Investigational
Medicinal Chemistry & Pharmacology

Research Article Open Access

Testicular antioxidants and testosterone enhancing effects of the hydro-ethanolic extract of *Rauvolfia vomitoria* (Apocynacaea) in male Wistar rats

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Abstract

Background: Natural products such as extracts of plants have been seen as a possible alternative to conventional therapies in the treatment and management of male infertility. This study aimed at investigating the testicular antioxidant and testosterone enhancing ability of the hydro-alcoholic extract of *Rauvolfia vomitoria* in male rats.

Methods: Twenty-four male rats were divided into four groups of 6 rats each, treated daily with either vehicle (distilled water; 5 mL/kg) or hydro-ethanolic extract of *R. vomitoria* (20 mg/kg, 40 mg/kg, and 100mg/kg) for 60 days, and body weights recorded once every three days. At the end of the treatment, each animal was sacrificed, and reproductive organs were dissected out and weighed. Serum from capillary blood was used for testosterone quantification, while testicular homogenates were used for the estimation of antioxidant biomarkers.

Results: Treatment with *R. vomitoria* extract did not alter the animal's body weight. Instead, the extract at the dose of 40 mg/kg significantly increased ($P< 0.05$) the weights of all reproductive organs investigated. The plant extract also increased serum testosterone concentrations significantly ($P< 0.05$), with the highest effect observed in the animals treated with a dose of 40 mg/kg. Testicular antioxidant markers, thiobarbituric acid substances, glutathione, and catalase were equally improved ($P< 0.05$) by treatment with the plant extract at the dose of 40 mg/kg.

Conclusion: Hydro-ethanolic extract of *R. vomitoria* portrayed beneficial pharmacological properties on reproductive organs, testicular antioxidants, and testosterone concentrations in male rats. These pharmacological activities support the traditional use of the plant in the management of male fertility disorders.

Keywords: male infertility; male rat; oxidative stress; *Rauvolfia vomitoria*; testosterone.

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Citation on this article: Kada AS, Aneng E, Mbouh M, Manfo FPT, Ngimgoh SN, Nantia EA. Testicular antioxidants and testosterone enhancing effects of the hydro-ethanolic extract of Rauvolfia vomitoria (Apocynacaea) in male Wistar rats. Investigational Medicinal Chemistry and Pharmacology (2021) 4(2):54; Doi: https://dx.doi.org/10.31183/imcp.2021.00054.

Background

Androgens are the group of sex hormones responsible for the male characteristics. The major male sex hormone is testosterone, which can be reduced to a more potent/active androgen called dihydrotestosterone [1]. In general, androgens are essential for the development of the male external genitalia, the male secondary sexual characters and in the regulation of erectile response [2,3]. The absence of androgens at early developmental phase results in lack of virilization, lack of pubertal growth spurt, incomplete sexual development, and male infertility. Testosterone deficiency in adults may result in loss of libido and sexual activity. The incidence of sexual dysfunction resulting from hormonal imbalance is estimated to be 20-25% with hypogonadism being the most frequent dysfunction. Androgen deficiency increases with age in healthy men [2]. Androgen deficiency results from the inability of the testes to produce physiological levels of testosterone which may be due to a disturbance occurring at the hypothalamic pituitary-testicular axis or oxidative stress [3-5]. Reactive oxygen species (ROS) are unstable and very reactive by-products of normal metabolism. Even if low and controlled concentrations of these ROS play an important role in sperm physiological processes such as capacitation, acrosome reaction, and signaling processes of fertilization, their excess can cause damaging effects on the principal biomolecules including enzymes and proteins of the steroidogenic pathways and the resulting in androgen deficit caused by ROS can lead to male infertility [4,6-8].

Androgen deficiency or insufficiency is managed by testosterone replacement therapy [7]. However, some evidences have pointed out adverse of androgen replacement therapy including increase cardiovascular morbidity, increased incidence of prostate cancer and erythrocytosis [9]. The search for medicinal products for proper management of androgen deficiency with minor/less adverse outcomes is therefore highly encouraged. Many plants have been traditionally used in the control of various male reproductive related dysfunctions [10,11] with a goal of obtaining bioactive products from the pharmacopeia.

Rauvolfia vomitoria (Apocynacaea) is a plant used in many countries within the tropics in Africa including Cameroon, for the treatment ailments such as hypertension, mental illness, stomach-ache, measles, sleep disorders, diarrhoea, and malaria. It is also used for treatment of male reproductive dysfunctions, especially impotence. Extract from the plant is claimed to exhibit aphrodisiac activity [12-14]. Previous studies in animal model showed that the aqueous extract of *R. vomitoria* enhanced sperm parameters and testicular testosterone [15]. A recent study on the ethanolic extract of this plant showed increased sexual behaviour and sperm characteristics in male rats [16]. The commonly used forms of the plant according to some traditional healers are aqueous and hydro-alcoholic preparations in folk medicine and previous studies though they have demonstrated certain benefit of *R. vomitoria* on male reproductive function, no study has yet been carried out to assess any contribution of the plant for improvement of the antioxidant status of the testis. As serum testosterone is generally used as clinical parameter for evaluation of the male reproductive function, assessing the effect of *R. vomitoria* on serum testosterone could further contribute for understanding of its beneficial effect on reproductive function. This may also help defining possible correlating points with the previously observed testicular testosterone enhancing activity of the plant. The current study thus aimed at investigating the testicular antioxidant and testosterone enhancing activity of the hydro-ethanolic extract of *R. vomitoria* in male Wistar rats.

Methods

Collection of the Plant material and preparation of the hydro-alcoholic extract

The roots of *R. vomitoria* were collected in the locality of Bafut (North-west region of Cameroon) in October 2019. The plant was identified by Dr Tacham Walter Dam, a botanist in the Faculty of Science of the University of Bamenda, and a specimen stored at the Cameroon National Herbarium under the identification No HNC/16887. The plant roots were dried under a shade and grinded into powder. Twenty grams of powder were macerated in 200 mL of 70% ethanol for 48 hours and the mixture was filtered using Whatman filter paper number 1. The filtrate was evaporated at 45°C and a brown coloured the extract was obtained extract obtained. The extraction yield was 17.8%.

Experimental animals

Twenty-four male albino Wistar rats of approximately four weeks' old weighing 100±10g were obtained from the animal house of the Department of Biochemistry of the University of Bamenda. The rats were raised under normal laboratory conditions with a natural 12-hours light-dark cycle and were fed on fresh standard pellet and have received water *ad libitum*. Experimental animals were handled according to ethical guidelines of the Cameroon National Veterinary Laboratory as referenced by the approval and health control No 001/17 CCS/MINEPIA/RD-NW/DD-ME/SSV.

Chemical materials

All chemicals used in this study were analytical grade, these included Potassium dihydrogen phosphate, sodium potassium tartrate and potassium iodide (Guandong Guanghua chemical fractions co-ltd in China); Trichloroacetic acid (TCA), hydrogen peroxide, copper sulphate pentahydrate, sodium carbonate and sodium hydroxide (BDH chemicals Ltd Poole England); 2,2-dithio-5,5-dibenzoic acid and thiobarbituric acid (TBA) (Sigma-Aldrich Chemie Co-Ltd spruce street Germany); and Diazepam (5 mg/mL) (Peark Pharmacuticals P Ltd, India). Commercial ELISA kit for the quantification of testosterone, manufactured by Omega diagnostic, was purchased from Biopharcam S.A (Bafoussam, Cameroon).

Animal grouping and treatment

Animals were randomly distributed into four groups (groups 1 to 4) of six rats each, which were assigned to the following treatments: vehicle (group 1; distilled water, 5 mL/kg), *R. vomitoria* extract at the doses of 20 mg/kg (group 2), 40 mg/kg (Group 3) or100 mg/kg (group 4). The doses of the plant extract were defined from previous studies on animal model [15,16]. Administration of the vehicle or plant extract to the animals was done daily through oral intubation/gavage for 60 days, and their body weight recorded once after every 3 days. At the end of follow-up period, each rat was anaesthetized using diazepam (5 mg/mL), sacrificed and reproductive organs (testis, epididymis, ventral prostate, and seminal vesicles) dissected out and weighed. Capillary blood was collected into non-heparinized centrifugal tubes, centrifuged (3000 rpm, 15 min) and serum collected for evaluation of testosterone levels. Testes were homogenized (20% w/v) in Phosphate buffer (0.1 M, pH 7.2), centrifuged and supernatant collected for subsequently determination of oxidative stress biomarkers.

Determination of serum testosterone levels and testicular antioxidants biomarkers

Serum testosterone level was determined using ELISA kit, as per manufacturer's instructions. Reduced glutathione concentrations were measured in testicular homogenates using the method of Ellman [17]. The levels of TBARS were determined by the method of Wilbur et al. [18] while catalase (CAT) activity was evaluated by the method of Misra and Fridovish [19]. All antioxidants' parameters were corrected using protein levels of the testicular homogenate, evaluated by the method of Gornall et al. [20].

Statistical analysis

Data obtained were presented as mean ± standard deviation (mean ± SD) for each animal group. Normal distribution of data set was assessed using Kolmogorov-Smirnov test. Differences between means or treatments assessed by one-way analysis of variance (ANOVA), and pairwise comparison evaluated using Student Newman-Keuls test. Analyses were performed using the MedCalc® software Version 8.0.01, and p values <0.05 considered statistically significant.

Results

Effects of R. vomitoria on animal body weight

The body weight of the rats increased generally throughout the 60 days of treatment (Figure 1) and all doses of the plant extract did not affect the latter parameter when compared to the vehicle group (P>0.05).

Effects of R. vomitoria on reproductive organ weights

Table 1 represents the weights of reproductive organs of the male rats following treatment with the *R. vomitaria* extract. All doses of the extract investigated significantly increased (P<0.05) the relative weights of the prostate glands of the animals when compared to the vehicle group. The plant extract at the dose of 40 mg/kg also increased (P<0.05) the weight of the testis, epididymis, and seminal vesicles of animals.

Effects of R.vomitoria on the serum testosterone levels

All doses of the *R. vomitoria* extract significantly increased (P<0.05) serum testosterone concentrations when compared to the rats receiving distilled water, with the maximum androgenic effect observed in the group exposed to the dose40 mg/kg (Figure 2).

Effects of R. vomitoria on testicular oxidative stress markers

Oxidative stress parameters were assessed in the testes of the animals and results are presented in Table 2. The *R. vomitoria* extract displayed a bell pharmacological pattern with CAT activity and GSH levels, whereas an inverted bell pharmacological pattern was noticed for TBARS levels. The significant (P<0.05) effect was generally observed at the dose 40 mg/kg. The doses 100 mg/kg of the extract mainly showed a lowering (P<0.05) antioxidant effect on TBARS and GSH in male rats.

Discussion

Androgens are crucial for male sexual and reproductive function, and their biosynthesis and the male reproductive function in general are susceptible to be altered by excess free radicals from cellular oxidative stress. Antioxidants are substances capable of inactivating reactive oxygen species (ROS) and nitrite species (RNS) thereby preventing the generation of several oxidative stress linked ill-health conditions such as infertility [8]. Natural products such as extracts of plants are more and more targeted as possible alternative to conventional therapies in the treatment and management of male infertility [16]. In the present study, male rats were treated with different doses of hydro-alcoholic extract of *R. vomitoria* and the testicular antioxidant status and testosterone levels were evaluated.

Animals in all groups had a general increase in body weight throughout the treatment period. Body weight is considered as the first line sign of any adverse effect of any given treatment [21]. The absence of a negative effect of *R. vomitoria* extract on the animal body weights suggests innocuity of the extract, at least on the present physiological parameters. Similar findings were observed in previous studies with other plant extracts [16,22,23]. Treatment of animals with *R. vomitoria* extract for 60 days induced increase of weights of reproductive organs including testes, epididymis, ventral prostrate and seminal vesicles. These organs are responsible for appropriate and normal functioning of the reproductive function. The organs are involved in production sexual hormones and sperm cells (testis), storage and maturation of the germ cells (epididymis), or provision of nutrients to germ cells (prostrate and seminal vesicles) [24]. The extract of *R. vomitoria* contains ingredients that may help maintaining the testicular environment and preserve sperm cell integrity and functionality. Massoma et al. [15] also demonstrated an increase of the testis, prostrate and seminal vesicles in animal treated 21 days with the aqueous extract of *R. vomitoria* at the doses 100 and 200 mg/kg. The present findings showed that hydro-ethanolic extraction highly favoured the pharmacological effects of *R. vomitoria* on male rat reproductive organ weights. The increased reproductive organs weight could also imply stimulation of testosterone production, given that the latter organs are androgen-dependent [25]. This was further explored through assessment of testosterone levels in the animals.

The development, maintenance and functioning of male reproductive organs generally depend on normal level of androgens [3]. In the present study, serum testosterone concentrations were significantly increased in the animals upon the administration of *R. vomitoria* extract at all investigated doses. Similarly, the aqueous leaves extract of *Cardiospermum halicacabum* increased testosterone levels in male rats after treatment for 30 days [26]. The increase of testosterone levels could therefore support elevated weights of reproductive organs, which are androgen dependent [25]. The increased testosterone could as well be regarded as a biological indicator for the effectiveness of *R. vomitoria* extract in stimulating steroidogenesis, probably by increasing the testicular pool of cholesterol, which is a precursor of steroid hormones, through induction of genes coding for steroidogenic enzymes or steroidogenic acute regulatory (StAR) protein in testicular Leydig cells [22,23,27]. Testosterone is essential in formation of germ cells and the maintenance of spermatozoa [5]. Improvement of the testosterone levels together with that of reproductive organ weights are therefore credible mechanisms of increased sexual behaviour and sperm characteristics in male rats previously reported [16]. The

androgenic activity of the plant extract can also be explained by the presence of phenolic compounds and flavonoids present in the extract [28]. In fact, flavonols (sub-class of flavonoids) possess a 5,7-dihydroxychromen-4-one backbone which is beneficial in increasing StAR protein expression in the testes and as such contributes to increased testosterone production from testis Leydig cells [29]. The androgenic effect of the hydro-ethanolic extract of *R. vomitoria* was more important at the dose of 40 mg/kg compared to the highest dose of 100 mg/kg, this could be explained by a saturation of the receptors by the bioactive compounds present in the plant at the high dose (100 mg/kg). Similar reducing pharmacological effect at the high doses of the plant extracts was observed in previous studies [3, 22].

Multiple studies have indicated that ROS inhibits testosterone production in Leydig cells by dissipating mitochondrial membrane potential and reducing the expression and activity of testicular steroidogenic enzymes [30-32]. Thus, accumulation of ROS in the testis results in reduced levels of testosterone [33]. Decrease testosterone production by Leydig cells and increase generation of ROS in testicular tissue could result in decreased sperm motility. The lipid peroxidation of sperm membrane is the key mechanism of ROS-induced sperm damage that leads to infertility [34,35]. The antioxidant defence system of testicular tissue protects the testes against oxidative damage and can reduce free radicals and effectively decrease the damage of ROS on Leydig and spermatogenic cells [36]. Antioxidants such as catalase, SOD, GSH and glutathione peroxidase act as a defence system against reactive oxygen species [37]. Findings from this study revealed that administration of the hydro-ethanolic extract of *R. vomitoria* significantly decreased testicular TBARS concentrations, increased GSH levels and catalase activity. Decreased TBARS level is an indication of decreased lipid peroxidation in testicular tissues [38]. GSH displays its antioxidant activity by the reconstruction of thiol groups (-SH) in proteins and preventing cell membrane from lipid oxidation [39]. Maintaining the steady availability of GSH in animals positively affects the integrity of the Leydig cells and sperm quality [40]. On the other hand, catalase is one of the crucial antioxidant enzymes that mitigate oxidative stress to a considerable extent by destroying cellular hydrogen peroxide to produce water and oxygen [34,39]. The hydro-ethanolic extract of *R. vomitoria* could be beneficial in the management of the testicular function under oxidative stress situations.

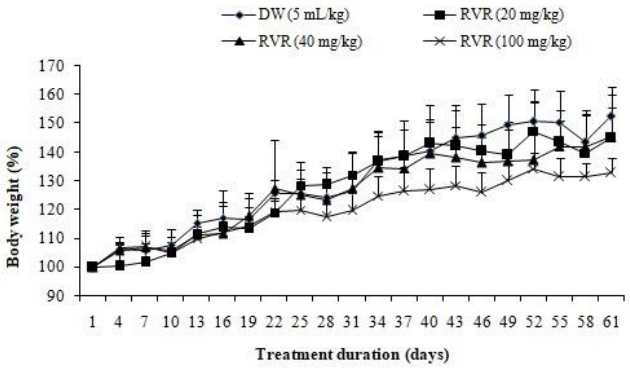

Figure 1. Change in relative body weights of animals during 60 days of treatment with *R. vomitoria* and distilled water.

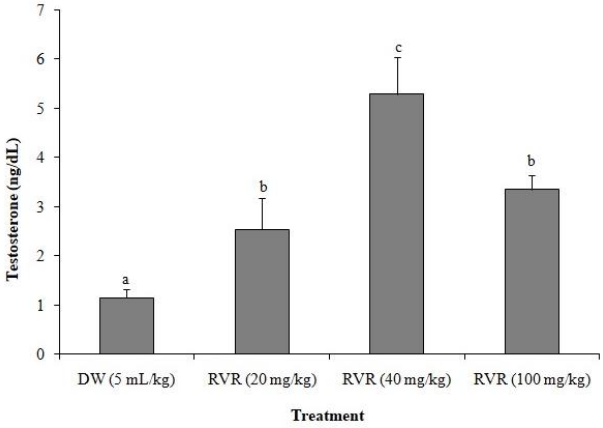

Figure 2. Serum testosterone levels among different groups of animals.
Values not sharing common superscript letters (a-c) differ significantly, P<0.05, Students-Newman-Keuls. DW: distilled water, RVR: *Rauvolfia vomitoria* extract.

Table 1. Relative weights of reproductive organs of animals.

	Treatment			
	Distilled water (5 mL/kg)	***R. Vomitoria* (20 mg/kg)**	***R. Vomitoria* (40 mg/kg)**	***R. Vomitoria* (100 mg/kg)**
Prostate (g/100 g bwt)	0.17 ± 0.07^{a}	0.21 ± 0.02^{ab}	0.25 ± 0.05^{b}	0.24 ± 0.03^{b}
Testis (g/100 g bwt)	0.52 ± 0.05^{a}	0.53 ± 0.04^{a}	0.62 ± 0.06^{b}	0.57 ± 0.02^{a}
Epididymis (g/100 g bwt)	0.40 ± 0.04^{a}	0.42 ± 0.05^{a}	0.53 ± 0.06^{b}	0.43 ± 0.05^{a}
Seminal vesicles (g/100 g bwt)	0.43 ± 0.09^{a}	0.49 ± 0.06^{a}	0.75 ± 0.11^{b}	0.47 ± 0.03^{a}

Values not sharing common superscript letters (a, b) differ significantly; P<0.05, Students Newman Keuls test.

Table 2. Testicular oxidative stress biomarkers of different animal groups.

	Treatment			
	Distilled water (5 ml/kg)	***R. Vomitoria* (20 mg/kg)**	***R. Vomitoria* (40 mg/kg)**	***R. Vomitoria* (100 mg/kg)**
Catalase activity (IU/mg of protein)	18.48 ± 0.85^{a}	18.50 ± 0.41^{a}	24.60 ± 0.81^{b}	20.10 ± 0.39^{a}
GSH (mmole/mg of protein)	4.79 ± 0.01^{a}	4.14 ± 0.03^{a}	5.69 ± 0.02^{b}	3.14 ± 0.06^{c}
TBARS (nmole/mg of protein)	1.04 ± 0.03^{a}	0.72 ± 0.02^{b}	0.54 ± 0.02^{b}	0.61 ± 0.01^{b}

Values not sharing common superscript letters (a-c) differ significantly, P<0.05, Students-Newman-Keuls. GSH: Reduced glutathione; TBARS: thiobarbituric acid reactive substance.

Conclusion

The hydro-alcoholic extract of *R. vomitoria* improves reproductive function in male rats by virtue of its positive effects on reproductive organ weights, testicular antioxidant parameters and testosterone levels. Though these findings call for further investigations on the action mechanisms of *R. vomitoria*, they support the use of the plant in the management of male reproductive ailments.

Abbreviations

GSH: Reduced glutathione,ROS: reactive oxygen species, RNS: nitrite species, *R. vomitoria* or RVR: *Rauvolfia vomitoria*, SOD: superoxide dismutase, StAR: steroidogenic acute regulatory, TBARS: thiobarbituric acid reactive substance.

Authors' Contribution

SAK designed and followed up the experiments, contributed in drafting the manuscript; EA and MM carried out experiments and contributed in drafting the manuscript; TPFM was involved in discussion of data, drafting and reviewing the manuscript; NSN contributed in laboratory analyses; AEN was involved in the designed and followed up the experiments, organized and revised the manuscript.

Acknowledgments

The authors express their gratitude to Che Gilga Allieh, Tashi Standley, Tekoh Theofelix and Kewir Tracy who assisted with the animal follow up and laboratory analyses. Dr Tacham Walter Dam from Department of Biological Sciences, University of Bamenda is also acknowledged for the botanical identification of the plant.

Conflict of interest

The authors declare no conflict of interest

Article history:
Received: 22 September 2021
Received in revised form: 12 October 2021
Accepted: 14 October 2021
Available online: 14 October 2021

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