International Scholars Journals

Advances in Food Science and Technology ISSN: 6732-4215 Vol. 3 (9), pp. 001-011, September, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Effect of Hibiscus *sabdariffa L*. on circulating levels of reproductive hormones in rabbits *(Oryctolagus cuniculus)*

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Accepted 15 January, 2015

Effect of *Hibiscus sabdariffa L. (*family malvecea*)* calyx anthocyanin was investigated on circulating levels of reproductive hormones in rabbits (*Oryctolagus cuniculus*). Forty (40) rabbits (twenty each for male and female) weighing 1.2 ± 0.2 kg were grouped into two control (water control, anthocyanin (delphinidin-3-monoglucoside) standard) and two experimental groups (whole extract (calyces of *H. sabdariffa L.*) and anthocyanin extract of calyces of *H. sabdariffa L*). While the water control group received 1.0 ml of water at 0900 h everyday for 28 days at 14:10 light/dark regime, the anthocyanin control and experimental group were administered 200 mg/kg oral doses of the different preparations. Blood samples were drawn prior to administration (day zero) and every seven days at 0900 h for the estimation of serum prolactin, follicle stimulating hormone and testosterone using ELISA. 28-day daily oral administration of delphinidin-3-monoglucoside and anthocyanin-rich extract of *H. sabdariffa L.* to rabbits is associated with high circulating prolactin in males and non-lactating female rabbits. This may be potentially important in phytotherapuetic induction of milk production in lactating animals. This treatment regime is also associated with lowered circulating FSH in males and female rabbits. Long-term reduction in circulating FSH levels might play important role in failed follicular development in developing female animals and gonadal atrophy in matured animals.

Key words: *Hibiscus sabdariffa*, anthocyanins, serum prolactin, follicle stimulating hormone, testosterone.

INTRODUCTION

Historically, plant has played significant roles in health (Vogel and Vogel, 1997). Phytochemical screening procedures have unveiled the chemicals responsible for these functions (Faraz et al., 2003). Structural analysis of these phytochemicals has provided the basis for their phytotherapeutic potencies (Stintzing et al., 2002). This has also increased the relevance of plant in drug developments. One of the plants that have been given extensive studies in the last two decades is *Hibiscus sabdariffa.* It was originally cultivated for fibre and then its brilliant coloured petals were harvested for food and drink purpose. Its medicinal values were first traced to its ability to reduce hypertension in population observed to have daily consumption of the aqueous extract of its calyces. This discovery led to uncovering its cardio-protective property then its hypocholestrolemic property (Chen et al., 2003). These properties have been traced to its anti-oxidant properties (Passamonti et al*.,* 2003). Recently, anticancer, anti-inflammatory, hepatoprotective and its ability to improve visual acuity has been widely reported. Phytochemical screening and structural analysis has identified a class of flavonoids called anthocyanin as responsible for these activities (Hou et al., 2005). Delphinidin-3-monoglucoside, malvidin-3-mono-glucoside and their methyl-derivatives have been isolated in tissues of *H. sabdariffa*. Pharmacodynamics and pharmacokinetics studies have identified aglycone derivatives of these anthocyanins in sera and urine samples which widen the range of biological activities expected for *H. sabdariffa*

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Abbreviation: FSH, Follicle stimulating hormone.

extract (Kahkonen and Heinonen, 2003; Galvano et al., 2004).

Akpantah et al. (2003) reported the ability of flavonoids to increase testosterone levels in rats. Esomonu et al. (2005) and Oluyemi et al. (2007a) claimed increased erythropoiesis in rat models administered flavonoids extract of *Garcinia kola*. Since testosterone increases erythropoietin secretion, flavonoids-induced erythropoietin cannot be disclaimed. Thus, erythropoietin and testosterone were the earlier hormones discovered to be modulated by flavonoids. Oluyemi et al. (2007b) then reported increased in sperm count in animals treated with flavonoids extract of *G. Cambogia.* Okasha et al. (2008) reported anthocyanin-induced increase in serum prolactin level of lactating albino rats. These findings positively correlate flavonoids (especially anthocyanins) administration with reproductive hormone level alteration.

This study was undertaken to study the extent to which different anthocyanin preparations of *H. sabdariffa* alter the basal levels of selected reproductive hormones: prolactin (PRL), follicle stimulating hormone (FSH) and testosterone (T) in rabbit model.

MATERIALS AND METHODS

Animals

A total of 40 rabbits (twenty (20) each for male and females) weighing 1.2 ± 0.2 kg from the same litter were housed singly in metabolic cages. They were kept under 14:10 light/dark regime for one month before the administration. The animals were fed on rat chow and water *ad libitum.*

Plant sample

Dry calyces of *H. sabdariffa* was obtained from a local market in Benin City, Edo State, Nigeria and authenticated at the Plant Science and Biotechnology Department, University of Benin, Edo State, Nigeria. The calyces were extracted in acid water (distilled water containing 0.01 M HCL) overnight with shaking. The anthocyanin-rich extract was concentrated to dryness on speed vac. 5 g of the anthocyanin-rich extract was dissolved in 50 ml of deionized, distilled water for anthocyanin purification.

Semi- purification of *Hibiscus sabdariffa Linn* anthocyanin using solid phase

Extraction

Five gram C18 cartridge (Sigma Aldrich) was washed with methanol, the cartridge was washed with acidified deionized distilled water to remove the methanol. 25 ml of the anthocyanin-rich extract was forced through the cartridge and then washed with acidified water to remove sugar and other polar compounds in the extract. Anthocyanin component was eluted with acidified ethanol (0.01% HCl) and collected in a 150 ml flask. The ethanol was totally removed in a rotary evaporator at 40°C under vacuum. The concentrated extract was redissolved in acidified water. The extract was stored at -4°C until use as described by Schwarz et al. (2004).

Experimental design

There were two controls and two experimental groups. Water control (five animals each for male and female), delphinidin-3-monoglucoside control (standard anthocyanin control) (five animals each for male and female), whole extract of *H. sabdariffa* L. calyx (five animals each for male and female each) and anthocyanin isolate of *H. sabdariffa* L. (five animals each for male and females) 200 mg/kg body weight of delphinidin-3-monoglucoside and whole extract of *H. sabdariffa* calyx and anthocyanin isolate of *H. sabdariffa* calyx were administered orally for 4 weeks.

Blood sample collection

Blood samples were collected from the ear veins of animals at 7-day intervals at 0900 h throughout the experimental period.

Hormonal assay

The hormones were estimated using the standard protocols of ELISA kits as designated below:

Prolactin (Fortress Diagnostics Limited, BT41 IQS, UK), Follicle Stimulating Hormone (ANOGEN, Mississauga, CANADA. Cat No. EL10013) and Testosterone (Immuno Biological Laboratories, Hamburg, Germany. Cat no. DB52181).

Statistical analysis

The values were recorded as mean ± standard error of mean.

RESULTS AND DISCUSSION

The effect of administration of whole extract of *H. sabdariffa* and its anthocyanin isolate (anthocyanin extract) was investigated on serum levels of follicle stimulating hormone (FSH), prolactin (PRL) and testosterone in rabbit model (male and female). These effects were compared with groups administered delphinidin-3-monoglucoside (anthocyanin standard) and water as placebo (water control).

Prolactin

The effect on serum prolactin in males is shown on Figure 1. Water control group showed fairly constant prolactin level for 28 days, anthocyanin extract showed the highest on PRL on day 7 (1.20 ± 0.05 ng/mL) but decreased from day 14 to day 28. Animals administered whole extract showed a similar response at day 7 (0.10 ± 0.04 ng/mL) but maintained a steady level from day 14 to 28. Anthocyanin control peaked on day 28 after a steady rise from day 7.

Figure 2 showed the effect of anthocyanin administration on female circulating prolactin. Groups administered water showed fairly constant circulating prolactin (day 0 = 0.7 ng/mL, day 28 = 0.8 ng/mL). The group administered whole extract of *H. sabdariffa*, peaked on day 7

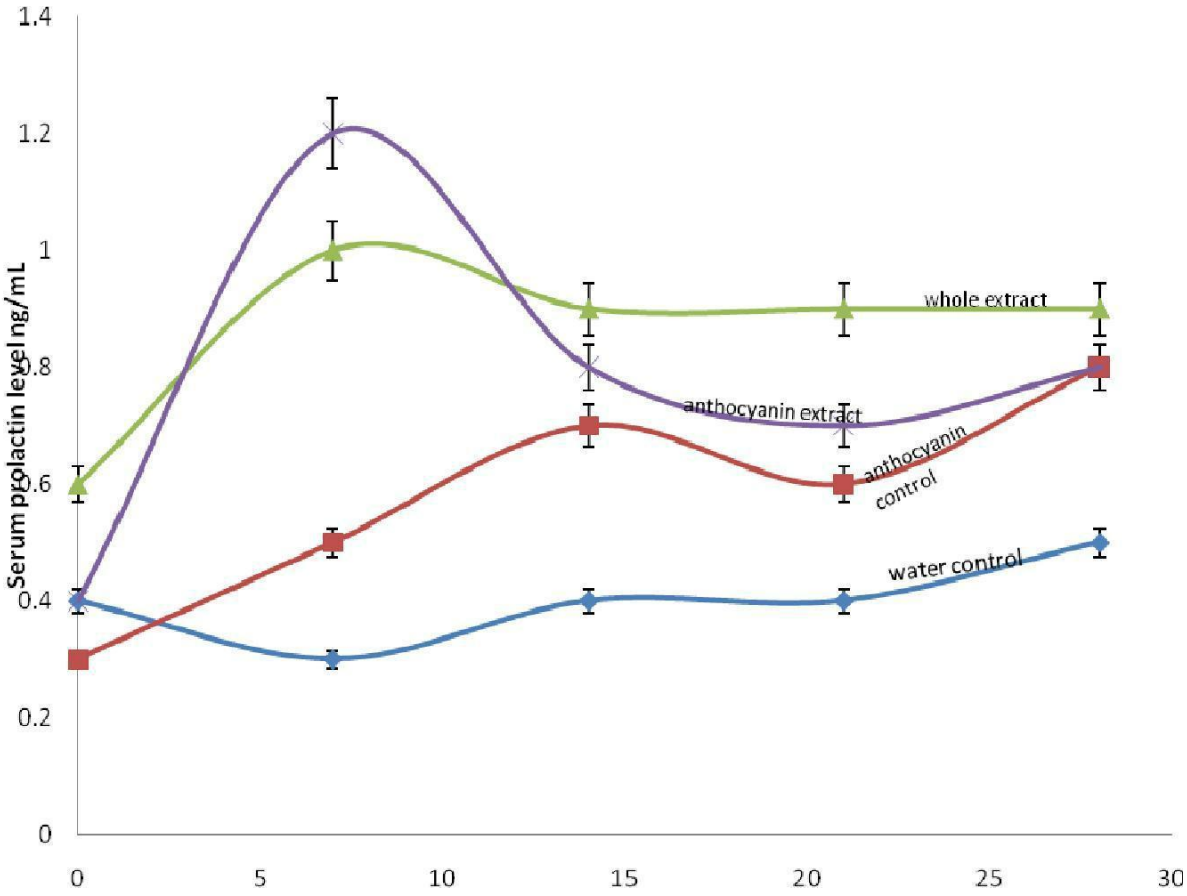

Figure 1. Serum prolactin of males administered 200 mg/kg body weight oral doses of anthocyanin preparations. Water control group showed fairly constant prolactin level for 28 days, anthocyanin extract shows the highest on PRL on day 7 (1.20 ± 0.05 ng/mL) but decreased from day 14 to day 28. Animals administered whole extract showed a similar response at day 7 (0.10 ± 0.04 ng/mL) but maintained a steady level from day 14 to 28. Anthocyanin control peaked on day 28 after a steady rise from day 7.

(2.3 ± 0.3 ng/mL) and gradually decreased to (1.5 ± 0.2 ng/mL) on day 21 and rose to 1.9 ± 0.3 ng/mL on day 28. Anthocyanins extract group peaked on day 7 (2.1 ± 0.4 ng/mL) and decreased steadily till day 28 (1.4 ± 0.2 ng/mL). Standard anthocyanin group peaked on day 14 (1.7 ± 0.1 ng/mL) and decreased steadily till day 28 (1.2 ± 0.2 ng/mL).

The data showed that anthocyanin administration is associated with increased serum prolactin as reported by Okasha et al. (2008). Our data however revealed a unique pattern; mixture of different anthocyanins comparatively increased serum prolactin in male rabbits under sub-acute administration but failed to maintain the level with time. While the whole extract can maintain the level under similar conditions, purified single anthocyanin type had longer lag period of prolactin release but it revealed time-dependent increase in prolactin secretion. The data recorded for water control group (female) showed slightly higher circulating prolactin level compared with similar treatment for males. This confirms that female animals have higher circulating prolactin than males (Colao and Lombardi, 1998). When administered anthocyanin preparation, female rabbits showed higher prolactin-releasing response compared to male rabbits.

Serum prolactin increases as a result of increased synthesis and secretion of prolactin in the anterior pituitary in response to hormonal factors such as thyrotropin-releasing hormone and oxytocin, physiological signals such as sleep, stress, pregnancy and tactile signals during weaning (Brook and Hindmarch, 2001), whereas, reduction in the circulating prolactin can be traced to the activity of prolactin-inhibiting hormone, dopamine and tissue-specific prolactin clearance by ovary, liver, kidney and mammary gland during lactation (Colao and Lombardi, 1998; Brook and Hindmarch, 2001; Wass and Shalet, 2002). The concentration of prolactin in the serum at any time is therefore influenced by the factors inducing synthesis, those inhibiting synthesis and turnover rate at the target cells (Wass and Shalet, 2002). For anthocyanin to increase circulating prolactin, it must have

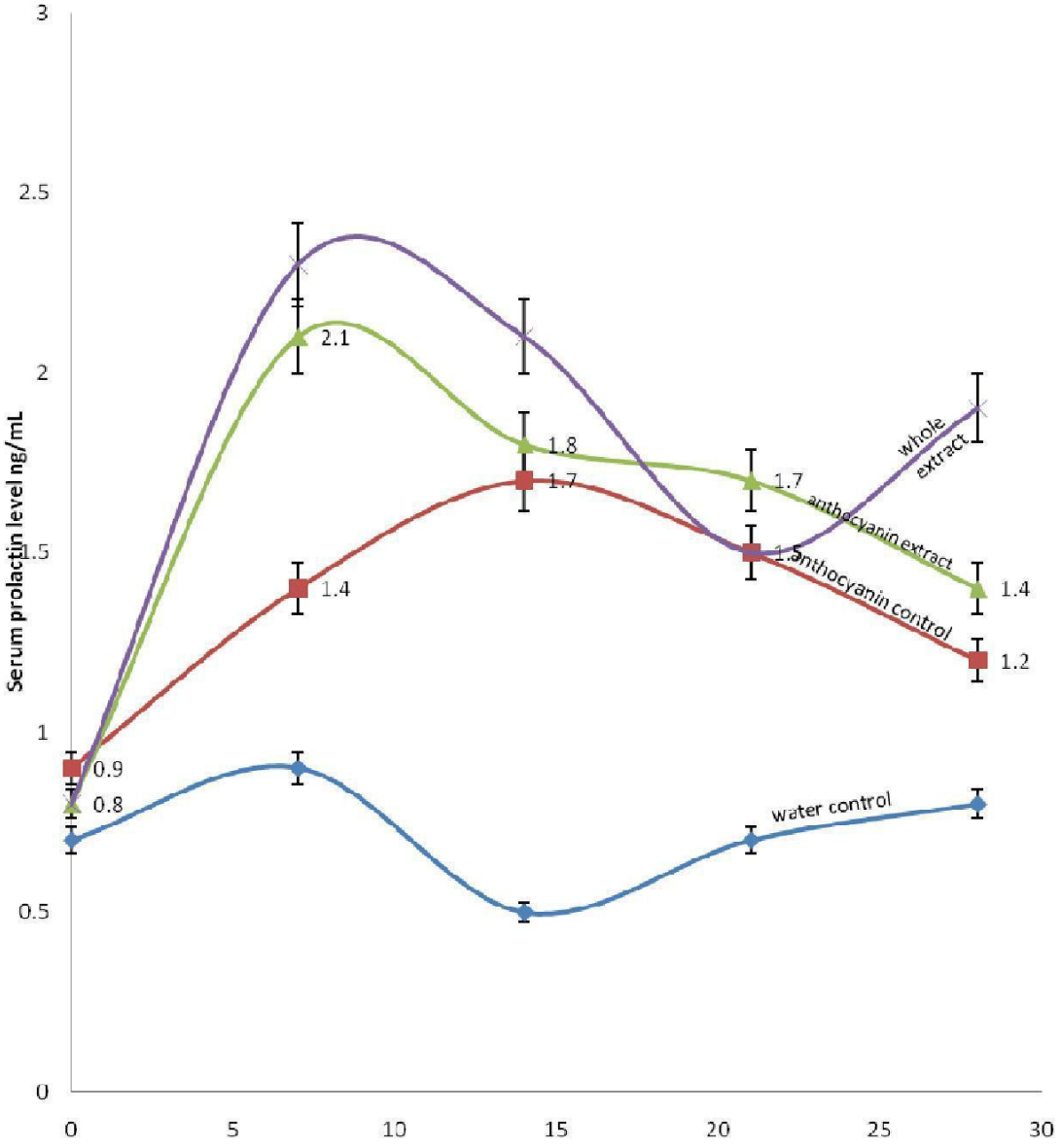

Figure 2. Serum prolactin (PRL) of female rabbits administered 200 mg/kg body weight oral doses of anthocyanin preparations with time. Groups administered water showed fairly constant circulating prolactin (day 0 = 0.7 ng/mL, day 28 = 0.8 ng/mL). The group administered whole extract of *H. sabdariffa*, peaked on day 7 (2.3 ± 0.3 ng/mL) and gradually decreased to (1.5 ± 0.2 ng/mL) on day 21 and rose to 1.9 ± 0.3 ng/mL on day 28. Anthocyanins extract group peaked on day 7 (2.1 ± 0.4 ng/mL) and decreased steadily till day 28 (1.4 ± 0.2 ng/mL). Standard anthocyanin group peaked on day 14 (1.7 ± 0.1 ng/mL) and decreased steadily till day 28 (1.2 ± 0.2 ng/mL).

interfered with one or more of these processes. It is widely reported that anthocyanin increase prolactin secretion by antagonism to D_2-doparminergic receptor. Recent findings have indicated that anthocyanin activates monoamine oxidase (Byambaa et al, 2005; Yadav et al., 2008) thus, increasing the catabolism of dopamine. The whole-extract also contains other phytochemicals especially the phenolic compounds which are structurally and electrically analogous to phenothiazines and butyrophenones (therapeutic antagonists of dopamine receptor) thus, phenolics may act as potent antagonist. Also, inhibitory role of phenol during protein synthesis has been reported and possibly they could inhibit prolactin recaptor synthesis therefore, reducing its effect on target cells and clearance. Also, flavonoids and phenolics are analogous to thyroxine hormones; these compounds would possibly compete with thyroxine hormones for cytoplasmic receptors and act as antagonists. This exerts a feed-back effect on the thyroid gland to produce more thyroxine, which is signalled by thyrotropin-releasing

hormone (Cooper, 2003; Hegedus, 2004; Roberts, 2004). This signal could also increase prolactin secretion (Cooper, 2003). The combination of these factors may account for increased circulating prolactin in groups administered anthocyanin. Anthocyanin control offers less prolactin-releasing effect, its values fluctuates possibly as a result of the absence of phenolic compounds which could have acted synergistically with anthocyanin. A long-term assessment of effect of anthocyanin on prolactin is necessary in that prolactin is known to decrease gonadal development and hyperprolactinemia is connected with galactorrhea, oligomenorrhea, decreased libido, decreased potency in males, sub-fertility and clinical signs and symptoms related to oestrogen and androgen deficiency.

Follicle stimulating hormone

Follicle stimulating hormone (FSH) is a glycoprotein produced in the anterior pituitary in response to gonadotrophin-releasing hormone (Litwack and Schmidt, 2001a). The principal function of FSH is to stimulate gametogenesis, follicular development in females and spermatogenesis in males (Litwack and Schmidt, 2001b). In females, FSH acts on immature follicular cells of the ovary and induces development into mature follicle and oocyte capable of steroidogenesis. Steroidogenic functions in females depend on other hormonal factors such as luteinizing hormone which stimulates androgen formation in gonads while FSH stimulates the conversion of androgens to estrogens. In males, FSH acts on the sertoli cells, stimulating the synthesis and release of androgen-binding protein (Litwack and Norman, 1997). Decrease in FSH on day 7 of administration is possibly connected to the surge of prolactin on this same day as previously observed and reported by Nordio et al. (1989). Since ovarian inhibin and oestrogen are known negative feed-back regulators of hypothalamic release of gonadotrophin-releasing hormone and anterior pituitary secretion of FSH (Litwack and Schmidt, 2001a), increased prolactin secretion in response to anthocyanin administration possibly interfered with gonadal inhibin and oestrogen secretion. The expected response should be an increased FSH secretion; however, the response was a reverse. To explain this observation, decrease in circulating follicle stimulating hormone would be traced to the net effect of synthesis and clearance and since steroids reduce follicle stimulating hormone synthesis, it must be assumed that steroids are possibly responsible for decrease in circulating FSH level. Anthocyanin administration increases circulating testosterone levels as reported by Oluyemi et al. (2007b). Also, since it has been established that ovarian and testicular maturation and steroidogenic functions are enhanced in the presence of FSH and lowered circulating FSH has been reported under anthocyanin administration, it could be argued that circulating steroids may have a minor role to play in reduced circulating FSH as shown on Figures 3 and 4. It must be noted however, that there is extra-gonadal steroidogenesis as seen in the liver, adipose tissue, skin and hypothalamus, which are known to synthesise estradiol by the combinatorial activities of 5-alpha reductase and P-450 aromatase (Brown and Goldstein, 1999). Anthocyanin is known to induce cytochrome P-450-monooxygenase; it might also induce the synthesis of the monooxygenases needed for extra-gonadal steroidoge-nesis (Bittman, 1997). Anthocyanidins (deglycosylated form of anthocyanin) have aromatic nuclei similar to those of the steroid hormones with this, they mimic steroid hormones. This may possibly play important role in reducing FSH level on day 7 because before absorption, some of the anthocyanin is hydrolysed by intestinal glycosidases and absorbed in form of anthocyanidins (Passamonti et al., 2003).

Testosterone

Testosterone is both a hormone and a prohormone. In male animals, the highest percentage of plasma testosterone is produced by the leydig cells. This hormone is essential to growth and functions of reproductive organs most importantly, sertoli cells for spermatogenic functions. Figure 5 showed the effects of different anthocyanin-based treatments on serum testosterone in male rabbits. The water-control group showed fairly constant testosterone levels throughout the experimental period. For anthocyanin control group, testosterone levels decreases from day 0 to day 21 and increased to the peak on day 28. Anthocyanin extract shows decreased serum testosterone from day 0 to day 7 and increased steadily to the peak value on day 28. This trend is similar to that of group administered whole-extract. All the treatment groups showed initial reduction in circulating testosterone which may be connected to the decreased circulating follicle stimulating hormones previously reported (Figure 4). This claim can further be established by connecting increased circulating testosterone to increased circulating follicle stimulating hormone. Follicle stimulating hormone is a known regulator of testicular development and function, increases spermatogenesis and steroidogenesis. The increase in the circulating testosterone level in male can also be traced to the direct effect of anthocyanin on testicular steroidogenesis and feed-back inhibition on follicle stimulating hormone synthesis in the pituitary; anthocyanin can be absorbed in the gastro intestinal system as anthocyanidins. Anthocyanidins are deglycosylated anthocyanins with hydrophobic properties and steroid nuclei which can mimic the androgens. In this form, anthocyanidins may act as antagonist to androgen receptor functioning as feed-back regulator of follicle stimulating hormone secretion in the pituitary. Also, the mimicry may also cause binding of anthocyani-

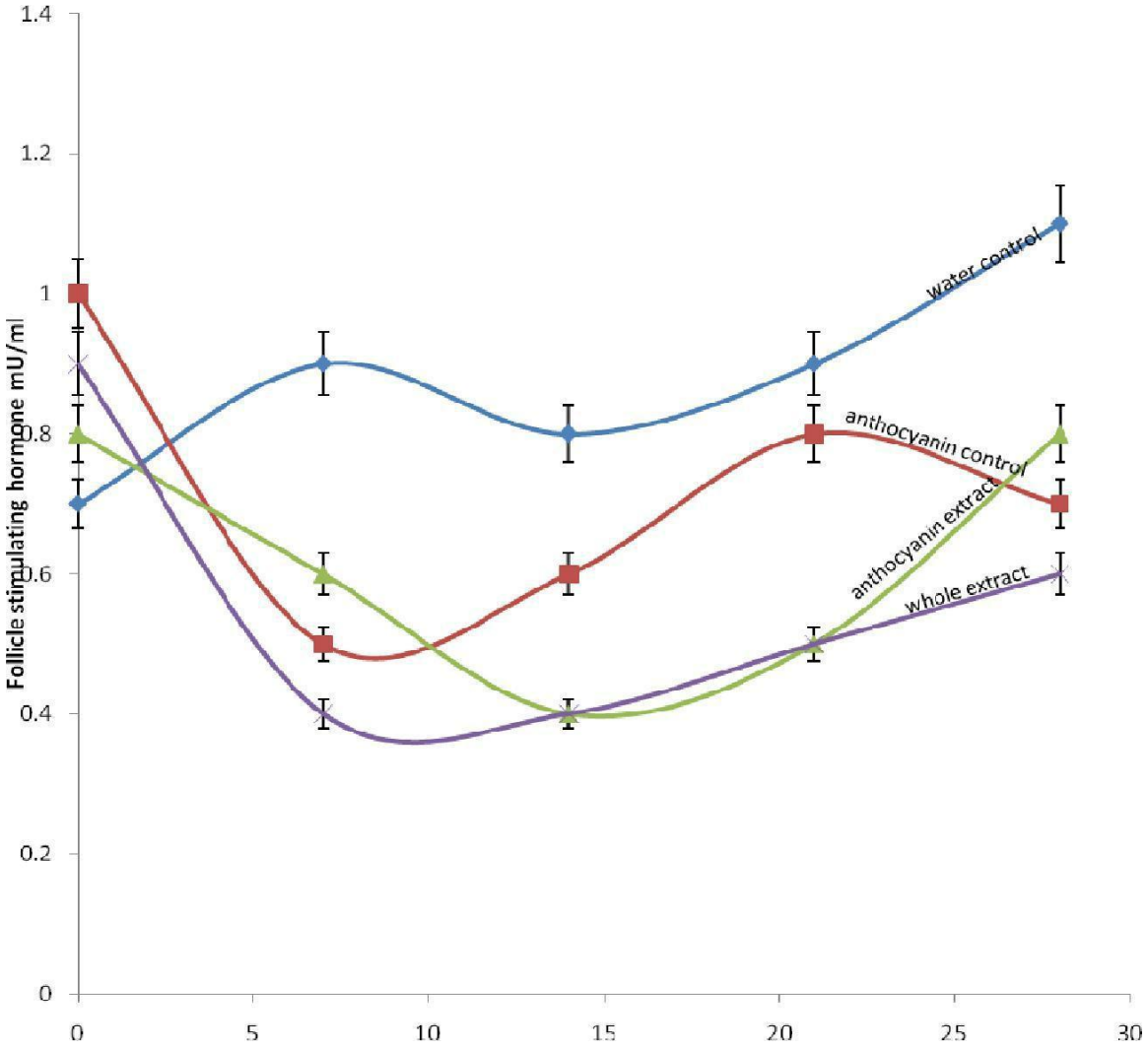

Figure 3. Serum levels of follicle stimulating hormone (FSH) in female rabbits administered 200 mg/kg body weight oral doses of anthocyanin preparations with time. Follicle stimulating hormone level increased gradually from day 0 (0.70 ± 0.02 mU/mL) to the peak value on day 28 (1.1 ± 0.1mU/mL) for water control group. Circulating follicle stimulating hormone level decreased in response to administration of anthocyanin-based preparation. The decrease is most prominent on day 7, meanwhile, on day 14, circulating follicle stimulating hormone increased in anthocyanin control and whole extract-administered groups till day 28 for whole-extract group. Group administered with anthocyanin extract showed minimum serum FSH level on day 14 (0.40 ± 0.01mU/mL) but increased gradually till day 28 (0.8 ± 0.1mU/mL).

dins to the steroid receptor and in saturation state, androgens are retained in circulation. Preferentially, circulating testosterone may then act on kidney when converted to 5-β-dihydrotestosterone to produce erythropoietin which in turn stimulates erythropoiesis. Ability of anthocyanin to stimulate erythropoiesis has been reported (Esomonu et al., 2005; Oluyemi et al., 2006). Circulating testosterone can also be taken up by muscle cells causing increased mitochondrial oxygen uptake and fat catabolism. Anti-obesity property of flavonoids had also, been previously reported (Oluyemi et al., 2006).

In female rabbits, testosterone level did not have a definite pattern over the period of this study (Figure 6). Testosterone level for the water control group oscillated from day zero to day 28. It reached the peak value on day 7 and dropped to the minimum on day 14. It then rose steadily from day 21 to day 28. To explain this fluc-tuation in the female circulating level of testosterone, the organs and enzymatic machinery available for the synthe-sis, uptake, function and metabolism must be understood. Ovaries are principally involved in steroid synthesis in female animals which ensures maturation and function of secondary sex characteristics. This hormone is also synthesised during pregnancy by placenta which functions

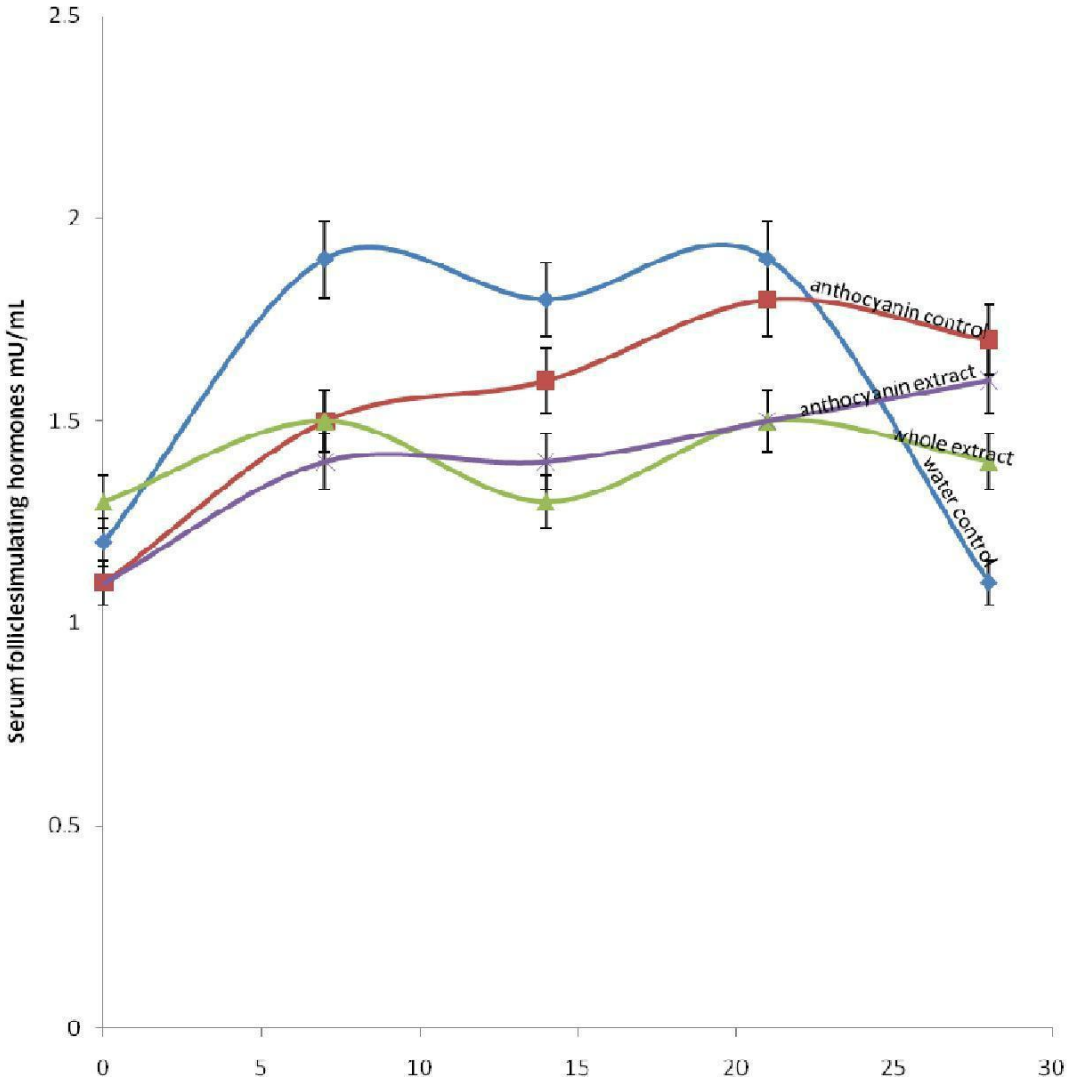

Figure 4. Serum levels of follicle stimulating hormone (FSH) in male rabbits administered 200 mg/kg body weight oral doses of anthocyanin preparations with time. In water control group, FSH rose steadily from day 0 to day 7, remained fairly constant till day 21 and decreased rapidly to the lowest on day 28. Groups administered with anthocyanin-extract showed gradual rise from day zero to day 28. Serum FSH of anthocyanin control increased gradually from day 0 to day 21 and decreased slightly on day 28.

basically in the maintenance of pregnancy. Research evidences have indicated the role of adrenal gland in the synthesis of testosterone. This hormone has been widely reported in the alteration of sexual behaviour in females and its fluctuation in the water control would be a possible indication of rapid changes in this behaviour at basal level (Rommers et al., 2001; Gacek, 2002 and Rommers et al., 2002). Thegroup administered anthocyanin extract maintained a higher serum testosterone from day 7 to day 21 above the water control, possibly indicating the ability of anthocyanin to either potentiate ovarian and adrenal testosterone synthesis, or interferes with its tissue-uptake or metabolism. Ovarian steroidogenesis is enhanced by luteinizing hormone; this hormone had the second highest circulating concentration (Figure 2) on day 7 of anthocyanin extract treatment. This may account for the high level of circulating testosterone in female rabbits. Also, structural mimicry between anthocyanidins derivative of anthocyanin and testosterone could account for reduced tissue-uptake and metabolism therefore causing persistence in the circulating levels of female rabbit testosterone. Persistence of testosterone in circulation may account for the sharp decline in the circulating LH from day 14 to day 28 (Figure 2) and FSH levels from day 7 to day 21 (Figure 3). Increased testosterone in anthocyanin control group from day 21 to day 28 could be as a result of the homogeneity of the anthocyanin (delphinidin-3-monoglucoside); indicating that higher

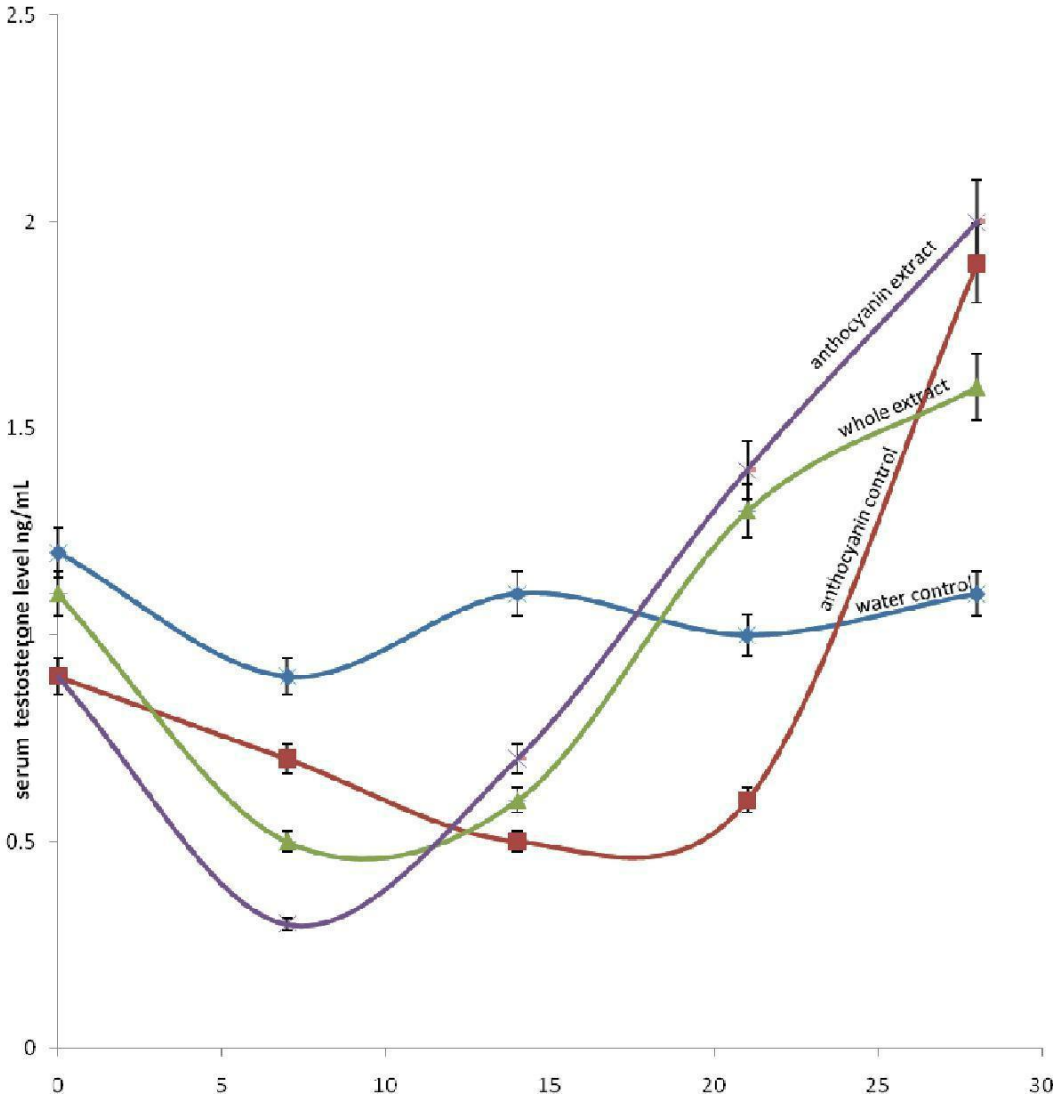

Figure 5. Serum levels of testosterone in male rabbits administered 200 mg/kg body weight oral doses of anthocyanin preparations with time. The water-control group showed fairly constant testosterone levels throughout the experimental period. For anthocyanin control group, testosterone levels decreased from day 0 to day 21 and increased to the peak on day 28. Anthocyanin extract showed decreased serum testosterone from day 0 to day 7 and increased steadily to the peak value on day 28. This trend is similar to that of group administered whole-extract.

testosterone synthesis is achieved with anthocyanin mixture rather than pure extracts.

Conclusion

28-day daily oral administration of delphinidin-3-mono-glucoside and anthocyanin-rich extract of *H. sabdariffa* to rabbits is associated with high circulating prolactin in males and non-lactating female rabbits. This may be potentially important in phytotherapuetic induction of milk production in lactating females but long-term research is needed to exclude the risk of hyperprolactinemia, which is associated with galactorrhea and secondary amenorrhea (females), impotence and hypogonadism (males). This treatment regime is also associated with lowered circulating FSH in males and female rabbits. Although, inverse quantative relationship has been established between circulating levels of FSH and LH, however, long-term reduction in circulating FSH levels might play important role in failed follicular development in developing female animals and gonadal atrophy in matured animals. Therefore, long-term assessment of anthocyanin-based treatment on circulating FSH is needed. The treatment protocol is also associated with short-term increase in circulating testosterone.

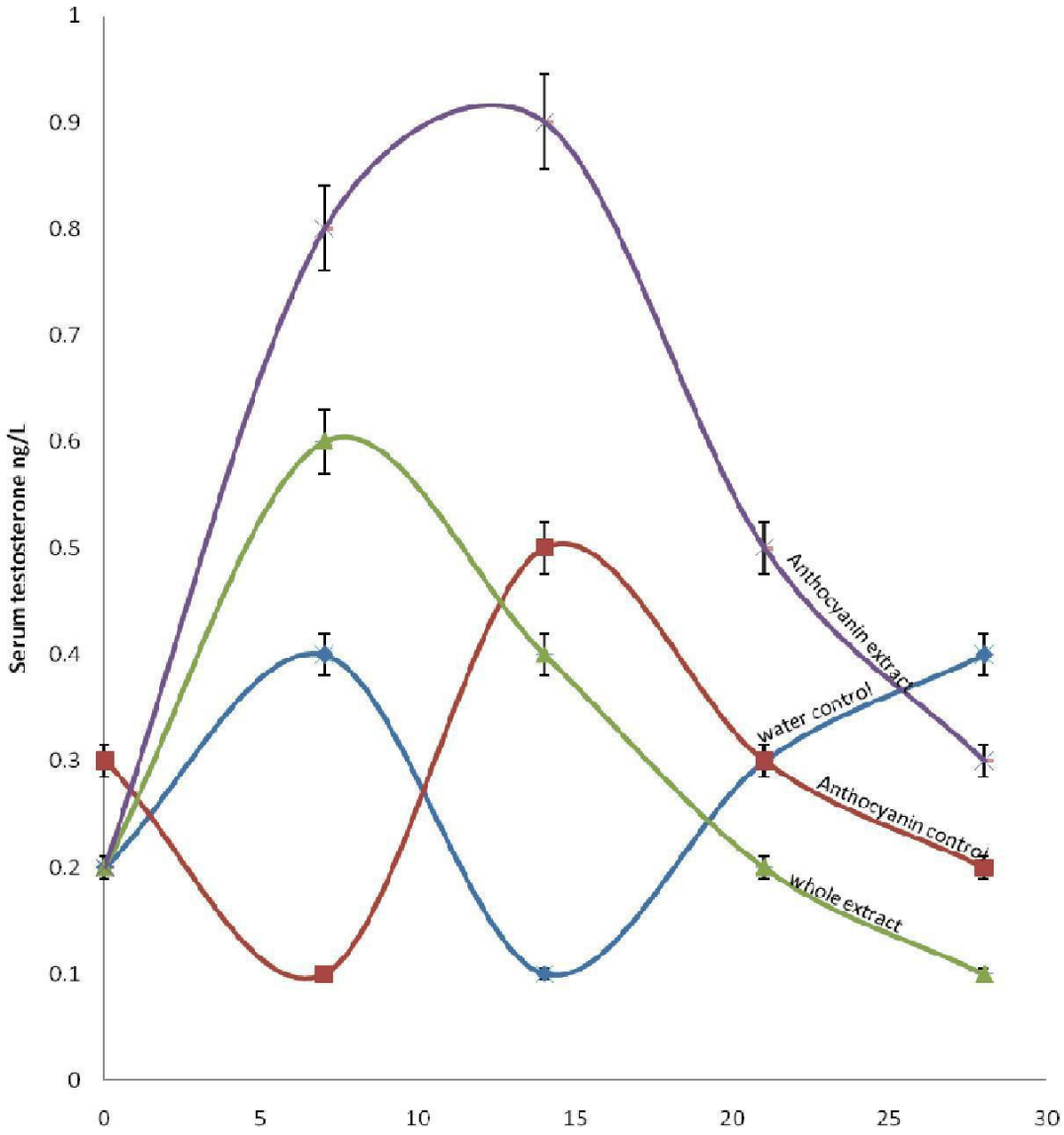

Figure 6. Serum levels of testosterone in female rabbits administered 200 mg/kg body weight oral doses of anthocyanin preparations with time. Testosterone level for the water control group oscillates from day zero to day 28. It reached the peak value on day 7 and drops to the minimum on day 14. It then rose steadily from day 21 to day 28. Testosterone level for the anthocyanin extract increased from day 0 to day 14 and decreased steadily till day 28. The whole extract had similar pattern with the anthocyanin extract.

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