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

Omowumi T. Kayode, Abolanle A.A. Kayode, Charles O. Nwonuma

Department of Biochemistry, Landmark University, Omu Aran, Kwara, Nigeria
Department of Pharmacognosy, University of Ibadan, Ibadan, Oyo, Nigeria

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.

Keywords
Alcoholic bitters, Cholesterol, Testosterone, Antioxidant enzymes, Testicular function.
Corresponding author: Omowumi T. Kayode (kayode.omowumi@lmu.edu.ng)

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**Introduction**

Infertility describes prolonged difficulty at achieving success with conception between a male and female having unhindered access to sexual activity\(^1\).

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 inadequacies\(^1\).

For centuries, plants and plant-based products have been utilized as an important and safe natural source of medicines for treating different health challenges\(^2\). 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 plants\(^5\). 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\(^6\). 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 triglycerides\(^8\).

**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 Tietz\(^9\).

**Protein determination**

The total protein concentration was estimated in the testes homogenates using the Biuret method\(^1\). 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:

\[
\text{Total protein concentration (g/dL)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \times 6
\]
Lipid profile assay
Determination of total cholesterol and triglyceride concentration were carried out using enzymatic kits procured from Randox laboratories [1,12].

Enzyme activities
HMG-CoA reductase activity. HMG-CoA reductase activity was measured in testes homogenate using the procedure of Rao and Ramakrishnan [1]. 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 hydroxylamine (2 mol/L) reagent of pH 5.5 for HMG-CoA and with 0.5 mL of freshly prepared hydroxylamine (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 5 minutes at 4°C and then centrifuged. The enzyme activity in the supernatant solutions was determined using spectrophotometer. The Adreno-Chrome 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 protocol [15]. The breakdown of H2O2 on addition of the enzyme is followed by absorbing the decrease in light absorption of peroxide solutions 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/ml [16].

Statistical analysis
Data were expressed as mean ± 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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Testosterone (pg/mL)</th>
<th>Follicle stimulating hormone (pg/mL)</th>
<th>Luteinizing hormone (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.54 ± 0.01 a</td>
<td>0.02 ± 0.0 a</td>
<td>0.06 ± 0.00 a</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.05 ± 0.2 b</td>
<td>0.01 ± 0.004 a</td>
<td>0.02 ± 0.00 a</td>
</tr>
<tr>
<td>Alomo</td>
<td>7.63 ± 0.04 c</td>
<td>0.17 ± 0.04 b</td>
<td>0.14 ± 0.03 c</td>
</tr>
<tr>
<td>Striker</td>
<td>4.22 ± 0.1 d</td>
<td>0.22 ± 0.0 b</td>
<td>0.09 ± 0.00 a</td>
</tr>
<tr>
<td>Orijin</td>
<td>4.23 ± 0.2 d</td>
<td>0.40 ± 0.1 c</td>
<td>0.33 ± 0.1 b</td>
</tr>
</tbody>
</table>

Data are mean of five determinations ± 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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol concentration (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>HMG-CoA reductase activity (µmol/g protein)</th>
<th>Protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.07 ± 0.4 a</td>
<td>0.53 ± 0.1 a</td>
<td>1.08 ± 0.02 a</td>
<td>5.59 ± 0.3 a</td>
</tr>
<tr>
<td>Ethanol</td>
<td>10.19 ± 0.6 a</td>
<td>0.66 ± 0.01 b</td>
<td>2.45 ± 0.3 b</td>
<td>4.20 ± 0.3 b</td>
</tr>
<tr>
<td>Alomo</td>
<td>15.98 ± 0.2 b</td>
<td>0.50 ± 0.04 a</td>
<td>3.19 ± 0.03 c</td>
<td>6.72 ± 0.4 c</td>
</tr>
<tr>
<td>Striker</td>
<td>15.76 ± 0.4 b</td>
<td>0.49 ± 0.04 a</td>
<td>3.63 ± 0.1 cd</td>
<td>6.23 ± 0.3 ac</td>
</tr>
<tr>
<td>Orijin</td>
<td>15.22 ± 0.1 b</td>
<td>0.27 ± 0.02 c</td>
<td>3.94 ± 0.01 d</td>
<td>6.02 ± 0.4 ac</td>
</tr>
</tbody>
</table>

HMG-CoA: β-Hydroxy-β-methylglutaryl-CoA reductase. Data are mean of five replicates ± 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.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Malondialdehyde (mmol/ml)</th>
<th>Superoxide dismutase (U/mg protein)</th>
<th>Catalase activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.30 ± 0.2 a</td>
<td>0.52 ± 0.1 a</td>
<td>5.59 ± 0.3 a</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.66 ± 0.4 b</td>
<td>0.66 ± 0.2 b</td>
<td>6.19 ± 0.3 c</td>
</tr>
<tr>
<td>Alomo</td>
<td>3.78 ± 0.2 c</td>
<td>1.48 ± 0.1 ac</td>
<td>6.72 ± 0.4 b</td>
</tr>
<tr>
<td>Striker</td>
<td>2.2 ± 0.2 a</td>
<td>1.35 ± 0.1 ac</td>
<td>6.23 ± 0.3 ab</td>
</tr>
<tr>
<td>Orijin</td>
<td>1.33 ± 0.1 d</td>
<td>1.12 ± 0.03 c</td>
<td>6.02 ± 0.4 ab</td>
</tr>
</tbody>
</table>

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

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 digestion

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 spermatogenesis

Malondialdehyde (MDA), which is a by-product of lipid peroxidation 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 humans. 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 function. 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 radicals. 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 radical. 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 fertility. 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 synthesis. 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 hormones.

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 Orjin bitters, https://doi.org/10.5256/f1000research.16648.d225117

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