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Comparative morphophysiological evaluation of the testis of adult Wistar rats fed low protein-energy diet and dosed with aqueous extracts of *Cuscuta australis*

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Summary: *Cuscuta australis* (*C. australis*) seed and stem are historically used by the local population as dietary supplement for the management of infertility. This study, therefore, evaluated the effect of orally administered aqueous extracts of *C. australis* seed and stem, 300mg/kg body weight/day for seven days, on the testis of the adult Wistar rat fed either low or normal protein-energy diets. The control group received water. The relative weight of the testis was non-significantly increased ($p>0.05$) in the Low Protein-energy diet-Water-treated (LPWA), Low Protein-energy diet-Seed-treated (LPSE) and Normal Protein-energy diet-Seed-treated (NPSE) groups relative to the Normal Protein-energy diet-Water-treated (NPWA). The weight of the testis was also non-significantly increased ($p>0.05$) in the Low Protein-energy diet-Stem-treated (LPST), but decreased in the Normal Protein-energy diet-Stem-treated (NPST), relative to LPWA and NPWA. Heights of germinal epithelium were significantly decreased ($p<0.05$) in the LPWA, LPSE and LPST relative to the NPWA, NPSE and NPST. Diet significantly influenced ($p<0.001$) the effect of stem extract on the height of germinal epithelium. The NPSE, LPSE, NPST, LPST and LPWA showed significantly decreased ($p<0.001$) plasma levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) relative to NPWA. The LPWA, LPSE and NPST also showed significantly decreased ($p<0.001$) levels of testosterone relative to NPWA and LPST. Diet significantly influenced ($p<0.001$) the effect of seed on the level of LH. Seed-diet interactions significantly affected the levels of FSH ($p<0.001$) and LH ($p<0.05$), but not testosterone. Diet significantly influenced ($p<0.001$) the effects of stem extract on the levels of FSH, LH and testosterone. Stem-diet interactions significantly affected ($p<0.001$) the levels of FSH, LH and testosterone. Our data suggest that the aqueous extract of *C. australis* stem is more potent than the seed extract and that dietary protein-energy intake may influence the efficacy of orally administered aqueous extracts of *C. australis*.

Keywords: *Cuscuta australis*, Protein-energy malnutrition, Testis, Plasma luteinizing hormone, Follicle stimulating hormone, Testosterone

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INTRODUCTION

Cuscuta australis (*C. australis*) is an annual parasitic vine, commonly called dodder, which wraps around other plants for nourishment and forms the major flora of the tropical East and West Africa, Sudan, Madagascar, Southern Europe, Japan and Australia (Maria, 1987). Welsh *et al.* (2009) reported one hundred and thirty five species and thirteen varieties of Cuscuta. The seed and the stem of *C. australis*, locally named "Omoonigelegele" in Yoruba, are historically used by the local population as dietary supplement for the management of infertility.

The principal flavonoids components of the seed of *C. australis* include quercetin, astragalin, kaempferol and hyperoside, with the total flavone amount being approximately 3.0% in the raw herb (Guo and Li, 1997; Ye *et al.*, 2002; 2005). The seed of *C. australis* predominantly contains both kaempferol and astragalin and the stem also contains a high level of kaempferol (Ye *et al.*, 2002). Although Qin *et al.* (2000) observed that flavonoids extracted from the seed of *Cuscuta chinensis* Lam (*C. chinensis*) increased the weights of testis, epididymis and pituitary gland, and stimulated testosterone and luteinizing hormone secretion both *in vitro* and in immature rats, there is no scientific report documenting the efficacy of *C. australis* and/or the effect of dietary components on the efficacy of *Cuscuta* species.

Malnutrition, according to Asadifar *et al*. (2005), affects absorption, protein binding, distribution, bio-

transformation and renal elimination of xenobiotics / drugs. Lee *et al*. (1997) observed that protein-energy malnutrition modulates the various microsomal cytochrome P450s, the key enzymes that control the rate of drug metabolism, differently. Nutrient restriction in the adult has also been reported to cause decreased gonadotropin production (Clarke and Henry, 1999).

The present study, therefore, was designed to evaluate the effect of aqueous extracts of *C. australis* on the structure and function of the testis of the adult Wistar rat as well as the influence of dietary protein-energy malnutrition on the efficacy of *C. australis* extracts.

MATERIALS AND METHODS

Extract preparations

Mature seeds and stems of *C. australis* were collected from Abeokuta, Ogun State, Nigeria. The samples were identified and deposited at the Herbarium of the Department of Botany, University of Ibadan, Ibadan, Nigeria as UIH-22351. One hundred and fifty grams (150g) of each sample was dried, powdered, decocted, and refluxed three times with 450mL of water, and then filtered. The filtrates were concentrated by rotary vacuum evaporation and then lyophilized with a freeze dryer. The yields of water extracts of the seeds and stems were known to be 15.33% and 13.33% (w/w) respectively. The lyophilized powder (300mg) was dissolved in one millilitre of distilled water before oral administration.

Experimental animals and their feeding

Thirty adult male albino Wistar rats, obtained from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria, were used for this study. These rats were aged between sixteen and twenty weeks and weighed between 200g and 250g. They were maintained in galvanized wire mesh cages in six groups of five animals each. Two feed formulations, Normal Protein-energy (NP, 16.55% total protein, 16.04 $mJkg^{-1}$ gross energy) and Low Protein-energy (LP, 6.21% total protein, 18.11 $mJkg^{-1}$ gross energy) diets (Akingbemi *et al*., 1996) were used throughout the experiment (Table 1). The diets and drinking water were provided *ad libitum*. All the rats were placed on the appropriate diet two weeks before the commencement of the experiment.

Experimental design

The Wistar rats were randomly assigned to the two dietary protein-energy groups: Normal Protein-energy (NP) and Low Protein-energy (LP) diets. Each dietary group was further divided into three subgroups, each containing five rats, as follows: (i) untreated control group that received vehicle, distilled water, only (NPWA, LPWA), (ii) treated group that received aqueous extract of Seed (NPSE, LPSE) and (iii) treated group that received aqueous extract of Stem (NPST, LPST). The untreated/control group of rats received distilled water (1ml/kg body weight) per os, while the treated group of rats received 300mg/kg body weight/day (Yen *et al*., 2007) of their respective extract for seven days.

Sample collection

Each animal was weighed, deeply anaesthetized with 25% Urethane (ethyl carbamate), 0.6ml per 100g given intraperitoneally, and 2ml of blood collected from the medial canthus of the eye into a Lithium-heparinized test tube prior to sacrifice on Day 8 of the experiment. The blood samples were centrifuged, the plasma decanted into Ependorf tubes and stored at -20^{o}C prior to subsequent hormonal assays which were usually conducted within 48 hours. Testes from each rat were weighed and grossly observed for lesions. Tissues of the testis were fixed by immersion in Bouin's fluid for 48 hours.

Histopathological preparations of the testis

Bouin's fluid-fixed testicular tissues were processed routinely and embedded in paraffin wax. Sections, 5µm thick, were cut and stained with haematoxylin and eosin for histopathology and morphometric measurements. In the morphometric measurements, twenty sections of seminiferous tubules that were round or nearly round were chosen randomly and measured in each treatment group. The height of seminiferous tubule epithelium and the tubular (lumen) diameter were measured using micrometric eyepiece 10× and objective 40× in a light microscope. The diameter of the lumen of the seminiferous tubule was measured across the minor and major axes, and the mean diameter obtained.

Hormonal assays

Plasma follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone levels were determined using commercially available radio-immunoassay kits (Fortress® Diagnostics, UK).

Statistical analysis

The data obtained were subjected to one-way Analysis Of Variance (ANOVA) and 2×2 Random Block Design ANOVA. The group means were separated by Duncan's Multiple Range Test (DMRT). Results were presented as group mean ± standard error of mean (SEM). The level of significance was $p<0.05$.

RESULTS

Control versus aqueous extracts of the seed and the stem:

Relative weight of testes

There were no significant differences ($p > 0.05$) between the mean relative weights of the testis in all the treatment groups (Table 2).

Histopathology of testes

The seminiferous tubules of all the low protein-energy diet-treated group (LPWA, LPSE and LPST) showed wide lumen and were lined by low height germinal epithelium, unlike the normal cyto-architecture observed in their respectively paired normal protein-energy diet-treated group (NPWA, NPSE and NPST) as shown in Figures 1 to 3.

Histomorphometry of testes

Heights of the seminiferous tubule epithelium

Heights of the seminiferous tubule epithelium were significantly decreased ($p<0.05$) in the LPWA, LPSE and LPST relative to the NPWA, NPSE and NPST (Table 2).

Diameter of the lumen of seminiferous tubules

There were no significant ($p>0.05$) differences between the diameters of the lumen of seminiferous tubules of NPWA and NPSE as well as between those of LPWA, LPSE and LPST. The tubular diameter of NPST, however, was significantly decreased ($p<0.01$) relative to NPWA, LPWA, NPSE, LPSE and LPST as shown in Table 2.

Hormonal assays

Follicle stimulating hormone (FSH)

The plasma levels of FSH were significantly decreased ($p<0.001$) in the NPST, LPST, NPSE, LPSE and LPWA relative to the NPWA (Table 3). The LPWA and LPSE showed the lowest FSH levels.

Luteinizing hormone (LH)

The plasma levels of LH were significantly decreased ($p<0.001$) in all the groups (NPSE, LPSE, NPST, LPST and LPWA) relative to the control (NPWA) group (Table 3). Luteinizing hormone level in the NPSE, though lower than in the NPWA, was significantly higher ($p<0.001$) than those of the NPST, LPST, LPWA and LPSE (Table 3).

Testosterone

Plasma levels of testosterone in the LPWA, LPSE and the NPST were significantly decreased ($p<0.001$) relative to the NPWA and the LPST as shown in Table 3. No significant differences ($p>0.05$) were observed between the plasma testosterone levels of the NPWA, the NPSE and the LPST (Table 3).

Control versus aqueous extract of the seed

Relative weight of testes

The aqueous extract of the seed significantly increased ($p<0.05$) the relative weight of the testis, independent of the dietary status (Table 4), in comparison to the control. However, the interaction

Table 1: Ingredient composition (g/kg fresh matter) of experimental diets.

Component	Low Protein-energy (6.21%)	Normal Protein-energy (16.55%)
Whole Maize	420	420
Maize starch	490	420
Fish meal	20	90
Palm oil	60	60
Vit./Min. mixture*	10	10

* Vitamin/Mineral/Trace elements mixture (Vitalyte®)
Source: Akingbemi *et al*. (1996)

Table 2: Relative weight (%) of testis, height of the epithelium of seminiferous tubule (μm) and diameter of the lumen of seminiferous tubule (μm) in the Wistar rat that received either *Cuscuta* seed or stem

Variable	NPWA	LPWA	NPSE	LPSE	NPST	LPST
Testis (ns)	0.951 ± 0.07	1 ± 0.035	1.038 ± 0.68	1.195±0.064	0.935± 0.024	1.20±0.201
HSE *	7.17 ± 0.37^{a}	6.21 ± 0.46^{b}	7.057 ± 0.14^{a}	5.81 ± 0.87^{b}	7.33 ± 0.18^{a}	5.36 ± 0.092^{b}
DSL***	9.72 ± 0.223^{c}	11.64 ± 0.78^{ab}	10.27 ± 0.23^{bc}	12.28 ± 0.65^{a}	8.18 ± 0.5^{d}	12.9 ± 0.29^{a}

Values in the same row with different superscripts are significantly different. ns = No significant difference; *$p<0.5$; ***$p<0.001$ DSL = Diameter of the lumen of seminiferous tubule. HSE = Height of the epithelium of seminiferous tubule. NPWA (Normal Protein-energy diet-Water). LPWA (Low Protein-energy diet-Water). NPSE (Normal Protein-energy diet-Seed). LPSE (Low Protein-energy. NPST (Normal Protein-energy diet-Stem). LPST (Low Protein-energy diet-Stem).

Table 3: Plasma concentrations of FSH, LH and testosterone of either *Cuscuta* seed or stem-treated Wistar rats fed either normal or low protein-energy diets.

Variable	NPWA	LPWA	NPSE	LPSE	NPST	LPST
FSH***(lU/L)	2.03 ± 0.159^{a}	0.200 ± 0.055^{d}	0.640 ± 0.136^{c}	0.18 ± 0.037^{d}	1.12 ± 0.086^{b}	0.560 ± 0.075^{c}
LH***(lU/L)	1.63 ± 0.156^{a}	0.260 ± 0.068^{c}	1.120 ± 0.153^{b}	0.34 ± 0.51^{c}	0.380 ± 0.049^{c}	0.220 ± 0.374^{c}
Testost (lU/L)***	3.00 ± 0.105^{a}	1.020 ± 0.262^{c}	2.46 ± 0.667^{ab}	1.68 ± 0.199^{bc}	0.64 ± 0.206^{c}	3.02 ± 0.520^{a}

Values in the same row with different superscripts are significantly different.. FSH=follicle stimulating hormone, LH=Leutenizing hormone, Testost= Testosterone, NPWA=Normal Protein-energy diet-Water. LPWA=Low Protein-energy diet-Water. NPSE=Normal Protein-energy diet-Seed. LPSE =Low Protein-energy diet-Seed. NPST=Normal Protein-energy diet-Stem, LPST=Low Protein-energy diet-Stem. Values in the same row with different superscripts are significantly different. ******* $P< 0.001$.

Table 4: Influence of diet and/ or *Cuscuta* extracts on the morphophysiology of the testis of adult Wistar rats.

Variables	Diet (D)						Extract (E)			D-E interaction	
	NP			LP							
	WA	SE	ST	WA	SE	ST	WA	SE	ST	SE	ST
Testes (%)	0.95	1.04	0.94	1	1.2	1.2	0.98	1.12*	1.1	ns	ns
HSE (µm)	7.17	7.04	7.33	6.21	5.81	5.36 ***	6.69	6.34	6.34	ns	ns
DSL (µm)	9.72	10.3	8.18	11.6	12.3**	12.9 ***	10.68	11.29	10.51	ns	**
FSH (lU/L)	1.63	1.12	0.38	0.26	0.34	0.22***	0.95	0.41***	0.84**	***	***
LH (lU/L)	2.03	0.64	1.12	0.2	0.18**	0.56***	1.12	0.73	0.3***	*	***
Testost (lU/L)	3	2.46	0.64	1.02	1.68	3.02***	2.01	2.07	1.83	ns	***

ns – No significant difference, *p<0.05, **p<0.01, ***p<0.001, NP (Normal protein-energy diet), LP (Low protein-energy diet), WA (Control), SE (Seed), ST (Stem), HSE (Height of seminiferous tubule epithelium), DSL (Diameter of seminiferous tubule lumen). FSH (Follicle stimulating hormone), LH (Leuteinizing hormone), Testost (Testosterone).

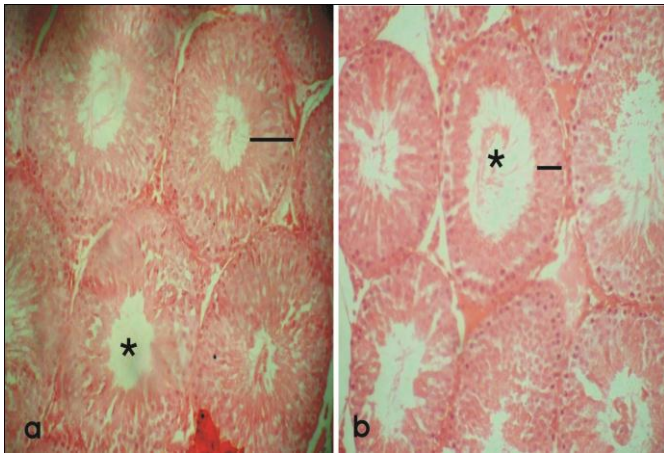

Fig. 1: Photomicrographs of the seminiferous tubules of Control groups of rats fed either normal (a) or low (b) protein-energy diets, NPWA and LPWA, respectively. Note the wide lumen of the seminiferous tubule (asterisk) lined by a low height germinal epithelium (bar) in the LPWA (b). Stain: H&E. Magnification: ×100.

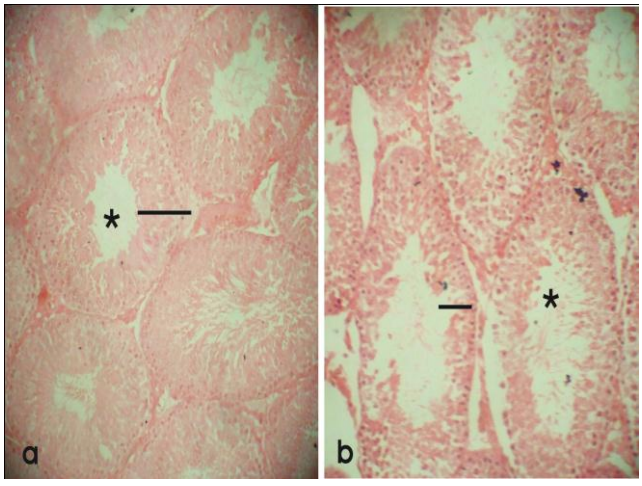

Fig. 2: Photomicrographs of the seminiferous tubules of *Cuscuta australis* seed-treated groups of rats fed either normal (a) or low (b) protein-energy diets, NPSE and LPSE, respectively. Note the wide lumen of the seminiferous tubule (asterisk) lined by a low height germinal epithelium (bar) in the LPSE (b). Stain: H&E. Magnification: ×100

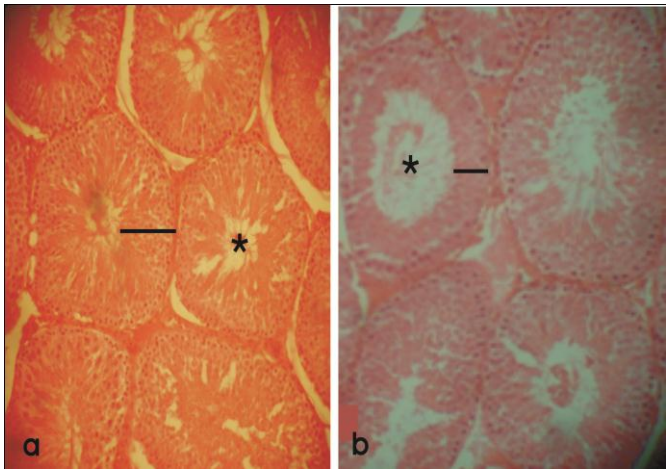

Fig. 3: Photomicrographs of the seminiferous tubules of *Cuscuta australis* stem-treated groups of rats fed either normal (a) or low (b) protein-energy diets, NPST and LPST, respectively. Note the wide lumen of the seminiferous tubule (asterisk) lined by a low height germinal epithelium (bar) in the LPST (b).Stain: H&E.. Magnification: ×100

between diet and seed extract had non-significant influence (p>0.05) on the weight of the testis as shown in Table 4

Histomorphometry of testes

Heights of the seminiferous tubule epithelium

The aqueous extract of the seed, protein-energy diet as well as the interaction between diet and seed extract had no significant influences (p>0.05) on the height of seminiferous tubule epithelium of the rat when compared to their respective control (Table 4).

Diameter of the lumen of seminiferous tubules

Diameters of the lumen of seminiferous tubules of the seed extract-treated groups were significantly (p<0.01) affected by the dietary status of the rat relative to their respective controls (Table 4). However, there were no significant (p>0.05) seed

extract as well as diet-seed extract interaction effects on the tubular diameters as shown in Table 4.

Hormonal assays

Follicle stimulating hormone (FSH)

Dietary status of the rat had no significant ($p>0.05$) effect on the plasma levels of FSH in the seed extract-treated group relative to their respective controls. However, aqueous seed extract significantly decreased ($p<0.001$) the level of FSH in the rat. The interaction between the seed extract and the protein-energy diet also significantly influenced ($p<0.001$) the plasma level of FSH (Table 4).

Luteinizing hormone (LH)

Aqueous seed extract treatment had no significant ($p>0.05$) effect on the plasma level of LH. However, plasma LH levels of the seed extract-treated rats were significantly influenced ($p<0.001$) by the dietary status of each group of rats as shown in Table 4. There was also a significant ($p<0.05$) diet-seed extract interaction effect on the plasma LH level (Table 4).

Testosterone

There were no significant ($p>0.05$) effects of the seed extract, the diet and the diet-seed extract interaction on the plasma testosterone level of the rat (Table 4).

Control versus aqueous extract of the stem

Relative weight of testes

The effects of both the stem extract and the diet on the relative weights of the testis were not significantly different ($p>0.05$) from those of the controls as shown in Table 4. Equally, the diet-stem interaction had no significant ($p>0.05$) effect on the relative weight of the testis (Table 4).

Histomorphometry of testes

Heights of the seminiferous tubule epithelium

Diet had a significant ($p<0.001$) effect on the height of the seminiferous epithelium of the stem extract-treated group relative to the control (Table 4). However, stem extract treatment and the interaction between the stem extract and protein-energy diet had no significant ($p>0.05$) influences on the heights of the seminiferous epithelium as shown in Table 4.

Diameter of the lumen of seminiferous tubules

Dietary status of the rat had a significant ($p<0.001$) effect on the diameter of the lumen of the seminiferous tubule of stem extract-treated group (Table 4). The tubular diameter was also significantly influenced ($p<0.01$) by the interaction between the stem extract and the dietary protein-energy (Table 4). Stem extract, however, had no significant ($p>0.05$) effect on the diameter of the tubular lumen (Table 4).

Hormonal assays

Follicle stimulating hormone (FSH)

Dietary status of the stem extract-treated rat significantly influenced ($p<0.001$) the plasma level of FSH as shown in Table 4. Stem extract also significantly decreased ($p<0.01$) the plasma level of FSH. The interaction between diet and stem extract was equally significant ($p<0.001$) as shown in Table 4.

Luteinizing hormone (LH)

The plasma LH level of the stem extract-treated rat was significantly influenced ($p<0.001$) by the dietary status of such rat (Table 4). The plasma level of LH was also significantly influenced ($p< 0.001$) by both the stem extract treatment and the protein-energy diet-stem extract interaction (Table 4).

Testosterone

The dietary status of the stem-treated rat significantly influenced ($p<0.001$) the plasma testosterone level (Table 4). The interaction between the stem extract and the diet also significantly influenced ($p<0.001$) the level of plasma testosterone (Table 4). However, there was no significant ($p>0.05$) effect of the stem extract on the plasma testosterone level.

DISCUSSION

The morphological indices of the testis in the normal protein-energy diet-treated group were not disrupted by the orally administered aqueous extract of either *C. australis* seed or stem in this study. This may be indicative of the safety of this herb as a traditional dietary supplement for the management of infertility, as was earlier reported for the seed of *C. chinensis* by Zheng *et al.* (1998), especially in an adequately nourished population. However, the reduced height of the seminiferous tubule epithelium and the accompanying increase in the diameter of the lumen of the seminiferous tubule in the low protein-energy diet-Cuscuta-treated group means that aqueous extracts of *C. australis* should be used with caution in the presence of severe protein-energy malnutrition. Protein deficiency and/or specific deficiencies in the intake and utilization of nutrients, according to Glass *et al*. (1979), McDonald (1980), Kalla *et al*. (1990), Gonzalez-Reimers *et al.* (1994) and Karaca *et al.* (2003) can retard the maturation, growth and function of the reproductive organs in experimental animals.

The hormonal assay profile showed that the testosterone level in this study was both extract and diet dependent. *Cuscuta* stem extract increased plasma testosterone level in the presence of protein-energy malnutrition. It, however, decreased the plasma testosterone level in the presence of adequate protein-energy nourishment. The reverse is the case

for the seed extract diet-paired match. These observed extract and diet-dependent effects may be attributable to the report by Ye *et al*. (2002) of the presence of a higher concentration of the bioactive substance, kaempferol, in the *C. australis* stem than in the seed. Qin *et al.* (2000) observed that kaempferol, a flavonoid, potentates production of testosterone in animals with sub-normal testosterone level.

The significant reductions of plasma LH and FSH levels in all the *C. australis* extract-treated groups (NPST, LPST, NPSE and LPSE) relative to NPWA are similar to the observation of Qin *et al* (2000) that the flavonoids of *C. chinensis* seed suppressed lutenizing hormone level most especially in ageing rats. The decreased gonadotropin concentration in LPWA group corroborates the reports of Guaragna *et al.* (1986), Vawda and Mandlwana (1990), Cameron and Norbisch (1991), Clarke and Henry (1999) and Karaca *et al.* (2003) that malnutrition is associated with changes in circulating androgen and gonadotropin levels in animal and man.

The significantly increased level of testosterone in the LPST group may be due to a synergistic interplay between low dietary protein-energy level and the stem extract components. Protein-energy malnourishment in the LPST group of rats, like the stress associated with hormonal imbalance in the ageing rats reported by Qin *et al*. (2000), may have induced an initial subnormal testosterone level and its associated stress. The testosterone stress, in turn, may have triggered a feed-back mechanism for the bioactive substance(s) of *C. australis* stem to stimulate an increased production of testosterone similar to the effect of *C. chinensis* in the ageing rat reported by Qin *et al.* (2000).

This study attempts to evaluate the influence of dietary protein-energy on the effects of aqueous extracts of *C. australis* seed and stem on the structure and function of the testis of the Wistar rat. To our knowledge, this is the first report on comparative effects of the stem and seed of *C. australis* and provides a fresh insight into the traditional use of this parasitic vine as a dietary supplement for the management of infertility. Our work has shown that the aqueous extract of *C. australis* stem stabilised the morphophysiology of the testis better than the seed extract. It has also shown that the potency of both extracts is influenced by the quality of dietary protein-energy intake. These findings may be of value in the traditional management of male reproductive failures.

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