

## **In vivo effects of *Eurycoma longifolia* Jack (Tongkat Ali) extract on reproductive functions in the rat**

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### **Abstract:**

An aqueous extract of *Eurycoma longifolia* (Tongkat Ali; TA) roots is traditionally used to enhance male sexuality. Because previous studies are limited to only few sperm parameters or testosterone concentration, this study investigated the *in vivo* effects of TA on body and organ weight as well as functional sperm parameters in terms of safety and efficacy in the management of male infertility. Forty-two male rats were divided into a control, low-dose (200 mg kg<sup>-1</sup> BW) and high-dose (800 mg kg<sup>-1</sup> BW) group ( $n = 14$ ). Rats were force-fed for 14 days and then sacrificed. Total body and organ weights of the prostate, testes, epididymides, gastrocnemius muscle and the omentum were recorded. Moreover, testosterone concentration, sperm concentration, motility, velocity, vitality, acrosome reaction and mitochondrial membrane potential (MMP) were assessed. Whilst TA decreased BW by 5.7% ( $P = 0.0276$ ) and omentum fat by 31.9% ( $P = 0.0496$ ), no changes in organ weights were found for the prostate, testes and epididymides. Testosterone concentration increased by 30.2% ( $P = 0.0544$ ). Muscle weight also increased, yet not significantly. Whilst sperm concentration, total and progressive motility and vitality increased significantly, MMP improved markedly ( $P = 0.0765$ ) by 25.1%. Because no detrimental effect could be observed, TA appears safe for possible treatment of male infertility and ageing male problems.

### **Introduction**

The World Health Organization (WHO) defines traditional medicine as ‘the knowledge, skills and practice of holistic care, recognized and accepted for its role in the maintenance of health and the treatment of diseases, based on indigenous theories handed down from generation to generation for its beliefs and experiences’ (WHO, 2000). An estimated 60–80% of the population in nonindustrialised countries rely upon traditional medicine for their basic healthcare needs due to its accessibility and affordability. In the United States and Europe, approximately 34% of people are using traditional medicine exclusively or in conjunction with ‘modern medicine’ (Eisenberg et al., 1993; Vincent & Furnham, 1999). Reproduction is a dual commitment (Mbizvo & Bassett, 1996), and in 2003, more than 186 million couples in developed countries were reported to be suffering from infertility (WHO, 2003). Given that the male factor contributes approximately 30–50% to fertility-related cases

(Hull et al., 1985) and that almost 50% of male infertility cases are classified as idiopathic (Sherins, 1995), investigating alternative therapies to manage male infertility may prove cost-effective and may provide the patient with a holistic approach to medicine.

In Malaysia and South-East Asia, the root of *Eurycoma longifolia* Jack, an evergreen plant, belongs to the family Simaroubaceae. Locally in Malaysia, the plants are known as Tongkat Ali, which literally means 'Ali's walking stick' (Jaganath & Ng, 2000). Its medicinal value relates to the ground where it is found, and higher concentrations of phytochemical compounds are found in the Malaysian Peninsular Tongkat Ali (TA) when compared with TA found elsewhere, such as in Thailand, Vietnam or Indonesia. The roots contain a wide variety of chemical compounds including eurycomaride, tannins, high molecular weight polysaccharides, glycoproteins and mucopolysaccharides, as well as alkaloids of the quassinoid group (Jiwajinda et al., 2001; Miyake et al., 2009). Compounds of the latter group, for example, quassin can be used in food because of its extremely bitter taste (50 times more bitter than quinine) (Council of Europe, 1991).

The process of consuming the root also follows the traditional way, wherein the roots are cut into smaller portions, boiled and consumed as a tea but produces bitterness (Bhat & Karim, 2010). Therefore, traditional healers suggest mixing the extract (chipped roots) with honey, sugar syrup or dates for reducing the bitterness (Bhat & Karim, 2010). However, a few experienced traditional healers claim that 'the more the bitterness' – the 'better' is the efficacy.

Concoctions and extracts of the Tongkat Ali roots are most popular and famous for numerous ailments including malaria (Chan *et al.*, 1986; Kardono *et al.*, 1991), ulcers (Tada *et al.*, 1991), fevers and as an aphrodisiac (Ang & Sim, 1997, 1998a,b; Ang & Ngai, 2001) as it has androgenic and profertility properties (Hamzah & Yusof, 2003; Tambi & Kadir, 2006; Tambi & Imran, 2010). Nevertheless, studies investigating the *in vivo* effects of treatment with this extract on male reproductive functions, particularly its effects on spermatozoa, are limited to sperm motility and sperm concentration or to the serum testosterone concentration. Therefore, this study aimed at investigating the effect of a patented, water-soluble extract of Tongkat Ali in a broader manner on general wellbeing, organ and total body weight, sperm concentration and viability. In addition, sperm motility, various sperm velocity parameters and acrosome reaction and mitochondrial membrane potential as functional sperm parameters were investigated in an animal model using rats.

## **Materials and methods**

### **Animals used and ethical clearance**

For all experiments conducted within this study, ethical clearance from the Institutional Review Board at the University of the Western Cape, Bellville, South Africa, was obtained. Forty-two adult male Sprague–Dawley rats weighing between 300–350g were kept in standard cages within the animal facilities of the Department of Medical Bioscience with temperature ranging between 20 and 23 °C.

Animals were fed with standardised rat feed and water *ad libitum*, and standard laboratory conditions 12-h light and 12-h dark were maintained.

### **Force feeding and sacrificing of rats**

A powdered, patented (patent number: W00217946) and standardised water-soluble extract of Tongkat Ali (TA; Physta<sup>TM</sup>) was provided by Biotropics Malaysia Berhad (Kuala Lumpur, Malaysia). The powdered extract was dissolved in normal tap water and force-fed to the animals in a volume of 200 ml to a concentration of 200 mg kg<sup>-1</sup> body weight (low dose) and 800 mg kg<sup>-1</sup> body weight (high dose). The group of rats that served as control was given the same amount of tap water. The rats were segregated into a control and two experimental groups with 14 animals per group and treated for 14 days. Body weights of rats were recorded, and the overall well-being of the animals was monitored throughout the feeding process. At the end of the feeding period, animals were sacrificed by means of cervical dislocation, and the testes, epididymis, seminal vesicles and prostate gland were excised immediately, cleared of adhering fat and extraneous tissue, and the respective organ weights recorded.

### **Lean muscle mass**

The gastrocnemius muscle of the male rats was isolated for the control and dosage groups to determine whether treatment with the TA extract had an influence on lean muscle mass.

### **Fat mass**

The omentum fat from the greater curvature of the stomach was isolated in male rats of the control and dosage groups to determine whether treatment with the TA extract had an effect on adipose tissue mass.

### **Isolating the epididymides**

The cauda epididymis was immediately isolated after cervical dislocation of the animals and placed in a Petri dish containing 1 ml DMEM (Dulbecco's modified Eagle's medium, Gibco, Auckland, New Zealand) supplemented with 1% BSA (Bovine Serum Albumin, Sigma, St. Louis, USA). One millilitre of the above medium was placed in a second Petri dish, and a section of the cauda epididymis was isolated in this dish that remained in an incubator (Lasec, Cape Town, South Africa) at 37 °C to allow the spermatozoa to 'swim out' into the medium for approximately 10 s.

### **Sperm count**

The sperm count was estimated using the Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel). One drop of cauda epididymal spermatozoa was diluted 1 : 100 in DMEM supplemented with 1% BSA and placed in the centre of the lower disc and further examined with a microscope (Zeiss, Oberkochen, Germany) using the 209 objective under phase contrast. The number of spermatozoon was calculated by counting five rows of ten squares, and an average concentration was calculated in millions per millilitre.

### **Determination of sperm motility and vitality**

Following the 'swim out' of spermatozoa into the medium, a 20- $\mu$ l sample was taken from the periphery of the sample and placed onto a slide (Leja slides, Nieuw Ver- nep, the Netherlands), and the velocity curve line (VCL), velocity straight line (VSL) and velocity average path (VAP) ( $\mu\text{m s}^{-1}$ ), as well as the percentages of total and progressive motile spermatozoa were determined using the Motility/Concentration module of the Sperm Class Analyzer version 4.1.0.1 (Microptic S.L., Barcelona, Spain) with the 49 objective under phase contrast.

### **Determination of sperm vitality**

The DUO-VITAL kit (Cantoblanco, Madrid, Spain) was used for sperm vitality assessment in the rat. Five microlitre of diluted sperm sample was aspirated and added to a clean slide. Subsequently, 1 $\mu$ l of the red and 1 $\mu$ l of the green fluorochrome solution were added to the sample and mixed together. The sample was then covered with a clean coverslip and observed with the 259 objective under fluorescence (Zeiss, Oberkochen, Germany). A total of 100 spermatozoa were counted and calculated as a percentage. Sperm cells that fluoresced green were 'scored' as live, and cells that fluoresced red were 'scored' as dead. Cells that fluoresced as green-red were 'scored' as dead due to incipient loss of membrane integrity.

### **Assessment of the acrosomal status**

The status of the acrosome was assessed as described by Larson & Miller (1999). In brief, 50 $\mu$ l of rat epididymal spermatozoa of the control and treatment groups obtained with the 'swim out' method was fixed for 10 min with 50 $\mu$ l of 4% p-formaldehyde (Sigma-Aldrich, St. Louis, USA) solution in PBS (Oxoid, Hampshire, UK) at pH 7.4 and centrifuged at 2500 g for 5 min at room temperature. The supernatant was then removed and the sample resuspended in 50  $\mu$ l of 10 mM ammonium acetate (Sigma-Aldrich) at pH 9.0. Subsequently, a droplet of the spermatozoa was smeared on slides and air-dried. Afterwards, spermatozoa were stained for 2 min at room temperature with 0.22% Coomassie Brilliant Blue G-250 (Sigma-Aldrich) solution prepared in 50% methanol (Sigma-Aldrich) and 10% acetic acid (Merck, Gauteng, South Africa). Stained slides were washed with distilled water, air-dried, mounted with a drop of glycerol, covered with a coverslip and observed under a bright-field microscope (Lasec, Cape Town, South Africa). A total of 100 spermatozoa were counted, and those with intact acrosome exhibited blue stain over both dorsal (convex) and ventral (concave) surfaces of the spermatozoon head, whilst spermatozoa stained only over the ventral surface were regarded as acrosome reacted. The percentage of acrosome-reacted spermatozoa was calculated.

### **Determination of the mitochondrial membrane potential ( $D_{wm}$ )**

The percentage of spermatozoa with intact mitochondrial membrane potential ( $D_{wm}$ ) was determined by means of the DePsipher kit (R&D Systems, Abingdon, UK) 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 millilitre 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. Afterwards, 50  $\mu$ l of this supernatant was added to 50- $\mu$ l washed spermatozoa and incubated for 20 min at 37 °C. After incubation, 10  $\mu$ l of each sample was analysed at 1000 $\times$  magnification using an epifluorescence microscope (Zeiss). A total of 100 spermatozoa were counted, and those exhibiting a green fluorescence within their mid-pieces were regarded as having disturbed Dw<sub>M</sub>, whilst those spermatozoa showing red fluorescence were regarded as having intact Dw<sub>M</sub>. The percentage of spermatozoa with intact Dw<sub>M</sub> was calculated.

### **Determination of serum testosterone concentration**

For the determination of the serum testosterone concentration, the Testosterone ELISA kit (DRG Instruments GmbH, Marburg, Germany) was used and the manufacturer's procedure was followed. In brief, 25  $\mu$ l of the standards, controls and respective dosage groups was dispensed into the wells of the 96-well plate, which was provided with the kit. Next, 200  $\mu$ l of conjugate enzyme was added to the wells containing the standards and samples. The plate was then placed on a stirrer for an hour. After this step was completed, a washing buffer was prepared by adding 30 ml of buffer to 1170 ml distilled water. The contents of the wells were briskly shaken out after stirring and then thoroughly submerged and washed three times with the diluted washing buffer. Following this step, the plate was tapped dry on tissue paper so that no droplets remained in the wells. Subsequently, 200  $\mu$ l of substrate at 37 °C (tetramethylbenzidine) was added to the wells. The plate was then incubated for 15 min at room temperature. Afterwards, a 100  $\mu$ l of stop solution (0.5 M H<sub>2</sub>SO<sub>4</sub>) was added to stop the enzymatic reaction, and the plate was then inserted into a plate reader (DRG Instruments Microtiter Plate Reader) to determine the absorbance of each well at 450  $\pm$  10 nm. Finally, concentrations were calculated according to the standard curve.

### **Statistical evaluation**

All statistical calculations were performed using the MedCalc statistical software (version 12.2.1; Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov–Smirnov test, parametric tests (paired Student's *t*-test and ANOVA for trend analysis) were used for further analysis. A *P*-value of <0.05 was considered significant.

### **Results**

Tables 1 and 2 depict the summary statistics of the bodily parameters including serum testosterone concentrations and the sperm parameters taken in this study.

Following the treatment of the animals, no changes could be found for the weights of the testes, epididymides, prostate and the gastrocnemius muscle. Yet, remarkable and significant changes, respectively, were observed for the body weight, the omentum fat mass and the serum testosterone concentration. Whilst in direct comparison with the control, the body weight for both treatment concentrations (200 and 800 mg kg<sup>-1</sup>) showed significant decreases (Fig. 1), this

was not the case for the omentum fat mass (Fig. 2). Treatment with 800 mg TA/kg body weight resulted in a decrease in the body and omentum fat weight of 5.7% and 31.9% respectively. An ANOVA trend analysis, however, showed significant results for the body weight ( $P = 0.0276$ ) and the omentum fat mass ( $P = 0.0496$ ). In turn, the serum testosterone concentration increased by 30.2% in the high-dose group (800 mg kg<sup>-1</sup>). Although the ANOVA trend analysis did not give a significant result ( $P = 0.0544$ ), this increase is remarkable.

For the semen parameters, sperm concentration, vitality as well as total and progressive motility, significant improvements were observed (Figs 3–6). The increases in the nominal values from the control to the high-dose group were 54.9%, 21.9%, 3.5% and 8.3% respectively. For all these parameters, significant trends were found (sperm concentration:  $P < 0.0001$ ; vitality:  $P = 0.0156$ ; total motility:  $P = 0.0458$ ; progressive motility:  $P = 0.0232$ ).

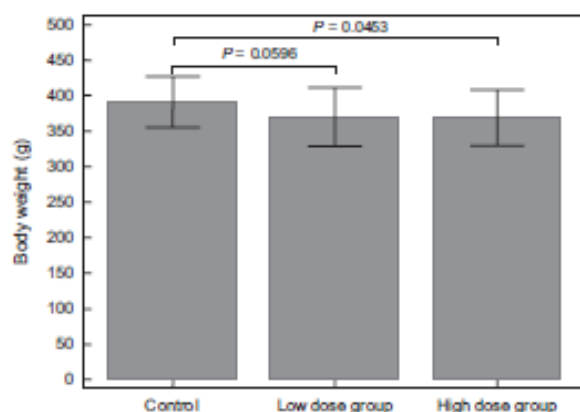
For the sperm parameters analysed in this study, significant trends towards increased values after the treatment could only be observed for VAP ( $P = 0.0219$ ), VCL ( $P = 0.0298$ ) and VSL ( $P = 0.0107$ ). The respective per cent increases from the control group to the high-dose group were 11.2%, 10.6% and 13.2% respectively. For the percentage of spermatozoa with intact mitochondrial membrane potential, a remarkable increase of 25.1% from the control to the high-dose value could be observed (Fig. 7). However, the ANOVA trend analysis did not yet reach significance level ( $P = 0.0765$ ). On the other hand, acrosome reaction as determined by the percentage of nonacrosome-reacted spermatozoa did not seem to be affected (ANOVA trend analysis:  $P = 0.2186$ ).

**Table 1** Summary statistics of bodily parameters including the serum testosterone concentration taken in this study

Parameter	<i>n</i>	Mean ± SD	Median	Range
Body weight (control) [g]	24	391.39 ± 36.03	397.05	312.00–452.09
Body weight (low dose) [g]	24	369.87 ± 41.01	369.00	289.09–438.84
Body weight (high dose) [g]	24	369.09 ± 38.97	374.38	263.45–430.89
Testes (control) [g]	24	1.72 ± 0.11	1.68	1.56–1.94
Testes (low dose) [g]	24	1.84 ± 0.18	1.80	1.56–2.18
Testes (high dose) [g]	24	1.75 ± 0.15	1.75	1.25–1.97
Prostate (control) [g]	24	0.67 ± 0.14	0.69	0.37–0.95
Prostate (low dose) [g]	24	0.57 ± 0.12	0.56	0.38–0.85
Prostate (high dose) [g]	24	0.65 ± 0.16	0.65	0.29–0.98
Epididymis (control) [g]	24	0.62 ± 0.08	0.60	0.43–0.79
Epididymis (low dose) [g]	24	0.62 ± 0.06	0.62	0.50–0.70
Epididymis (high dose) [g]	24	0.58 ± 0.06	0.58	0.38–0.68
Omentum fat (control) [g]	10	0.47 ± 0.16	0.42	0.27–0.70
Omentum fat (low dose) [g]	10	0.35 ± 0.26	0.22	0.14–0.93
Omentum fat (high dose) [g]	10	0.32 ± 0.23	0.25	0.13–0.84
Gastrocnemius muscle (control) [g]	10	2.16 ± 0.37	2.25	1.46–2.70
Gastrocnemius muscle (low dose) [g]	10	2.28 ± 0.17	2.34	1.93–2.47
Gastrocnemius muscle (high dose) [g]	10	2.23 ± 0.19	2.17	2.00–2.55
Testosterone concentration (control) [ng ml <sup>-1</sup> ]	10	0.86 ± 0.34	0.83	0.35–1.54
Testosterone concentration (low dose) [ng ml <sup>-1</sup> ]	10	0.77 ± 0.29	0.75	0.23–1.20
Testosterone concentration (high dose) [ng ml <sup>-1</sup> ]	10	1.12 ± 0.52	0.89	0.43–1.74

**Table 2** Summary statistics of sperm parameters taken in this study

Parameter	n	Mean ± SD	Median	Range
Sperm concentration (control) [ $10^6 \text{ ml}^{-1}$ ]	10	4.74 ± 0.81	4.90	3.80–6.00
Sperm concentration (low dose) [ $10^6 \text{ ml}^{-1}$ ]	10	6.56 ± 1.09	6.80	4.80–7.80
Sperm concentration (high dose) [ $10^6 \text{ ml}^{-1}$ ]	10	7.34 ± 0.89	7.20	6.20–8.80
Vitality (control) [%]	24	53.00 ± 20.30	56.00	10.00–80.00
Vitality (low dose) [%]	24	68.75 ± 15.27	73.00	46.00–90.00
Vitality (high dose) [%]	24	64.58 ± 23.94	68.50	23.00–96.00
Total motility (control) [%]	24	94.60 ± 7.07	97.90	69.70–99.50
Total motility (low dose) [%]	24	98.43 ± 1.59	98.95	94.60–100.00
Total motility (high dose) [%]	24	97.88 ± 1.18	98.15	95.50–99.50
Progressive motility (control) [%]	24	87.63 ± 13.42	92.85	44.20–99.50
Progressive motility (low dose) [%]	24	95.30 ± 3.41	96.10	89.30–99.50
Progressive motility (high dose) [%]	24	94.93 ± 3.69	95.25	80.60–99.50
VAP (control) [ $\mu\text{m s}^{-1}$ ]	24	123.53 ± 29.28	128.80	52.90–158.30
VAP (low dose) [ $\mu\text{m s}^{-1}$ ]	24	140.10 ± 26.73	139.30	107.90–194.50
VAP (high dose) [ $\mu\text{m s}^{-1}$ ]	24	137.42 ± 21.37	137.90	101.60–181.40
VCL (control) [ $\mu\text{m s}^{-1}$ ]	24	337.36 ± 82.95	331.50	184.20–484.30
VCL (low dose) [ $\mu\text{m s}^{-1}$ ]	24	383.25 ± 80.78	360.20	295.00–565.20
VCL (high dose) [ $\mu\text{m s}^{-1}$ ]	24	372.94 ± 62.78	365.75	283.30–498.20
VSL (control) [ $\mu\text{m s}^{-1}$ ]	24	59.97 ± 15.20	60.90	23.00–85.10
VSL (low dose) [ $\mu\text{m s}^{-1}$ ]	24	70.36 ± 17.45	67.65	46.40–109.80
VSL (high dose) [ $\mu\text{m s}^{-1}$ ]	24	67.91 ± 12.83	70.25	48.10–94.70
Intact $\Delta\psi_m$ (control) [%]	14	44.07 ± 21.72	37.50	22.00–97.00
Intact $\Delta\psi_m$ (low dose) [%]	14	52.93 ± 22.68	53.50	21.00–97.00
Intact $\Delta\psi_m$ (high dose) [%]	14	55.14 ± 15.73	51.50	32.00–78.00
Not AR reacted (control) [%]	14	83.07 ± 7.75	84.00	70.00–93.00
Not AR reacted (low dose) [%]	14	81.14 ± 5.91	82.50	72.00–90.00
Not AR reacted (high dose) [%]	14	86.43 ± 5.06	87.50	77.00–93.00

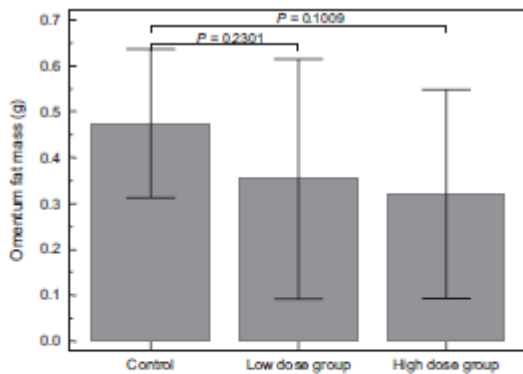


**Fig. 1** Effect of different concentrations of TA on total body weight (mean ± SD) *in vivo*. A significant (ANOVA trend analysis:  $P = 0.0276$ ) decrease in total body weight can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The decrease from the control to the high-dose group is significant ( $P = 0.0435$ ).

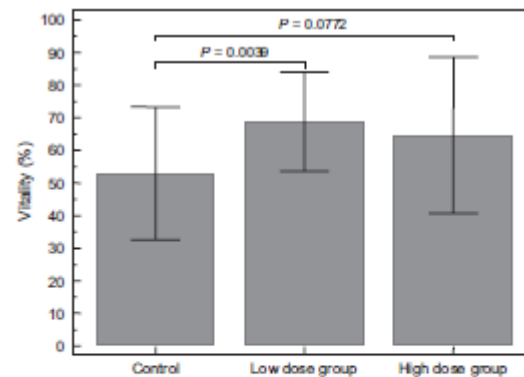
## Discussion

To date, only a small number of studies are published on traditional or complementary medicine, mostly coming from China, India or Japan. In Chinese medicine, more than 450 clinical trials are published investigating the efficacy of traditional medicines on ailments like asthma, cancer or diabetes mellitus (Yuan & Lin, 2000; Pach *et al.*, 2002), and the number of studies investigating the effect of plants or their extracts on male fertility potential is much less (50–60), with only few Asian plants or their extracts being tested for their effect on male fertility (Zheng & Zhang, 1997; Kumi-Diaka & Townsend, 2001; Liu *et al.*, 2004; Tambi & Imran, 2010).

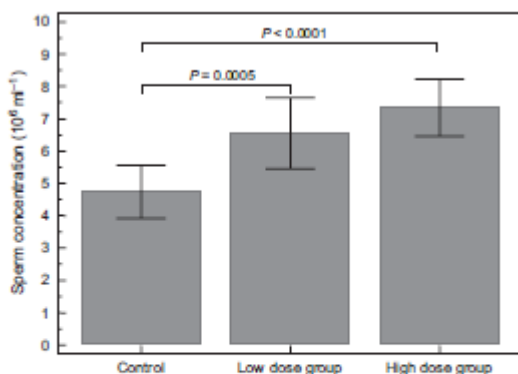
In South-East Asia, the *Eurycoma longifolia* (Tongkat Ali) is very popular and numerous publications on the various effects of this natural remedy are available. With regard to the aphrodisiac and profertility properties of the root extract of Tongkat Ali, some reports on its ability to increase serum testosterone levels have been published (Chan *et al.*, 2009; Zanolli *et al.*, 2009; Tambi *et al.*, 2012; Henkel R, Solomon M, Erasmus N, unpublished). Other studies showed that Tongkat Ali increased sexual motivation and performance (Ang & Sim, 1997, 1998a,b; Ang & Ngai, 2001).



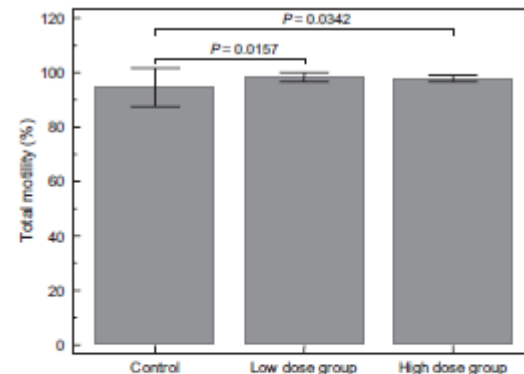
**Fig. 2** Effect of different concentrations of TA on the omentum fat mass (mean  $\pm$  SD) *in vivo*. A significant (ANOVA trend analysis:  $P = 0.0496$ ) decrease in total mass of the omentum fat can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. Although the effective decrease from the control to the high-dose group is 31.9%, the differences are not significant.



**Fig. 4** Effect of different concentrations of TA on the sperm vitality (mean  $\pm$  SD) *in vivo* after swimming out of the epididymis. A significant (ANOVA trend analysis:  $P = 0.0156$ ) increase in the sperm vitality can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The effective increase from the control to the high-dose group is 21.9%.



**Fig. 3** Effect of different concentrations of TA on the sperm concentration (mean  $\pm$  SD) *in vivo* after swimming out of the epididymis. A highly significant (ANOVA trend analysis:  $P < 0.0001$ ) increase in the sperm concentration can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The effective increase from the control to the high-dose group is 54.6%.

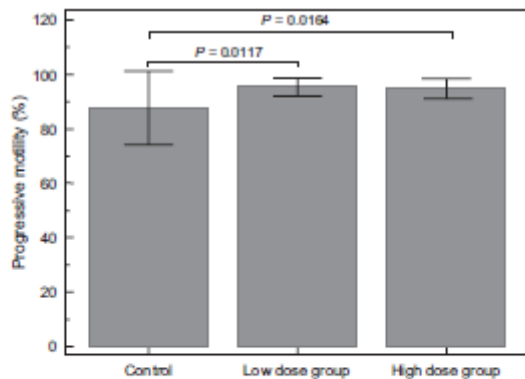


**Fig. 5** Effect of different concentrations of TA on the sperm total motility (mean  $\pm$  SD) *in vivo* after swimming out of the epididymis. A significant (ANOVA trend analysis:  $P = 0.0458$ ) increase in the sperm total motility can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The effective increase from the control to the high-dose group is 3.5%.

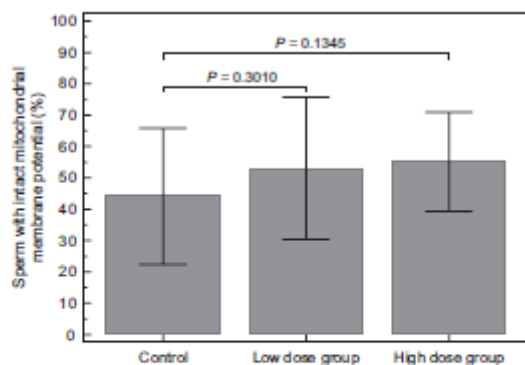
Because studies on the efficacy of treatment with this plant are limited to sperm motility and concentration as well as testosterone levels, this study is aimed at shedding more light on the effects of Tongkat Ali on functional sperm parameters not previously discussed, as well as the general well-being, body and organ weight of the treated animals. As expected and previously reported (Chan *et al.*, 2009; Zanolli *et al.*, 2009; Tambi *et al.*, 2012; Henkel *et al.*, unpublished), treatment of the rats caused a dose-dependent increase in the serum testosterone concentration. The actual biochemical mechanism for this



testosterone-enhancing effect is still not known in detail. However, reports by several authors (Ali & Saad, 1993; Sambandan *et al.*, 2006; Asiah *et al.*, 2007) indicate that a bioactive eurypeptide of 4.3 kDa with aphrodisiac properties may be present in the extract, which may activate CYP17 (17  $\alpha$ -hydroxylase/17,20 lyase). This enzyme increases the conversion of pregnenolone and 17-OH-pregnenolone to yield more dehydroepiandrosterone (DHEA) as well as the metabolism of progesterone and 17-OH-progesterone to 4-androstenedione and testosterone, which accounts for the enhanced total testosterone and dehydroepiandrosterone levels found in human trials (Ali & Saad, 1993).



**Fig. 6** Effect of different concentrations of TA on the sperm progressive motility (mean  $\pm$  SD) *in vivo* after swimming out of the epididymis. A significant (ANOVA trend analysis:  $P = 0.0232$ ) increase in the sperm progressive motility can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The effective increase from the control to the high-dose group is 8.3%.



**Fig. 7** Effect of different concentrations of TA on the percentage of spermatozoa with intact mitochondrial membrane potential (mean  $\pm$  SD) *in vivo* after swimming out of the epididymis. A remarkable (ANOVA trend analysis:  $P = 0.0765$ ) increase in the sperm progressive motility can be seen in the low-dose ( $200 \text{ mg kg}^{-1}$ ) and high-dose ( $800 \text{ mg kg}^{-1}$ ) groups at higher concentrations of TA. The effective increase from the control to the high-dose group is 25.1%.

Studies by Chan *et al.* (2009) showed that the bioavailability of a standardised methanol extract in rats of the quassinoids Eurycomanol and eurycomanone was not detected in rat plasma, but after chronic ingestion of the extract, that eurycomanone alone was detected in testes homogenate of the treated animals. As testosterone is required in maintaining normal sexual function and is vital to spermatogenesis (Yakubu & Afolayan, 2009), the elevated levels of plasma

testosterone in rats treated with 200 mg kg<sup>-1</sup> of BW of a methanol extract in conjunction with the detection of eurycomanone may further suggest that the quassinoid passed through the blood–testis barrier and acted on the Leydig cells to initiate testosterone production. The resultant testosterone could then have elicited further effects on the Sertoli cells by attaching to androgen-binding protein (ABP), which may have enhanced germ cell development, thereby increasing the sperm count in the epididymis (Rommerts, 1992).

This increase in serum testosterone concentration seems to have a significant effect on the body as the body weight of the rats also decreased significantly in a dose-dependent manner. This decrease appears to have occurred by a decrease in fat mass as demonstrated for the omentum fat, which decreased by 32% between the control and high-dose group. In contrast, only a slight, but not significant increase in lean muscle mass could be observed. These results are consistent with those of a study on the activity of ageing male seniors (Henkel *et al.*, unpublished). After 5 weeks of treatment with Tongkat Ali, a significant increase in total (ANOVA trend analysis:  $P = 0.0195$ ) and free (ANOVA trend analysis:  $P = 0.0001$ ) testosterone concentrations and muscular force, determined by the handgrip test (ANOVA trend analysis  $P = 0.0602$ ), was observed.

Whilst the decrease in fat mass occurred rapidly, possibly because of a change in the hormonal status from a catabolic state, dominated by cortisol, towards an anabolic state, dominated by testosterone (Adlercreutz *et al.*, 1986), the increase in lean muscle mass appears to be a gradual process. Given the short treatment period in the present study, a significant increase in lean muscle mass may only be achieved over a longer treatment period. Furthermore, Hamzah & Yusof (2003) showed that the androgenic effect of increased serum testosterone is increased muscle mass, therefore increased potential for generating greater force in the muscles, evidenced by enhanced strength.

Although no or only little effect on the reproductive organs, the testes, prostate and epididymis, respectively, for the sperm parameters investigated, significant increases were observed for all parameters taken in the study except for the acrosome reaction and the mitochondrial membrane potential. Whilst no effect could be seen for the percentage of acrosome-reacted spermatozoa, the trend found in the ANOVA trend analysis for MMP shows borderline significance ( $P =$  results can also be attributed to the increase in serum testosterone concentration and not only confirm the report by Tambi & Imran (2010) that sperm concentration and general motility increase, but also show that motility parameters such as sperm velocity and even the mitochondrial membrane potential, which closely correlated with sperm motility (Kasai *et al.*, 2002) and has been regarded as highly sensitive sperm parameter (Marchetti *et al.*, 2002), can also be positively influenced by a Tongkat Ali treatment.

Particularly, the results for sperm vitality and acrosome reaction obtained in this *in vivo* study appear to be in contrast to those reported by Erasmus *et al.* (2012). Whilst these

authors reported a significant decline in sperm vitality and significantly higher percentages of acrosome-reacted spermatozoa after *in vitro* exposure of human spermatozoa to increasing concentrations of the extract, the *in vivo* treatment reveals a significant improvement in sperm vitality and no negative effect on acrosome reaction, an essential functional sperm parameter respectively. This apparent discrepancy can be explained by the different experimental designs, *in vitro* vs. *in vivo*.

Animal studies on subacute toxicity have shown an LD<sub>50</sub> for the alcohol and the water extract of 2000 and 3000 mg kg<sup>-1</sup> respectively. These studies further indicated that administration with the alcohol extract of up to 200 mg kg<sup>-1</sup> daily was not toxic, and other studies found no toxic effects up to 350 mg kg<sup>-1</sup> daily (Satyavivad *et al.*, 1998; Kuo *et al.*, 2003). Toxicological studies in mice showed that dosages of the aqueous Tongkat Ali extract below 1000 mg kg<sup>-1</sup> body weight caused no toxicity (Azimahtol, 2002). On the other hand, in rats, treatment with Tongkat Ali extract caused hepatotoxicity at dosages above 1200 mg kg<sup>-1</sup> body weight (Shuid *et al.*, 2011). It has also been shown that oral administration of Tongkat Ali is about 100 times less toxic than intraperitoneal injections, probably due to an elimination of the cytotoxic compound in the liver or poor absorption into the blood (Satyavivad *et al.*, 1998; Razak & Aidoo, 2011). Nevertheless, Erasmus *et al.* (2012) also came to the conclusion that the extract has no deleterious effects on functional sperm parameters at therapeutically used concentrations (<2.5 lg ml<sup>-1</sup>). In turn, this is supporting *in vivo* data by Tambi & Kadir (2006) that the extract is not toxic.

In conclusion, this study clearly demonstrates the androgenic and profertility effects, even in terms of sperm functions. The study also supports earlier reports on the safety of the treatment with Tongkat Ali, especially if the therapeutic dosages used for human treatment are taken into consideration. Taken together, this makes this traditional remedy an alternative option in treating and managing idiopathic male infertility. Given the observed testosterone-enhancing effects in the present study, TA may further provide relief to seniors suffering from ageing males' symptoms, thereby improving the quality of life of these patients. Nevertheless, the biochemical mechanism of action of the Tongkat Ali extract warrants further investigation.

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