INTRODUCTION

Sexual dysfunction is a serious medical and social problem that occurs in 10-25% of men and 25-63% of women. Among men aged 40-70 years, estimated 34.8% have moderate to complete erectile dysfunction. This condition can be managed via psychotherapeutic and pharmacotherapeutic approaches. Free radicals are present in seminal plasma, some of the most prevalent reactive oxygen species (ROS) are hydroxyl, superoxide, and hydrogen peroxide radicals. During oxidative stress, excessive production of the ROS or free radicals in seminal plasma tends to have a destructive effect of the sperm cells and in turn induce oxidative stress induced male infertility. Moreover, antioxidants in seminal plasma aid in scavenging the harmful effect of the ROS via the donation of electrons to the electron-impaired radicals, thereby reduce their influence. Spermatozoa in mammals are rich in polyunsaturated fatty acids (PUFA) as a result of that they are very prone to membrane lipid

13, 14-Epoxyoleanan-3-ol-acetate: A Male Fertility-Enhancing Constituent from Hexane Fraction of Momordica charantia L. (Cucurbitaceae)

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ABSTRACT

Objectives: Male infertility has been associated with oxidative stress-induced and/or microbial induced in some men. The use of medicinal plants to overcome oxidative stress-induced infertility cannot be over emphasized. Hence, the aim of this research was to isolate the antilipid peroxidation (an index of usage for treating oxidative stress-induced male infertility) bioactive principle from Momordica charantia using bioactivity-guided isolation.

Materials and Methods: n-Hexane fraction from the crude ethanol extract obtained by Soxhlet extraction of aerial parts (without fruit) of bitter melon, M. charantia, was assessed for in vitro lipid peroxidation, followed by bioactivity-guided isolation of bioactive principles using in vitro lipid peroxidation as an index of aphrodisiac and male fertility enhancer.

Results: Fractionation of the active n-hexane fraction using vacuum liquid chromatography (VLC) gave five pooled fractions on the basis of their thin layer chromatography (TLC) characteristics (n-hexane: EtOAc, 2:3, sulphuric acid spray). In vitro activity of the most active VLC fraction C was less than that of the positive control, vitamin E. Further fractionation of VLC-C by open column chromatography on silica gel led to the isolation of a compound which was purified by preparative-TLC. The purified compound, 10 mg/mL (R f 0.54, TLC silica gel, n-hexane: ethyl acetate; 2:3) was equipotent with vitamin E (25 mg/mL) in reducing peroxidation of polyunsaturated fatty acids in vitro. Structural elucidation by NMR (1H, 13C) and mean mass spectroscopy confirmed the identity of the new bioactive compound as 13, 14-epoxyoleanan-3-ol-acetate.

Conclusion: This study scientifically validates the traditional claim of M. charantia as an aphrodisiac or male fertility enhancer and suggests that 13, 14-epoxyoleanan-3-ol-acetate might be responsible for the observed activity.

Key words: Momordica charantia, n-hexane fraction, in vitro lipid peroxidation assay, 13, 14-epoxyoleanan-3-ol-acetate, vitamin E, VLC
peroxoide ion and ROS attack. Moreover, a balance is maintained between the amount of ROS produced and that scavenged arises, when this disturbed equilibrium toward pro-oxidants in semen and vaginal secretions can induce an oxidative stress on spermatozoa, which in turn can cause its damage and cause infertility.6 Theoretically, oxidative stress-induced sperm cells results in decreased sperm motility, presumably by a rapid loss of intracellular adenosine triphosphate leading to axonemal damage, decreased sperm viability and increased midpiece morphology defects, with deleterious effects on sperm capacitation and acrosome reaction.5 However, the key mechanism of ROS-induced sperm damage leading to infertility is principally induced by the effect peroxidation of sperm membrane lipid.6 Among the medicinal plants used in treating numerous diseases including male sexual dysfunction and infertility is Momordica charantia L. (Cucurbitaceae).6-10 It is a climbing vine commonly found in the tropics and subtropics. It is also a tropical vegetable employed in ethnomedicine for the treatment of various diseases including diabetes, malaria and dysentery, and as a stomachic, stimulant, emetic, antilulcer, and laxative.11 Previously, M. charantia has been investigated for analgesic and antipyretic,7,8 antimicrobial,9 and anti-HIV activities. Bioactive compounds responsible for the widely investigated antidiabetic and hypoglycemic activities have been linked to cucurbitanetriterpenoids.11-13 The cucurbitane-type triterpenoids have been extensively isolated from various parts of M. charantia.11-15 Until date, literature information is unavailable on male fertility enhancing the potential of M. charantia, and only a mention of traditional use of the Nigerian plant as an aphrodisiac is known.7,9,16 We therefore investigated in vitro fertility activity (using lipid peroxidation as index) of the most active hexane fraction of the aerial parts (it contain no fruit) by lipid peroxidation assay, and isolated the bioactive constituent to rationalize the traditional claim of the plant as an aphrodisiac plant by the people of Esan community in Edo State, Nigeria.

MATERIALS AND METHODS
Plant collection and authentication
M. charantia used in the research was collected from wild in the Ewu community of Esan-Central Local Government Area of Edo state (Nigeria) in January 2012 and authenticated at the herbaria in Paxherbal Laboratories, Ewu (Nigeria) by Professor J. C. Okafor and the Federal Forestry Research Institute of Nigeria, Ibadan, Oyo State (FHI 109577). Voucher specimens were also deposited in these herbaria and at the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Nigeria.

Extraction and solvent partitioning
1.2 kg of the dried aerial part of the plant was extracted to exhaustion with absolute ethanol in a Soxhlet apparatus. The extract was reduced in vacuo to yield a residue that was refrigerated at 4°C until needed. The crude ethanol extract was partitioned into n-hexane, chloroform, and water using a separatory funnel and total yield of the fractions was determined.

Chromatographic studies
Vacuum liquid chromatography (VLC): n-Hexane fraction (21.00 g) was packed onto a sintered glass Buchner filter funnel, loaded with silica gel for analytical thin layer chromatography (TLC) without binder and eluted with n-hexane, chloroform, and methanol mixtures to yield 17 fractions (x 200 mL each). The fractions were bulked according to their TLC profile (n-hexane: EtOAc, 2:3; H2SO4 spray reagent) into 5 main fractions (A-E), weighed and bio-assayed in vitro.

Bottom of form
Column chromatography (CC)/preparative-TLC: Conventional open CC (30 cm long and 5 cm diameter) of the most active VLC fraction C was done. The silica gel used was 70-230 mesh size (0.063-0.200 mm) particle size. The silica gel was loaded on top of the column and allowed to settle. VLC fraction C (3 g) was diluted, adsorbed onto silica gel and poured on top of the column which was eluted with n-hexane, chloroform, ethyl acetate, and methanol mixtures. Eluates (151 fractions x 10 mL) were collected into test tubes and bulked according to their TLC characteristics (silica gel, n-hexane: ethylacetate, 2:3; H2SO4 spray reagent) into 5 main fractions CC (A-E), dried, and weighed. The most active fraction, i.e. CC-D (0.63 g), was subjected to prep-TLC (commercial type) with n-hexane: ethyl acetate (4:6). The bands were scrapped, eluted with methanol, filtered, and evaporated in a fume closet to yield needle-shaped crystals.

Spectroscopic studies
1D NMR (1H and 13C NMR) as well as 2D NMR (DEPT, HMBC) experiments were performed on the isolated compound. Mean mass spectroscopy (MS) was recorded on an Agilent Technologies S973 network mass selective detector and the spectrum was compared with database NIST02 reference spectra library.

In vitro lipid peroxidation assay
Fresh but frozen Titus fish (Scomber japonicus) were purchased from the Ekpoma market in Benin City, washed, and fleshy muscular parts will be used. The tissue homogenate was prepared as described earlier as the modified method of Luotola and Luotola.17 Twenty grams of raw fish muscle tissue was added and the mixture was cooked at a temperature of 100°C for 15 min followed by thoroughly blending in an electric blender (10% w/v). This, in turn, was filtered and unbroken cells along with cell debris were removed by centrifugation at 1500 rpm for 15 min. The supernatant thus obtained was termed as a homogenate and used for the in vitro lipid peroxidation study. A lipid peroxidation study was conducted almost immediately after the homogenate preparation. 1 mL of each VLC fraction (100 mg) was added to 1 mL of the fish homogenate and mixed together. Thiobarbituric (TBA) reactivity in the homogenate
was determined by following a modified method of Luotola and Luotola. 3 mL of 20% trichloroacetic acid was added, mixed, and centrifuged for 15 min. Then, TBA (0.67% w/v, 1 mL) was added to 2 mL supernatant, mixed, and kept in a boiling water bath for 10 min, which, after, cooled down to room temperature. TBA chromogen (intensity of the pink coloured complex) was measured at 532 nm against blanks using an ultraviolet spectrophotometer (Thermo electron, Genesys 20 model). Vitamin E was used as the reference. A graph of absorbance against concentration will be plotted using the data obtained for pure vitamin E. Thiobarbituric acid reactive substances (TBARS) of the extract were evaluated from the standard curve against concentration will be plotted using the data obtained for pure vitamin E. Thiobarbituric acid reactive substances (TBARS) of the extract were evaluated from the standard curve and expressed as nmol TBARS per mg of tissue. This procedure was repeated for the positive control (vitamin E, 25 mg/mL), negative control (5% Tween 80, 1 mL), column fractions, and the isolated compound (10 mg/mL). All results were replicated three times and the mean was determined.

**Statistical analysis**

All data collected from the entire study was analyzed using Microsoft excel and Statistical Package for Social Sciences (SPSS) version 17 (when needed). All values in the test were presented as mean ± standard error of mean (SEM). Statistical differences between the means of the various groups were evaluated by One-Way ANOVA and tested at 0.05 level of significance. The results were considered statistically significant if the p values were 0.05 or less.

**RESULTS**

**Lipid peroxidation of bulked VLC fractions of the hexane fraction**

From an earlier study described by Adedokun et al., the n-hexane fraction was the most active aphrodisiac agent, in vivo and in vitro of the three fractions from the crude ethanol extract of *M. charantia*. The result of lipid peroxidation of the entire bulked VLC fractions (VLC-A to VLC-E) is shown in Table 1.

The values above represent the mean ± SEM of 5 replicates. Values with superscripts indicate significant different relative to the negative control (5% Tween 80) at p≤0.05 across the column for each time using One-Way ANOVA (non-parametric). From Table 1, VLC-C showed the highest degree of inhibition of polyunsaturated fatty acids in the fish tissue over time with lowest and highest amounts of malondialdehyde observed at 0 min and 240 min as 98.00 ± 0.01 and 171.00 ± 0.08 respectively, significantly different from negative control at p≤0.05 at a similar time interval as shown in Table 1.

**Lipid peroxidation of isolated compound and vitamin E**

The result of comparative lipid peroxidation study of isolated compound X (10 mg/mL) with both vitamin E (26 mg/mL) as well as the negative control (5% Tween 80) is shown in Figure 1. No significant difference was observed in the activity of compound X (10 mg/mL) and positive control (vitamin E) 26 mg/mL using an in vitro model.

**Spectroscopic analysis of compound X**

Compound X was subjected to spectroscopic analysis to identify the nomenclature of the unknown bioactive compound, NMR studies (1H, 13C, DEPT, and HMBC) and MS, as shown in Figures 2-6.

**DISCUSSION**

Bioactivity-guided studies by VLC with the n-hexane, chloroform, and methanol mixtures gave five bulked VLC fractions: -VLC-A (2.8 g, 13.4%), VLC-B (2.6 g, 12.5%), VLC-C (3.1 g, 14.7%), VLC-D (11.6 g, 55.2%), and VLC-E (0.8 g, 4.0%). In in vitro aphrodisiac screening of the VLC fractions (100 mg/ mL each), ability of the fractions to reduce lipid peroxidation significantly increased with time (Table 1). The potency of the fractions can be ranked as the following: VLC-C > VLC-D > VLC-A > VLC-B > VLC-E. Of all five VLC fractions, VLC-C had the highest potential in reducing the destruction of sperm, giving 171 nmoles malondialdehyde per gram of fish tissue in 240 min, which is less than 28 nmoles malondialdehyde per gram of fish tissue produced by the positive control, vitamin E, implying 16% potency.

VLC-C, upon open CC and elution with n-hexane, ethyl acetate, methanol mixtures gave six bulked fractions CC (A-E) of which

<table>
<thead>
<tr>
<th>Sample</th>
<th>n moles of malondialdehyde/gm tissue/time</th>
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<tr>
<td></td>
<td>0 min</td>
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<tr>
<td>VLC-A (100 mg/mL)</td>
<td>300.00 ± 0.01</td>
</tr>
<tr>
<td>VLC-B (100 mg/mL)</td>
<td>327.00 ± 0.21</td>
</tr>
<tr>
<td>VLC-C (100 mg/mL)</td>
<td>98.00 ± 0.01*</td>
</tr>
<tr>
<td>VLC-D (100 mg/mL)</td>
<td>206.00 ± 0.08</td>
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<tr>
<td>VLC-E (100 mg/mL)</td>
<td>647.00 ± 0.01</td>
</tr>
<tr>
<td>Vitamin E (positive control, 25 mg/mL)</td>
<td>25.17 ± 0.02*</td>
</tr>
<tr>
<td>5% Tween 80 (negative control, 1 mL)</td>
<td>275 ± 0.05</td>
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The values above are mean of three replicates. n= 3. n ± SEM. Samples with superscript * indicate significant difference at p≤0.05 relative to negative control using One-Way ANOVA (Kruskal-Wallis test) while samples with no superscript * indicate no significant difference at p<0.05 relative to negative control using One-Way ANOVA (Kruskal-Wallis test). VLC: Vacuum liquid chromatography.
CC-D (1 g) was the most active in vitro. A bioactive compound (50 mg) was finally isolated from CC-D by prep-TLC (hexane: ethyl acetate, 6:4). It gave Rf 0.54 (TLC silica gel, n-hexane: ethyl acetate; 2:3, brown on spraying with concentrated H2SO4). In vitro antilipid peroxidation bioassay of this compound showed a high degree of reduction of the peroxidation of PUFA that was stable over the experimentation period and parallel that of 25 mg of vitamin E (α-tocopherol) producing 30 nmoles malondialdehyde per gram fish tissue in 240 min (Figure 1).

Spectroscopic information from NMR studies (1H, 13C, DEPT, and HMBC) and MS suggested the bioactive compound to be a pentacyclic triterpene, 13, 14-epoxyoleanan-3-ol-acetate (M+ 470, C31H50O3) as shown in Figures 2-7 below. This is the first time this compound has been reported in M. charantia and according to the literature, no direct pharmacological property has been associated with this compound.

To date, only the structurally-related oleanan compounds, soyasaponins I-III have been isolated from the Japanese M. charantia. However, Venktesh et al. recently reported the isolation of 3-hydroxy-21-normethyl-19-vinylidenylursane, an in vivo aphrodisiac compound, from a Bombacaceae plant, Durio zibethinus fruit. A wide variety of cucurbita triterpenes and triterpene saponins have been isolated from M. charantia. Notable among these triterpenes are di- and tri-hydroxycucurbitadienes with antiobiotic activity, momordicolide (10E)-3-hydroxypentadeca-10-en-9-olide and momordicophenolide A, cucurbitane-type triterpenoid saponins such as momordicosides M, N, O, L, F1, F2, G, L, A, K, U, V, and W. However, none of these triterpenes is associated with aphrodisiac activity.

According to Kumar et al. M. charantia has been reported to negatively affect fertility in both male and female animals, without mention of any active constituent. The present study has established improvement in sexual activity of normal male rats by M. charantia. Apart from M. charantia, other Nigerian medicinal plants with reported in vitro lipid peroxidation activity are Syzygium aromaticum flower bud and Fadogia agrestis stem and Terminalia catappa seeds from elsewhere. Reviews on aphrodisiac and male fertility enhancing plants have been published. antioxidant activity of M. charantia might be connected with its ability to reduce lipid peroxidation, and hence protect sperm. It therefore has potential for treating sperm-related male infertility. M. charantia is one of a traditional aphrodisiac plant that has not yet been fully explored. This study is a continuation of our investigation into the aphrodisiac activity of M. charantia.

**CONCLUSION**

This investigation establishes in vitro male fertility enhancing activity for the aerial part of M. charantia and suggested bioactivity to be due to a pentacyclic triterpene 13, 14-epoxyoleanan-3-ol-acetate isolate from the most active n-hexane fraction for the first time. It has not been previously isolated from Cucurbitaceae or elsewhere. The in vitro aphrodisiac activity of the compound compared with that of vitamin E. Its structure was confirmed by NMR, MS, and by comparison with a computer database. The study further lends credence to the ethnopharmacological claim...
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Figure 3. $^{13}$C NMR of isolated compound X

Figure 4. DEPT of isolated compound X

Figure 5. HMBC of isolated compound X
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