



RESEARCH ARTICLE

Alcoholic bitters modulates sex hormones and some biochemical parameters of testicular function in male Wistar rats [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Alcoholic bitters have been acclaimed to boost sexual function and fertility in animals but there is no reported scientific evidence that evaluated its effects on the normal functioning of the testes. This study was therefore conducted to assess the effect of some alcoholic bitters on testicular function indices of male Wistar rats.

Methods: A total of 25 male Wistar rats were assigned into five groups of five animals each and treated with distilled water, ethanol, Alomo, Striker and Orijin Alcoholic Bitters at 0.2, 0.2, 0.2, 0.16 and 0.3 ml/kg body weight respectively for 28 days. The animals were thereafter sacrificed and the serum obtained was used for the determination of sex hormones. Assessment of testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), cholesterol, and malondialdehyde (MDA) concentrations as well as the activity of β -Hydroxy β -methylglutaryl-CoA reductase (HMG-CoA reductase), superoxide dismutase (SOD) and catalase (CAT) were carried out using standard methods.

Results: There were significant ($p < 0.05$) increases in protein, cholesterol, testosterone, FSH and LH, as well as in the activity of HMG-CoA reductase, SOD and CAT in all the groups of animals administered the alcoholic bitters, whereas concentration of MDA was significantly reduced ($p < 0.05$). Concentration of triglycerides was not significantly different ($p > 0.05$) from those of the control animals.

Conclusion: The alcoholic bitters enhanced the normal functioning of the testes, the antioxidant enzymes and the release of the reproductive hormones. This may partly explain its use in boosting sexual function and fertility in male rats.

Open Peer Review

Reviewer Status

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1. Gerhard Haidl , University Hospital Bonn, Bonn, Germany		
2. Quadri O. Nurudeen , Al-Hikmah University, Ilorin, Nigeria		
Any reports and responses or comments on the article can be found at the end of the article.		

Keywords

Alcoholic bitters, Cholesterol, Testosterone, Antioxidant enzymes, Testicular function.

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Introduction

Infertility describes prolonged difficulty at achieving success with conception between a male and female having unhindered access to sexual activity¹.

A male's inability to fertilize the female's ovum for upwards of twelve months of unrestricted intercourse categorizes him as infertile. This challenge is represented globally across continents where up to about fifteen percent of heterosexual couples are infertile and forty percent of these represents male factor infertility and sexual inadequacies¹.

For centuries, plants and plant-based products have been utilized as an important and safe natural source of medicines for treating different health challenges². Various plant extracts have been acclaimed to have aphrodisiac potentials and utilized traditionally to enhance sexual performance and fertility^{3,4}. Some of these extracts are produced, packaged and sold as bitters drink, which has gained global popularity in recent times.

Bitters are liquid preparations (regularly alcoholic) produced from herb and root concentrates of tropical and subtropical plants⁵. They have been utilized over several decades for their acclaimed set of medicinal benefits, such as treatment of kidney and bladder dysfunction, regulation of blood pressure, management of indigestion, menstrual cramps, ulcers, gastritis, insomnia, stress, depression, excessive weight and sexual inadequacies^{4,6}. The most commonly consumed alcoholic bitters in Omu aran, Nigeria are Alomo, Orijin and Striker bitters. The rising popularity of these bitters despite their severe bitter taste and appalling scent could be attributed to the alleged preferences of being effective medicinally, cheap and readily available.

This study therefore was aimed to study the effects of these alcoholic bitters on some testicular indices in order to justify its traditionally acclaimed use as an aphrodisiac and pro-fertility agent.

Methods

Alcoholic bitters

Bottles of Alomo, Orijin and Striker were purchased from the main market in Omu Aran, Kwara State, Nigeria.

Experimental animals

Twenty five healthy, adult male Wistar rats (110–130 g) which made up five groups of five animals each, were obtained from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State. The animals were housed in plastic rat cages placed in well-ventilated rooms (photo-period: 12 h light and 12 h dark cycle; temperature: 25–27°C; humidity: 45–55%) and fed with standard rat pellet and water *ad libitum*.

Reagents and chemicals

Reagent kits used for the assay for total cholesterol (Lot no: 416569) and triglyceride (Lot no: 435212) were products of Randox Laboratory Limited, UK. The assay kits for testosterone, follicle-stimulating hormone (FSH) and leutinizing hormone

(LH) were products of Monobind Inc, Lake Forest, CA, USA. All other reagents used were of analytical grade and were prepared in volumetric flask using glass-distilled water.

Experimental design

The Wistar rats were assigned into one of five groups consisting of five animals each as shown below:

- Group A is the control group which received 0.2ml/kg body weight distilled water;
- Group B received 0.2ml/kg body weight ethanol
- Group C received 0.2ml/kg body weight Alomo bitters
- Group D received 0.16ml/kg body weight Strikers bitters
- Group E received 0.3ml/kg body weight Orijin bitters

Administration of the herbal bitters, ethanol and water was performed orally once daily for 28 days, after which the rats were sacrificed by placing them in a jar containing diethyl ether. Testicular tissue was excised and used for the biochemical assays.

Serum and sample collection

Blood samples were collected by venal puncture into plain sample bottles subsequent to sacrifice of the rats, then centrifuged at 5000rpm and the serum was stored at 20°C prior to the subsequent hormonal assay.

One half of the testes for each rat were homogenized in ice-cold 0.25M sucrose solution. The homogenates were diluted using a dilution factor 1:9 w/v ratio. The diluted homogenates were further centrifuged at 5000 rpm for 10 minutes and then stored for biochemical assays.

Total lipid was extracted from the second half of the testicular tissue by a mixture of chloroform methanol (2:1, v/v) as described by Folch *et al.*⁷. Aliquots of total lipid extract were used for quantitative analysis of cholesterol and triglycerides⁸.

Determination of serum hormones

The serum concentrations of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were quantitatively determined from the serum by adopting the procedure highlighted by Tietz⁹.

Protein determination

The total protein concentration was estimated in the testes homogenates using the Biuret method¹⁰. Twenty microlitres of serum were mixed with 1000 µl of Biuret reagent (6 mmol/L potassium iodide, 21 mmol/L potassium sodium tartarate, 6 mmol/L copper sulphate, and 58 mmol/L sodium hydroxide). Thereafter, the mixture was incubated for 10 min at 37°C and the absorbance taken at 546 nm. Bovine serum albumin was used as the standard protein and the total protein was subsequently calculated using the formula:

Total protein concentration (g/dL) = (Absorbance of sample / Absorbance of standard) × 6

Lipid profile assay

Determination of total cholesterol and triglyceride concentration were carried out using enzymatic kits procured from Randox laboratories^{11,12}.

Enzyme activities

HMG- CoA reductase activity. HMG-CoA reductase activity was measured in testes homogenate using the procedure of Rao and Ramakrishnan¹³. The ratio of HMG-CoA to mevalonate was taken as an index of enzyme activity which catalyzes the conversion of HMG to mevalonate. The lower the ratio, the higher the enzyme activity. Equal volumes (0.5 mL each) of tissue homogenate and diluted perchloric acid (50 mL/L) were mixed together. This was allowed to stand for 5 minute and centrifuged at 2000 rpm for 10 min. This was filtered and 1 mL of filtrate was mixed with 0.5 mL of freshly prepared hydroxyl amine (2 mol/L) reagent of pH 5.5 for HMG-CoA and with 0.5 mL of freshly prepared hydroxyl amine (2 mol/L) reagent of pH 2.1 for mevalonate. After 5 minutes, 1.5 mL of ferric chloride reagent (prepared by dissolving 5.2 gm of trichloroacetic acid and 10 gm of ferric chloride in 50 mL of 0.65 mol/L HCl) was added to each of the test tube for HMG-CoA and mevalonate. The tubes were well shaken. Absorbance was read after 10 min at 540 nm versus a similarly treated arsenate blank using double beam UV-Visible spectrophotometer.

Superoxide dismutase (SOD). SOD was assayed using the standard method of Misra and Fridovich *et al.*¹⁴. Based on the inhibition of Epinephrine-Adrenochrome transition by the enzyme. A 0.5ml of testes homogenate was taken in a reaction vessel and then 0.5ml of distilled water was added to dilute the sample. To this 0.25ml of ice cold ethanol and 0.15ml of chloroform were added to precipitate the reaction mixture. The reaction mixture was well shaken for about 5minutes at 4°C and then centrifuged. The enzyme activity in the supernatant solutions was determined using spectrophotometer. The Adrenochrome produced in the reaction mixture, contains 0.2ml of EDTA (0.6mM), 0.4ml of Na₂CO₃ (0.25M) and 0.2ml of Epinephrine (3.0mM), final volume was adjusted to 2.5ml and then the absorbance readings were measured at 420nm in a UV-Visible recording Spectrophotometer. The Transition of Epinephrine to Adreno-Chrome was determined by the addition of the required quantity of enzyme to assess the enzyme activity expressed in terms of units/minute/mg protein.

Catalase (CAT). CAT was assayed using standard protocol¹⁵. The breakdown of H₂O₂ on addition of the enzyme is followed by absorbing the decrease in light absorption of peroxide solution in the UV region was determined. A 3ml of reaction mixture containing 1.9ml of phosphate buffer, [(0.05M) of pH 7.0], 1.0ml of substrate H₂O₂ and 0.1ml of testes homogenate was used in this assay. The activity was measured as change in optical activity/density at 240nm at 30sec interval for about 3 minutes. The CAT activity was expressed in terms of mole of H₂O₂ consumed/minute/mg protein.

Malondialdehyde (MDA) concentration. Thiobarbituric acid reacts with MDA giving rise to a high absorptivity adduct which

was assessed with a spectrophotometer at 531 nm. A standard graph was plotted, and concentration of MDA was expressed as nmol/ml¹⁶.

Statistical analysis

Data were expressed as mean \pm SEM of five replicates. The data were subjected to statistical analysis using Analysis of Variance and complemented with Duncan *post hoc*. The analyses were done with IBM SPSS statistics, version 22.0 (SPSS Inc; Chicago USA). Differences were considered statistically significant at $p < 0.05$.

Ethical approval

All procedures performed on the animal subjects were in accordance with the ethical standards of Landmark University Animal Care Committee (approval number LUAC-0031A). All efforts were made to ameliorate any suffering to the animals in the course of treatment by following careful intubation procedures and also by anaesthetising the animals prior to sacrifice to prevent their experiencing any form of associated pain.

Results

The serum level of testosterone, FSH and LH significantly increased ($p < 0.05$) in all bitters administered groups compared to the control group and the ethanol group (Table 1).

The administration of the Alomo, Striker and Orijin alcoholic bitters as well as ethanol to the animals resulted in a significant increase ($p < 0.05$) in protein concentration, HMG-CoA reductase activity, and cholesterol levels when compared to the control group. There was however no significant increases ($p > 0.05$) in the levels of the triglycerides (Table 2).

There was a significant increase ($p < 0.05$) in MDA concentration in the ethanol control group compared to the control group. However, the concentration of MDA in the bitters treated groups significantly reduced ($p < 0.05$) compared to the ethanol group (Table 3). Furthermore, there was a significant increase in the activities of SOD and catalase in the ethanol and bitters administered groups compared to the control group. The upregulation of enzyme activity was however significantly higher ($p < 0.05$) in the bitters group compared to the ethanol group.

Table 1. Reproductive sex hormones in the blood serum of Wistar rats treated with a variety of alcoholic bitters.

Groups	Testosterone (pg/mL)	Follicle stimulating hormone (pg/mL)	Luteinizing hormone (pg/mL)
Control	2.54 \pm 0.01 ^a	0.02 \pm 0.0 ^a	0.06 \pm 0.00 ^a
Ethanol	1.05 \pm 0.2 ^b	0.01 \pm 0.004 ^a	0.02 \pm 0.00 ^a
Alomo	7.63 \pm 0.04 ^c	0.17 \pm 0.04 ^b	0.14 \pm 0.03 ^a
Striker	4.22 \pm 0.1 ^d	0.22 \pm 0.0 ^b	0.09 \pm 0.00 ^a
Orijin	4.23 \pm 0.2 ^d	0.40 \pm 0.1 ^c	0.33 \pm 0.1 ^b

Data are mean of five determinations \pm SEM. Values with superscripts different from the control for each parameter are significantly different ($p < 0.05$)

Table 2. Biochemical parameters in Wistar rats treated with a variety of alcoholic bitters. Cholesterol and triglycerides were determined from total lipid extracted from testicular tissue; HMG-CoA reductase and protein concentration were determined from homogenized testicular tissue.

Groups	Cholesterol concentration (mmol/L)	Triglycerides (mmol/L)	HMG-CoA reductase activity ($\mu\text{mol/gprotein}$)	Protein concentration(mg/ml)
Control	10.07 \pm 0.4 ^a	0.53 \pm 0.1 ^a	1.08 \pm 0.02 ^a	5.59 \pm 0.3 ^a
Ethanol	10.19 \pm 0.6 ^a	0.66 \pm 0.01 ^b	2.45 \pm 0.3 ^b	4.20 \pm 0.3 ^b
Alomo	15.98 \pm 0.2 ^b	0.50 \pm 0.04 ^a	3.19 \pm 0.03 ^c	6.72 \pm 0.4 ^c
Striker	15.76 \pm 0.4 ^b	0.49 \pm 0.04 ^a	3.63 \pm 0.1 ^{cd}	6.23 \pm 0.3 ^{ac}
Orijin	15.22 \pm 0.1 ^b	0.27 \pm 0.02 ^c	3.94 \pm 0.01 ^d	6.02 \pm 0.4 ^{ac}

HMG-CoA: β -Hydroxy β -methylglutaryl-CoA reductase. Data are mean of five replicates \pm SEM. Values with superscripts different from the control for each parameter are significantly different ($p < 0.05$)

Table 3. Testicular antioxidant parameters in Wistar rats treated with a variety of alcoholic bitters.

Groups	Malondialdehyde (nmol/ml)	Superoxide dismutase (U/mg protein)	Catalase activity (U/mg protein)
Control	2.30 \pm 0.2 ^a	0.52 \pm 0.1 ^a	5.59 \pm 0.3 ^a
Ethanol	5.66 \pm 0.4 ^b	0.66 \pm 0.2 ^b	6.19 \pm 0.3 ^c
Alomo	3.78 \pm 0.2 ^c	1.48 \pm 0.1 ^{ac}	6.72 \pm 0.4 ^b
Striker	2.2 \pm 0.2 ^a	1.35 \pm 0.1 ^{ac}	6.23 \pm 0.3 ^{ab}
Orijin	1.33 \pm 0.1 ^d	1.12 \pm 0.03 ^c	6.02 \pm 0.4 ^{ab}

Data are mean of five replicates \pm SEM. Values with superscripts different from the control for each parameter are significantly different ($p < 0.05$)

Dataset 1. Data for assessment of various hormones in the serum and biochemical parameters in the testes of Wistar rats treated with: Group A (1) control group which received 0.2ml/kg body weight distilled water; Group B (2) received 0.2ml/kg body weight ethanol; Group C (3) received 0.2ml/kg body weight Alomo bitters; Group D (4) received 0.16ml/kg body weight Strikers bitters; Group E (5) received 0.3ml/kg body weight Orijin bitters

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Discussion

The administration of alcoholic bitters to the rats for the period of 28 days did not result in obvious physical toxicity to the animals. However, their behavioural pattern after the consumption of these bitters showed there was a sharp increase in their physical activity and agility, which was noticeable within the first few hours of the bitters and ethanol administration to the animals. This may be associated with the alcoholic content in these bitters. Another noticeable change was the improved appetite of these animals post-administration of the bitters, which supports a previous report that suggests that bitters promote appetite and digestion¹⁷.

The increased testicular protein concentration in the bitters-treated animals may indicate the upregulation of protein synthesis in the testes, which may positively enhance testicular function and spermatogenesis¹⁸.

Malondialdehyde (MDA), which is a by-product of lipid peroxidation¹⁹ was only elevated in the animals administered ethanol and not alcoholic bitters in the testicular tissue, suggesting that alcohol treatment alone may foster generation of reactive oxygen species leading to oxidative stress, a condition that precedes the onset of several diseases in humans²⁰. Of interest is the involvement of oxidative stress in the aetiology of testicular perturbation and altered spermatogenic function, which may adversely affect male fertility and sexual function²¹. The observed reduction of MDA for all the groups administered alcoholic bitters might indicate an antioxidative potential of the bitters.

Antioxidant enzymes SOD and catalase reduces oxidative stress by scavenging oxygen radical and converting it to hydrogen peroxide and oxygen and further reduction of the hydrogen peroxide to minimize tissue damage by the radicals²². The alcoholic bitters treatment resulted in increased activity of these enzymes compared to the group administered ethanol. The observed increase in the activity of the antioxidant enzymes SOD and catalase in this study might be related to the upregulation of proteins (a biomolecule class to which SOD and CAT belongs) by the bitters stated earlier which will in turn lead to effective mop-of circulating free radicals and hence reducing oxidative stress and protecting the tissue from highly reactive hydroxyl radical²². This further corroborates the inherent antioxidant property of these bitters.

Increased level of sex hormones (testosterone, LH and FSH) in the alcoholic bitters administered animals is probably a further indication of the ability of the bitters to mediate improved sexual function and fertility²³. Testosterone being the major male sex hormone will direct fortification of sexual prowess and function in animals. Furthermore, increased Luteinizing Hormone will likely contribute to the increased production of testosterone by the interstitial cells besides the other mechanism for its production via increased cholesterol synthesis²⁴. While increased FSH observed from this study by the bitters will enhance spermatogenesis by the sertoli cells leading to increased production of viable and motile spermatozoa

The observed increased cholesterol levels and the activity of HMG-CoA reductase in the testes after administration of the alcoholic bitters may shed light on the mechanism by which the bitters improved the synthesis of testosterone in the animal. HMG-CoA reductase is a major enzyme in the synthetic pathway for cholesterol and its increased activity will also lead to production of increased levels of testicular cholesterol, which is a precursor for synthesis of steroid sex hormones¹⁸.

Conclusion

The acclaimed aphrodisiac and fertility enhancement property of herbal bitters is supported by this study as evidenced by its ability to combat oxidative stress and enhance synthesis of sex hormones through cholesterol upregulation in the testes.

Data availability

F1000Research: Dataset 1. Data for assessment of various hormones in the serum and biochemical parameters in the testes of Wistar rats treated with: Group A (1) control group which received 0.2ml/kg body weight distilled water; Group B (2) received 0.2ml/kg body weight ethanol; Group C (3) received 0.2ml/kg body weight Alomo bitters; Group D (4) received 0.16ml/kg body weight Strikers bitters; Group E (5) received 0.3ml/kg body weight Orijin bitters, <https://doi.org/10.5256/f1000research.16648.d225117>²⁵.

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Version 1

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Quadri O. Nurudeen

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The conclusion that "The acclaimed aphrodisiac and fertility enhancement property of herbal bitters is supported by this study as evidenced by its ability to combat oxidative stress and enhance synthesis of sex hormones through cholesterol upregulation in the testes". The results in the study shows promising aphrodisiac potentials of the bitters, but not supporting the fertility enhancement property of the herbal bitters.

However, the article described what the authors achieved accurately, and clearly stated the problem being investigated. There is sufficient information presented for replication of the research. The article is interestingly novel and may be recommended for indexing after the minor corrections.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Reproductive Biochemistry and Toxicology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 June 2019

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Gerhard Haidl

Department of Dermatology, University Hospital Bonn, Bonn, Germany

This is, in principle, an interesting article on the effect of alcoholic bitters on male rat sexual hormones and fertility. The authors found an increase in the hormonal concentrations of FSH, LH and testosterone and conclude, that sexual desire and fertility have increased and improved, respectively. In this context, the antioxidant effect may indeed contribute to a better testicular function. However, for interpretation of changes in fertility evaluation of seminal parameters or – in this case even better – testicular biology is necessary. From the clinical experience we know that FSH and LH as well as testosterone can rise after stimulating treatment with antiestrogens, but normally elevated levels of FSH and LH indicate a testicular damage. As alcohol is not only toxic to the liver but also to the testis it is well imaginable that fertility has not improved but deteriorated.

Therefore, the conclusion that administration of alcoholic bitters may be helpful for fertility problems is than questionable. Without providing data on semen variables and testicular biopsy before and after treatment the manuscript should not be indexed.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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