13, 14-Epoxyoleanan-3-ol-acetate: A male fertility enhancing constituent from hexane fraction of *Momordica charantia* Linn (Curcubitaceae)

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**ABSTRACT**

**Introduction:** 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 is to isolate antlipid peroxidation (an index of usage in treatment of oxidative stress induced male infertility) bioactive principle from *Momordica charantia* using bioactivity-guided isolation.

**Materials and Methods:** *n*-Hexane fraction from 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 principle using *in vitro* lipid peroxidation as index of aphrodisiac as well as male fertility enhancer.

**Results:** Fractionation of active *n*-hexane fraction using vacuum liquid chromatography (VLC) gave five pooled fractions on the basis of their 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 positive control, vitamin E. Further fractionation of VLC-C by open column chromatography on silica gel led to isolation of a compound which was purified by preparative-TLC. The purified compound, 10 mg/mL (Rf 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 MS confirmed the identity of the new bioactive compound as 13, 14-epoxyoleanan-3-ol-acetate.

**Conclusion:** This study scientifically validates traditional claim of *M. charantia* as an aphrodisiac or male fertility enhancer as well as suggest 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

Running title: Isolation of antilipid peroxidation constituent from *n*-hexane fraction of *Momordica charantia* Linn (Curcubitaceae)

INTRODUCTION

Sexual dysfunction is a serious medical and social problem that occurs in 10 -25% of men and 25 -63% of women.¹⁻² Among men aged 40 -70 years, an estimated 34.8% have moderate to complete erectile dysfunction.² This condition can be managed via psychotherapeutic and pharmacotherapeutic approaches.¹,³ Free radicals are known to be present in seminal plasma, some of the most prevalent ROS are hydroxyl, superoxide and hydrogen peroxide radicals. During oxidative stress, excessive production of the ROS or free radicals in seminal plasma tend to cause destructive effect of the sperm cells and in turn induce oxidative stress induced male infertility. Moreover, antioxidants in seminal plasma aid in scavenging the deleterious effect of the ROS via donation of electrons to the electron impaired radicals, thereby reducing their influence.⁴ Spermatozoa in mammals are rich in (Poly Unsaturated Fatty Acids-PUFA) as a result they are very prone to membrane lipid peroxide ion as well as ROS attack. Moreover, a balance is maintained between the amount of ROS produced and that scavenged. arises when this disturbed equilibrium towards pro-oxidants in semen and vaginal secretions can induce an oxidative stress on spermatozoa, which in turn can cause its damage and cause infertility.⁴ Theoretically, oxidative stress induced sperm cell result to decreased sperm motility, presumably by a rapid loss of intracellular ATP 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).⁶⁻¹¹ It is a climbing vine commonly found in the tropics and subtropics. It is also a tropical vegetable employed in ethnomedicine for treatment of various diseases including diabetes, malaria and dysentery, and as a stomachic, stimulant, emetic, antibilious and laxative.¹² Previously, *M. charantia* has been investigated for analgesic and antipyretic,⁷,⁸ antimicrobial ⁹ and anti-HIV activities.¹⁴ Bioactive compounds responsible for the widely investigated antidiabetic and hypoglycaemic activities have been linked to cucurbitanetriterpenoids.¹²⁻¹³ The cucurbitane-type triterpenoids have been extensively isolated from various parts of *M. charantia*.¹²⁻¹⁶ To date, literature information is unavailable on male fertility enhancing potential of *M. charantia*, and only a mention of traditional use of the Nigerian plant as an aphrodisiac is known.⁷,⁹,¹⁷ 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 with a view to rationalizing 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**

*Momordica charantia* used for the research was collected from wild in 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, Nigeria (FHI 109577). Voucher specimens were also deposited in these herbaria and also at Department of Pharmacognosy herbarium, Faculty of Pharmacy, University of Benin, Nigeria.

**Extraction and solvent partitioning**

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

**Chromatographic studies**

**Vacuum Liquid Chromatography (VLC):** Hexane fraction (21.00g) was packed onto 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; H$_2$SO$_4$ spray reagent) into 5 main fractions (A-E), weighed and bio-assayed *in vitro.*

**Column chromatography (CC)/preparative-TLC:** Conventional open column chromatography (30 cm long and 5 cm diameter) of the most active VLC fraction C was done. The silica gel used was kieselgel 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 kieselgel 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; H$_2$SO$_4$ spray reagent) into 5 main fractions CC (A-E), dried and weighed. The most active fraction, CC-D (0.63 g) was subjected to prep-TLC (commercial type) with *n*-hexane: ethylacetate (4: 6). Bands were scrapped, eluted with methanol, filtered and evaporated in a fume cupboard to yield needle-shaped crystals.

**Spectroscopic studies**

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

**In vitro lipid peroxidation assay**

Fresh but frozen Titus fish (*Scomber japonicum*) was purchased from the Ekpoma market in Benin City, washed and fleshy muscular part will be used. The tissue homogenate was prepared as earlier described modified method of Luotola and Luotola. Twenty gram of raw fish muscle tissue was turned into a paste in a mortar. 200 mL distilled water 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 was in turn filtered and unbroken cells and cell debris removed by centrifugation at 1500 rpm for 15 min. The supernatant thus obtained was termed as homogenate and used for the *in vitro* lipid peroxidation study. Lipid peroxidation study was performed almost immediately after the homogenate preparation. 1 mL of each VLC fraction (100mg) 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 (TCA) was added, mixed and centrifuged for 15 min. 1 mL of 2-thiobarbituric acid (0.67% w/v) was added to 2 mL supernatant, mixed and kept in boiling water
bath for 10 min, cooled to room temperature. The TBA chromogen (intensity of the pink coloured complex) was measured at 532 nm against blanks using UV Spectrophotometer (Thermo spectronic, Genesys 20 model). Vitamin E was used as reference standard. A graph of absorbance against concentration will be plotted using the data obtained for the pure vitamin E. The 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 thrice and mean determined.

STATISTICAL ANALYSES
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±SEM (standard error of mean). Statistical differences between the means of the various groups were evaluated by one-way analysis of variance (ANOVA) and tested at 0.05 level of significance. The results were considered statistically significant if the p values were 0.05 or less.

<table>
<thead>
<tr>
<th>Sample</th>
<th>nmoles of malondialdehyde/gm tissue/time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>VLC-A (100mg/mL)</td>
<td>300.00±0.01</td>
</tr>
<tr>
<td>VLC-B (100mg/mL)</td>
<td>327.00±0.21</td>
</tr>
<tr>
<td>VLC-C (100mg/mL)</td>
<td>98.00±0.01*</td>
</tr>
<tr>
<td>VLC-D (100mg/mL)</td>
<td>206.00±0.08</td>
</tr>
<tr>
<td>VLC-E (100mg/mL)</td>
<td>647.00±0.01</td>
</tr>
<tr>
<td>Vitamin E (positive control, 25mg/mL)</td>
<td>25.17±0.02*</td>
</tr>
<tr>
<td>5% Tween 80 (negative control, 1mL)</td>
<td>275±0.05</td>
</tr>
</tbody>
</table>

RESULTS

Lipid peroxidation of bulked VLC fractions of hexane fraction

From an earlier study described by adedokun et al.\(^9\) \(n\)-hexane fraction was the most active aphrodisiac agent, in vivo and in vitro of the three fractions from the crude ethanol extract of *Momordica charantia*. The result of lipid peroxidation of the entire bulked VLC fractions (VLC-A to VLC-E) is shown in Table 1 below.

Table 1: *In vitro* lipid peroxidation of bulked VLC fractions of hexane fraction
The values above represent mean ± SEM of 5 replicates. Values with superscripts indicate significant different relative to negative control (5% Tween 80) at P≤0.05 across the column for each time using One way ANOVA (Non-parametric).

From Table 1 above, VLC-C showed the highest degree of inhibition of polyunsaturated fatty acids in the fish tissue over time with lowest and highest amount 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 similar time interval as shown in Table 1 above.

**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 negative control (5% Tween 80) is shown in Figure 1 below. No significant difference was observed in the activity of Compound X (10 mg/mL) and positive control (vitamin E) 26 mg/mL using *in vitro* model.

![Figure 1: In vitro lipid peroxidation of bioactive compound and vitamin E (25 mg/mL)](image)

**Spectroscopic analysis of Compound X**

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

![Figure 2: 1H NMR of isolated compound X](image)
Figure 3: \( ^{13} \text{C} \) NMR of isolated compound X

Figure 4: DEPT of isolated compound X

Figure 5: HMBC of isolated compound X.
DISCUSSION

Bioactivity-guided studies by Vacuum Liquid Chromatography (VLC) with 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 the in vitro aphrodisiac screening of the VLC fractions (100 mg/mL each), the ability of the fractions to reduce lipid peroxidation significantly increased with time (Table 1).

Potency of the fractions can be ranked as: VLC-C > VLC-D > VLC-A > VLC-B > VLC-E. Of all the five VLC fractions, VLC-C has the highest potential in reducing destruction of sperm giving 171 nmoles malondialdehyde per gram fish tissue in 240 min which is less than 28 nmoles malondialdehyde per gram fish tissue produced by the positive control, vitamin E, implying 16% potency.

VLC-C, upon open column chromatography and elution with hexane, ethyl acetate, methanol mixtures gave six bulked fractions CC (A-E) of which 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: ethylacetate, 6: 4). It gave Rf 0.54 (TLC Silica gel, hexane: ethyl acetate; 2:3, brown colour on spraying with concentrated H$_2$SO$_4$). In vitro antilipid peroxidation bioassay of this compound showed a high degree of reduction of peroxidation of polyunsaturated fatty acids (PUFA) which was stable over the experimentation period and also parallel that of 25 mg of vitamin E ($\alpha$-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 Figure 2 – 7 below. This is the first time this compound is being reported in *Momordica charantia* and according to literature, no direct pharmacological property has been associated with this compound. Although, Ratish Chandra Mistra *et al.* (2020) reported the presence of 13, 14-epoxyoleanan-3-ol-acetate in leaves of *Azadirachta indica*, and reported the compounds might be responsible for the antifungal property of the plant. To date, only the structurally-related oleanan compounds, goyasaponins I-III have been isolated from the Japanese *M. charantia*. However, Venkatesh *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 curcurbitane triterpenes and triterpene saponins have been isolated from *M. charantia*. Notable among these triterpenes are di- and tri-hydroxycucurbitadienes with antidiabetic activity, momordicolide (10E)-3-hydroxyl-dodeca-10-en-9-olide and momordicophenoide A, cucurbitane-type triterpenoid saponins such as momordicosides M, N, O, L, momordicosides F1, F2, G, L, A, K and momordicosides U, V, W. However, none of these triterpenes is associated with aphrodisiac activity. According to Kumar *et al.* *M. charantia* was reported to negatively affect fertility in both male and female animals, without mention of any active constituent. This 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 in the treatment of sperm-related male infertility. *M. charantia* is one of the traditional aphrodisiac plants not yet fully explored. This present 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 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 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 computer database. The study further lends credence to the ethno-pharmacological claim of the plant as an aphrodisiac by the people of Esan Central Local Government Area of Edo State in Nigeria. **ACKNOWLEDGEMENTS** We authors are grateful to Rev. Fr. Anslem Adodo and Paxherbal Laboratories, Ewu, Edo State, for access to laboratory facilities. We also appreciate the organizers of WANNPRES 2014 congress for the opportunity given to present some findings of this research. **CONFLICTS OF INTEREST** We the authors declare no conflict of interest.
REFERENCES