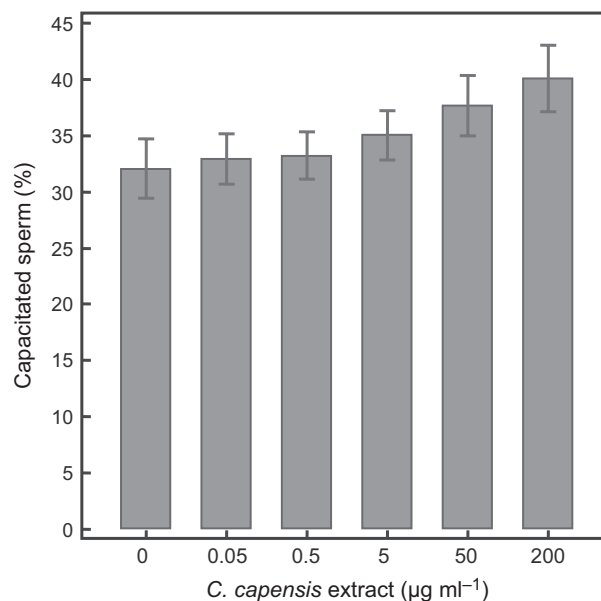
The use of herbal remedies to treat ailments is as old as the humankind itself. Several diverse lines of evidence

indicate that herbal medicines assimilate the oldest and most widespread form of medication (Vogel, 1991). Furthermore, the regulation and legislation of herbal medicines and traditional healing differs from country to country. In South Africa, the traditional health care service is regulated by the Traditional Health Practitioners Act No. 22 of 2007, which provides for a regulatory framework, which ensures the efficacy, safety and quality of the treatment. The high frequency of use of traditional remedies is due to the socio-cultural and socio-economical context in such countries. Yet, this type of medicine has barely been studied scientifically (Castleman, 1997; Halberstein, 2005). In spite of the great progress in modern Western medicine, plants or plant-based active substances are still used as a contribution in health care, even in Western medicine. It is estimated that an average of 25% of modern medicines originated directly or indirectly from medicinal plants (de Smet, 1997), sometimes even without the clinician knowing (Castleman, 1997). Reportedly, for anticancer and anti-infective drugs, this proportion is at over 60% (Cragg et al., 1997).

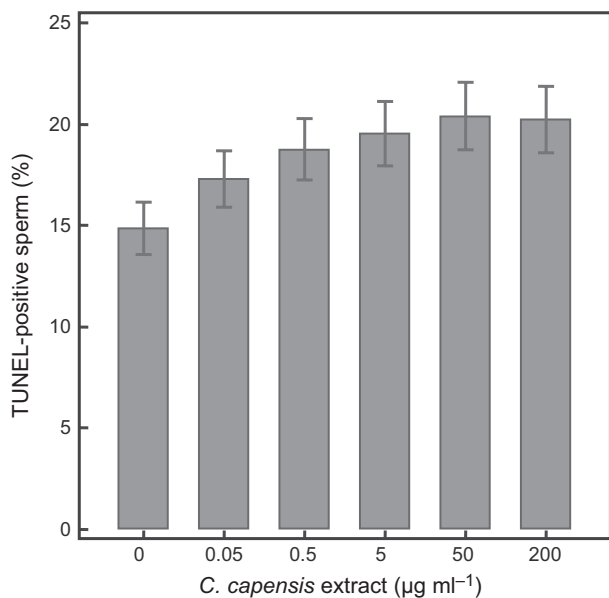
As the *C. capensis* rhizome extract (CRE) is used by traditional healers to treat male reproductive problems and no studies are available that investigated the effects of CRE



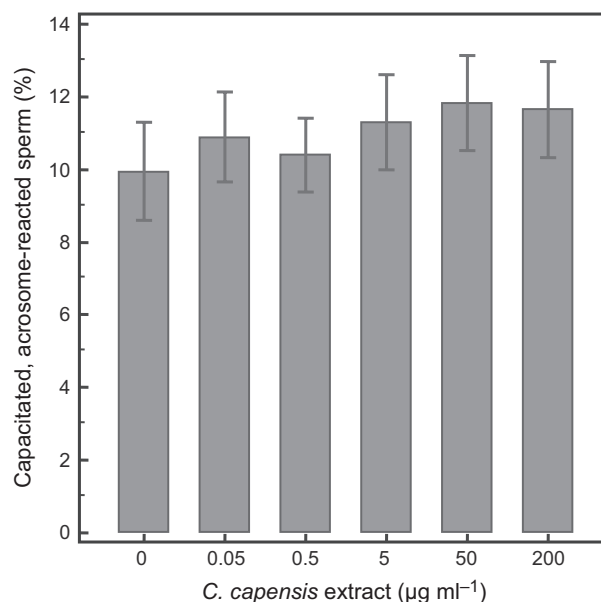
**Fig. 1** Effect of different concentrations of *Cissampelos capensis* extract on the percentage of ROS-positive spermatozoa. A significant, dose-dependent increase (ANOVA:  $P < 0.001$ ) towards higher percentages of ROS-positive sperm cells can be seen at higher concentrations of the *C. capensis* extract. The control differs significantly different from the incubation with 200 µg ml<sup>-1</sup> ( $P < 0.0001$ ).



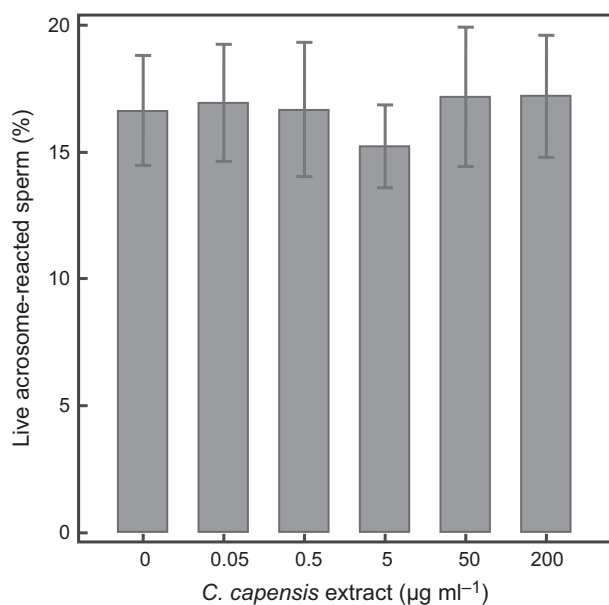
**Fig. 3** Effect of different concentrations of *Cissampelos capensis* extract on the percentage of capacitated spermatozoa *in vitro*. A significant, dose-dependent increase (ANOVA:  $P < 0.001$ ) towards higher percentages of capacitated sperm cells can be seen at higher concentrations of the extract. The control differs significantly different from the incubation with 200 µg ml<sup>-1</sup> ( $P < 0.0001$ ).



**Fig. 2** Effect of different concentrations of *Cissampelos capensis* extract on sperm DNA fragmentation *in vitro*. A significant, dose-dependent increase (ANOVA:  $P < 0.001$ ) towards higher percentages of spermatozoa with DNA damage can be seen at higher concentrations of the *C. capensis* extract. The control differs significantly different from the incubation with 200 µg ml<sup>-1</sup> ( $P < 0.0001$ ).



**Fig. 4** Effect of different concentrations of *Cissampelos capensis* extract on the percentage of capacitated, acrosome-reacted spermatozoa *in vitro* as determined by the CTC test. Increasing concentrations of the *C. capensis* extract seem to have only a marginal direct effect (ANOVA:  $P = 0.229$ ) on acrosome reaction because the control does not significantly ( $P = 0.0718$ ) differ from the incubation with 200 µg ml<sup>-1</sup>. Yet, a repeated measures analysis reveals a trend ( $P = 0.0030$ ).



**Fig. 5** Effect of different concentrations of *Cissampelos capensis* extract on the percentage of live, acrosome-reacted spermatozoa *in vitro* as determined by the Triple stain. Increasing concentrations of the *C. capensis* extract do not have an effect (ANOVA:  $P = 0.850$ ) on acrosome reaction. The control does not differ ( $P = 0.9846$ ) from the incubation with 200 µg ml<sup>-1</sup>.

on human spermatozoa, this study is the first one throwing some light on the effects of this extract on ejaculated human spermatozoa *in vitro*. This plant is the only endemic species in its family found in South Africa that grows during the winter season. The medicinal value of the rhizomes is mainly attributed to their alkaloid content; thus, it is available throughout the year. While the leaves contain mainly three alkaloids namely bulbocapnine, dicentrine and salutaridine with antimicrobial, antibacterial, antifungal and anti-inflammatory activity, respectively, the rhizomes contain mainly bisbenzyltetrahydroisoquinoline alkaloids, with cissacapine, 12-O-methylcurine and cycleanine as main alkaloids with average alkaloid yields of 15.3%, 35.9% and 46.3% respectively (de Wet *et al.*, 2011).

Generally, alkaloids can exhibit various effects on cells ranging from suppressing intracellular ROS production (Zhao *et al.*, 2012), antiproliferative effects (Slunska *et al.*, 2010), loss of mitochondrial membrane potential with intracellular ROS imbalance (Chiu *et al.*, 2012) to antioxidative effects (Jung *et al.*, 2009). For ergot (fungi of the genus *Claviceps* growing on cereals) alkaloids, which can cause ergotism, a significant inhibitory effect on calcium ionophore-induced acrosome reaction was shown *in vivo* on stallion sperm (Fayrer-Hosken *et al.*, 2012).

With respect to the bisbenzyltetrahydroisoquinoline alkaloids, not much has been published. Cycleanine has been shown to interact with the middle domain of

heat-shock protein 90α (Hsp90α) (Haginaka *et al.*, 2013), and has, like 12-O-methylcurine (Guedes *et al.*, 2002) and other alkaloids of this group (Tur *et al.*, 2000), calcium antagonist activities in smooth muscle cells (Martinez *et al.* (1998). This inhibition of calcium channels could be the reason why acrosome reaction did not or only marginally increased, although capacitation was significantly stimulated in the present study. In turn, bisbenzyltetrahydroisoquinoline alkaloids have been shown to selectively inhibit mitochondrial complex I (Granell *et al.*, 2004), which results in an intracellular increase in hydrogen peroxide and superoxide in various tissues (St. Pierre *et al.*, 2010). Even in human spermatozoa, disruption of the mitochondrial electron transport flow leads to the generation of ROS from complex I (Koppers *et al.*, 2008). It also appears that the dose-dependent increase in intracellular, intrinsic superoxide production as observed by the DHT test was sufficient to induce DNA damage, but not to inhibit sperm motility as reported by others (Koppers *et al.*, 2008). This would be consistent with the previous observations (Henkel *et al.*, 2005).

On the one hand, elevated ROS levels have repeatedly been associated with male infertility (Aitken *et al.*, 1989; Agarwal *et al.*, 2003; Henkel *et al.*, 2004, 2005), including loss of sperm motility and DNA damage. On the other hand, low levels of ROS are essential for normal sperm function in terms of the initiation of capacitation and acrosome reaction (O'Flaherty *et al.*, 2006a) as well as the relevant triggering and modulation of protein tyrosine phosphorylation involved in these events (O'Flaherty *et al.*, 2006b). In this case, increasing intracellular superoxide concentrations might have triggered capacitation. However, the subsequent steps in terms of the execution of acrosome reaction did not take place. This result can have different interpretations. Firstly, acrosome reaction was not distinctly stimulated by a specific trigger such as zona pellicida, calcium ionophore or low temperature. Secondly, bisbenzyltetrahydroisoquinoline alkaloids including 12-O-methylcurine and cycleanine have been described as potent Ca-antagonists (Martinez *et al.*, 1998; Guedes *et al.*, 2002). Because Ca<sup>2+</sup> influx triggers acrosome reaction (Storey *et al.*, 1992), the alkaloids might have stimulated capacitation but inhibited the initiation of acrosome reaction.

In a recent report (Haginaka *et al.*, 2013), it has been shown that biscoclaurine alkaloids including cycleanine, which is a compound of the *C. capensis* rhizomes, interact with the middle domain of heat-shock protein 90α (HSP90α), suggesting inhibitory functions of these alkaloids on this highly conserved molecular chaperone protein. HSP90α is an abundant cellular protein, which is induced under stress conditions (Richter & Buchner, 2001; Picard, 2002). With regard to the triggering of capacitation and initiation of acrosome reaction, one also

has to consider that HSP90 plays a significant role in the signal transduction pathways of a variety of cellular processes including spermatogenesis (Gruppi *et al.*, 1991).

This protein is a target for tyrosine phosphorylation in human sperm cells (Ecroyd *et al.*, 2003). In turn, tyrosine phosphorylation of this protein is an essential step in the activation of nitric oxide synthase in endothelial cells (Harris *et al.*, 2000; Brouet *et al.*, 2001), and ROS including NO have been shown to be a second messenger for sperm capacitation (de Lamirande & O'Flaherty, 2008). Moreover, bisbenzylisoquinoline alkaloids have been shown to suppress nitric oxide production in activated macrophages (Kondo *et al.*, 1993). Thus, HSP90 might be an important element of the signalling pathway of sperm capacitation, and an inhibition thereof by the *C. capensis* rhizome alkaloids would result in a blockage of capacitation. Yet, the opposite was the case, as a dose-dependent increase in the percentage of capacitated spermatozoa was observed. This result is corroborated by a significant and dose-dependent increase in sperm hyperactivation as indicated by all the relevant parameters involved (decreased VAP, VSL and LIN; increased BCF). The increase in the proportion of capacitated spermatozoa would then have to be attributed to elevated levels of intrinsic superoxide, which has also been demonstrated to trigger these processes (de Lamirande & O'Flaherty, 2008).

In conclusion, data presented in this study suggest that an aqueous extract of *Cissampelos capensis* rhizomes causes a dose-dependent increase in sperm intrinsic superoxide production leading to sperm capacitation and DNA fragmentation, while acrosome reaction, total motility, sperm viability and mitochondrial membrane potential appear not to be affected. These processes might have been triggered by bisbenzyltetrahydroisoquinoline alkaloids, which represent the main compounds of the extract. Further studies will have to elucidate the direct action of these alkaloids on sperm function as well as the therapeutic use and safety of this herbal extract for the treatment for male infertility.

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