



Research Article



# Bioactivity-guided Isolation of Anticancer Constituents of *Strophanthus hispidus* DC. (Apocynaceae) Whole plant

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## Abstract

**Background:** *Strophanthus hispidus* DC; a medicinal plant in the Apocynaceae family was reported to be effective in the management of cancer through an ethnobotanical survey conducted in Ibadan, Nigeria. This study explicitly explored the cytotoxic potential of the whole plant of *Strophanthus hispidus* leading to the isolation of cytotoxic secondary metabolites.

**Methods:** The crude extract of *S. hispidus* was prepared using the Soxhlet apparatus and concentrated using the rotary evaporator. The crude extract obtained from *S. hispidus* was then partitioned into *n*-Hexane, dichloromethane (DCM), ethyl acetate and butanol fractions for further study. Compounds were isolated by column chromatography, normal and reverse-phase high performance liquid chromatography, and structures were determined by spectroscopic methods. The crude extracts, fractions and isolated compounds were screened for cytotoxicity against breast (AU565, MCF-7), cervical (HeLa), and skeletal muscle (RD) with two normal cell lines: rat and human fibroblast (BJ and 3T3) for selectivity using the MTT assay. Doxorubicin and cycloheximide served as standards for the assay.

**Results:** The methanol extract and DCM fraction showed significant cytotoxicity with IC<sub>50</sub> values (Mean ± SEM) of 1.86 ± 0.01 µg/mL, 0.32 ± 0.01 µg/mL and 1.26 ± 0.06 µg/mL, 0.21 ± 0.06 µg/mL against AU565 and HeLa respectively. The bioassay-guided isolation of cytotoxic compounds from *S. hispidus* afforded two new compounds (**1**, **2**) alongside six other known compounds (**3**-**8**). The compounds were identified as 16-hydroxy-17-(1-hydroxyethyl)-13,17-dimethyl-5H-cyclopenta[a]phenanthrene-4,6-dione (**1**), 2-(4-ethoxy-3-methoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one (**2**); Stigmasterol (**3**); β-Sitosterol-β-D-glucoside (**4**); pityriacitrin (**5**); ursolic acid (**6**); helveticoside (**7**) and Urs-12-en-28-oic acid,2,3,23-trihydroxy-(2β,3β,4α) (**8**). Compounds **1**, **4**, **6**, **7** and **8** displayed significant cytotoxicity and selectivity towards cancer and normal cell lines, respectively.

**Conclusion:** This study further confirms that *S. hispidus* is cytotoxic with the DCM fraction being the most significantly cytotoxic and selective to normal cells. This is the first report on some characterized compounds from *S. hispidus*. In addition, these secondary metabolites can serve as leads in cancer drug development.

## Introduction

With the increasing demands for drugs for the treatment of cancer in low- and middle-income countries, new areas are provided for the utilization of medicinal plants and their products. Researches have shown that Nigeria is a country with a wide flora of medicinal plants with therapeutic values.<sup>1,2</sup> Cancer is ranked as the second leading cause of death<sup>3-5</sup> with several causes including environmental,<sup>6,7</sup> heredity<sup>8,9</sup> and lifestyle.<sup>10</sup> In Nigeria, the prevalent types of cancer include cervical, breast, and prostate.<sup>11</sup>

Natural products are known to possess a great range of bioactive constituents in the form of secondary metabolites for disease management.<sup>12,13</sup> Studies have shown useful

compounds such as vincristine and vinblastine from *Catharanthus roseus* as chemotherapeutic means of cancer treatment.<sup>14,15</sup> Several means of treatment using natural products from plants, animals and sometimes microorganisms have been evaluated.<sup>16,17</sup> An in-depth assessment of these could provide a clue to effective drugs for the treatment of diseases such as cancer.

*Strophanthus hispidus* DC belongs to the family of Apocynaceae with the common name Brown Strophanthus and the local name *Sagere*. This was obtained from a previous ethno-botanical survey conducted for plants used in the traditional treatment of cancer.<sup>18</sup> The plant, a climbing shrub which sometimes appears as a liana, is found in Western African countries including Nigeria,

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Ghana, and sometimes in other African countries such as Congo, Uganda and Cabinda.<sup>19-21</sup> Traditionally, the juice from the seeds are used as an arrow poison, also used to treat snake bites and skin infections.<sup>22</sup> The potency of the plant in the treatment of cardiovascular diseases<sup>23</sup> could be a result of the presence of cardenolide including K-strophanthoside, K-strophanthin- $\beta$  from *S. kombe*. Ursolic acid, a triterpene previously isolated from *S. speciosus* has been reported for great anticancer activity.<sup>24</sup> Boivinide A isolated from the ethanol extract of *S. boivinii* showed significant anti-proliferative activity against the human ovarian cancer cell line.<sup>25</sup>

The present study describes the isolation and identification of potent cytotoxic constituents from *S. hispidus* DC, and their cytotoxic properties against selected cancer cell lines using the MTT assay.

## Methods

### Plant collection and identification

Fresh whole plant of *S. hispidus* was obtained from Ipara-Remo in Ogun state, Nigeria between June and September. The plant material was identified and authenticated at the Forest Herbarium Ibadan, Nigeria, with a voucher number- FHI-112443 assigned. The plant specimen was then deposited in its Herbarium. According to the International Union of Conservation of Nature, *S. hispidus* was categorized as least concerned.

### Extraction and isolation

The air-dried and powdered plant material of *S. hispidus* (200 g) was extracted using a Soxhlet apparatus with MeOH in 4 cycles at 65 °C using 500 mL of MeOH at each cycle. The liquid extract (continuous hot extract) obtained was thereafter concentrated at a reduced pressure and temperature of 40 °C using the BUCHI rotary evaporator (R-100 rotavapor) which gave a greenish-brown paste. Approximately 2 Kg of *S. hispidus* yielded 135.4 g of crude extract. A 100 g of crude extract was reconstituted in 200 mL MeOH/ Water (3:1); thereafter, successive liquid-liquid extraction was carried out using *n*-hexane (300 mL x 4), dichloromethane (DCM) (300 mL x 4), ethyl acetate (EtOAc) and butanol (300 mL x 4) using a separating funnel.

The *n*-hexane fraction of crude extract of *S. hispidus* was purified using the column chromatographic method. Compounds-3 **SHH-14** (5 mg,  $R_f$  0.5) and -4 **SHH-32** (10 mg,  $R_f$  0.6) were obtained as pure isolates directly by the adsorption column chromatographic method (5 x 50 cm) using solvent systems *n*-hexane: ethyl acetate (80:20) and ethyl acetate: methanol (90:10) respectively. Fraction SHH-19 obtained from the preliminary column was loaded for further purification on column (5 x 30 cm) packed with 20 g of silica gel to obtain sub-fraction and a final purification on Japan Analytical Industry Co. Ltd preparative normal phase HPLC (LC-908W, SIL-60A) using *n*-hexane: EtOAc 70: 30 with 1% acetic acid to obtain compound-2, **SHH-42-4** (1.5 mg,  $T_R$  of 62 min,  $R_f$

0.5). The DCM fraction was purified using the adsorption column chromatographic method with elution carried out with solvent systems of *n*-hexane: DCM and then *n*-hexane: ethyl acetate in increasing polarity. A total of 126 fractions were obtained and pooled by TLC analysis into 25 major fractions. Further purifications were carried out using smaller column (2.5x30cm) and or high-performance liquid chromatography (HPLC) respectively. Compound-5 **ShD-58** (20 mg,  $R_f$  0.58), Compound-6, **ShD-9** (50 mg,  $R_f$  0.5), Compound-7, **ShD-21** (7 mg,  $R_f$  0.5), were isolated using solvent systems *n*-hexane: DCM 70:30, *n*-hexane: EtOAc 70:30; EtOAc: Methanol 90:10 with column size 5x50 cm respectively. Compound-8 **ShD-26-MF3**, was isolated by Japan Analytical Industry Co. Ltd preparative normal phase HPLC (LC-908W, SIL-60A) with DCM: MeOH 95:5 ( $T_R$  of 15 min,  $R_f$  0.8) while Compound-1, **ShD-MF-23B** was isolated on Japan Analytical Industry Co., Ltd semi-preparative reverse phase HPLC (ODS-H-80, MeOH: H<sub>2</sub>O 95:5 ( $T_R$  of 15 min,  $R_f$  0.54). The TLC plates were observed under UV light after development and then sprayed with sulphuric acid or Dragendorff's reagent to resolve the classes of compounds.

### General experimental procedure

NMR data was recorded on Bruker Avance III spectrometer operating at 500 and 400 MHz (<sup>1</sup>H) and 100 MHz (<sup>13</sup>C) using standard pulse sequences referenced to residual solvent signals. The HRMS were measured using LTQ Orbitrap spectrometer (Thermo Scientific, USA) equipped with a HESI-II source. The ultraviolet (UV) data were recorded and analysed on THERMO ELECTRON-VISION pro software V4.10 in MeOH. The infrared, IR spectrum was recorded using KBr pellets on BRUKER VECTOR 22 (FT-IR-1). Melting points were recorded on BUCHI M-560. Analytical TLC was carried out on a pre-coated silica gel aluminium plate 60 F<sub>254</sub> visualised at 254 nm (Merck). A Multiskan microplate spectrophotometer (Thermo Scientific) was used for the absorbance measurement for the MTT assay.

### Cell lines and cytotoxicity assay

The cancer cell lines purchased from American Type Culture Collection (ATCC), were seeded in 96-well plates at the density of 6x10<sup>4</sup> cells/mL followed by incubation at 37 °C overnight in a humidified CO<sub>2</sub> incubator. Cell culture material included Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and trypsin were obtained from Biosera (Nouaille, France). The plant extract, fractions and compounds were dissolved in DMSO and then added to the monolayer of cells at ten-fold concentration range of 1000 - 0.01 µg/mL followed by incubation at 37 °C for 24 h. Doxorubicin and cycloheximide were used as positive control while negative control contained the growth media and DMSO. After 48 h, 200 µL of MTT (0.5 mg/mL) was added to each well and then incubated for 4 h. Thereafter, 100

μL of DMSO was added to each well and then measured at an absorbance of 570 nm, using a microplate reader (Spectra Max Plus, Molecular Devices, CA, USA). Each experiment was carried out in triplicate and cell viability was calculated as

$$\text{Cell Viability \%} = \frac{A-B}{A} \times 100$$

where A represents the mean optical density of untreated cells while B is of treated cells respectively.<sup>26</sup>

### Data presentation and statistical analysis

Cytotoxicity was recorded as concentration causing 50% growth inhibition (IC<sub>50</sub>) for all cell lines with Microsoft Excel version 2016 using data from the three independent experiments. The results obtained were expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Version 5.01. (GraphPad Prism Software Inc., San Diego, CA). Data obtained were subjected to one-way analysis of variance (ANOVA) and then Tukey's test for statistical analysis with a *P* value of less than 0.05 considered statistically significant. The selective index was calculated as a ratio of IC<sub>50</sub> value of the normal cell to those of cancer cell lines.

### Results and Discussion

Breast and cervical cancers have been most commonly diagnosed in sub-Saharan African women over the last decade,<sup>27</sup> thus the choice of adenocarcinomas used for the study.

To explore the biological activity of *S. hispidus* with human cancer cells, we first extracted the whole plant with methanol, and then examined its cytotoxicity in terms of cell viability in four different cancer cells; AU565, MCF-7, HeLa, RD, and two normal cells; BJ and 3T3 for selectivity. After 72 hours of treatment, the MTT assay revealed that the crude extract drastically reduced the cell viability of the cancer cells in a dose-dependent manner with a range of IC<sub>50</sub> 0.32 to 1.86 μg/mL as shown in Table 1. The observations further confirmed the usage of *S. hispidus* in the management of cancer by traditional

medical practitioners.

For further investigations on the cytotoxicity of *S. hispidus*, the crude extract was partitioned into four different fractions based on the polarity of the solvents. To make a distinction for the fraction that exerted cytotoxicity, each fraction was tested against cell lines AU565 and HeLa, the *n*-hexane and DCM fractions showed significant cytotoxicity with IC<sub>50</sub> values (Table 1) for both cell lines. These fractions were then subjected to further investigations using repeated column chromatography and HPLC purification leading to the isolation of eight compounds. The compounds were identified as 16-hydroxy-17-(1-hydroxyethyl)-13,17-dimethyl-5H-cyclopenta[a]phenanthrene-4,6-dione (1); 2-(4-ethoxy-3-methoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one (2); stigmasterol (3)<sup>28</sup>; β-sitosterol-β-D-glucoside (Daucosterol) (4)<sup>29</sup>; pityriacitrin (5)<sup>30</sup>; ursolic acid (6)<sup>24</sup>; strophanthidin- digitoxoside (Helveticoside) (7)<sup>31</sup>; and Urs-12-en-28-oic acid, 2,3,23-trihydroxy-(2β,3β,4α) (8)<sup>32</sup> as shown in Figure 1.

In-depth spectroscopic analysis using 1D-(<sup>1</sup>H and <sup>13</sup>C), 2D-NMR, low and high-resolution EI-MS led to the elucidation of the structures of the compounds. Comparison with existing literature of known compounds were checked for isolated compounds. The present study is the first report of the chemical investigation of cytotoxicity of *S. hispidus*.

**Compound-1:** 16-hydroxy-17-(1-hydroxyethyl)-13,17-dimethyl-5H-cyclopenta[a]phenanthrene-4,6-dione: Off-white powder; EI-MS: *m/z* 344.2

corresponded to the molecular formula of C<sub>21</sub>H<sub>28</sub>O<sub>4</sub>; <sup>1</sup>H and <sup>13</sup>C NMR (Table 2)

IR (cm<sup>-1</sup>): 3341, 2946, 2871, 1651, 1053.

Melting point: 181.2 °C

**Compound-2:** 2-(4-ethoxy-3-methoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-chromen-4-one: Yellow powder; EI-MS: *m/z* 402.3 corresponded to the molecular formula of C<sub>21</sub>H<sub>22</sub>O<sub>8</sub>; <sup>1</sup>H and <sup>13</sup>C NMR (Table 3)

IR (cm<sup>-1</sup>): 3727, 3624, 2932, 1267, 1217

Melting point: 128.2 °C

The <sup>1</sup>H and <sup>13</sup>C NMR spectral assignments for compounds 3-9 were reported in the supplementary file as Tables S1 and S2.

Compound-1 was isolated as an off-white powder. The compound also elicits UV absorption at 220, 230 and 280 nm (Figure S1) which are the characteristic absorption bands of phenanthrene skeleton.<sup>33</sup> The 220 and 230 bands indicate π-π\* transition suggesting the presence of double bond-containing compounds. Also, 282 nm suggests the presence of n-π\* transition found due to the presence of the hydroxyl groups in the compound. The infrared absorption peak at 3341 represents the hydroxyl group, 2946 and 2871 cm<sup>-1</sup>, are typical of C-H stretching vibration, 1651, represents C=O stretch which is typical of an α,β-unsaturated keto-system and 1053 is typical of C-O stretch (Figure S2).

The HMBC spectrum of compound-1 (Figure S3),

**Table 1.** The *in vitro* cytotoxic activity of *S. hispidus* crude extract and fractions

| Sample       | AU565                          | HeLa                           | BJ                             |
|--------------|--------------------------------|--------------------------------|--------------------------------|
|              | IC <sub>50</sub> ± SEM (μg/mL) | IC <sub>50</sub> ± SEM (μg/mL) | IC <sub>50</sub> ± SEM (μg/mL) |
| ShCrude      | 1.86 ± 0.01 <sup>#</sup>       | 0.32 ± 0.01 <sup>#</sup>       | 4.19 ± 0.10 <sup>#</sup>       |
| ShH          | 10.72 ± 0.18                   | 11.91 ± 1.25                   | 14.26 ± 0.20                   |
| ShD          | 1.26 ± 0.06 <sup>#</sup>       | 0.21 ± 0.06 <sup>#</sup>       | 2.36 ± 1.10                    |
| ShE          | 8.13 ± 0.22                    | 1.6 ± 0.02                     | 4.23 ± 1.10                    |
| ShAq         | 18.55 ± 2.50                   | 1.7 ± 0.5                      | 3.1 ± 1.10                     |
| Doxorubicin* | 0.54 ± 0.04                    | 1.2 ± 0.01                     | 2.84 ± 0.02                    |

ShH: *Strophanthus hispidus* Hexane; ShD: *Strophanthus hispidus* DCM; ShE: *Strophanthus hispidus* Ethyl acetate fraction; ShAq: Aqueous fraction of *Strophanthus hispidus*. HeLa: Cervical carcinoma AU565: Breast adenocarcinoma. \*Standard drug for the study; # The mean difference is significant at *P* < 0.05 with respect to standard drug.

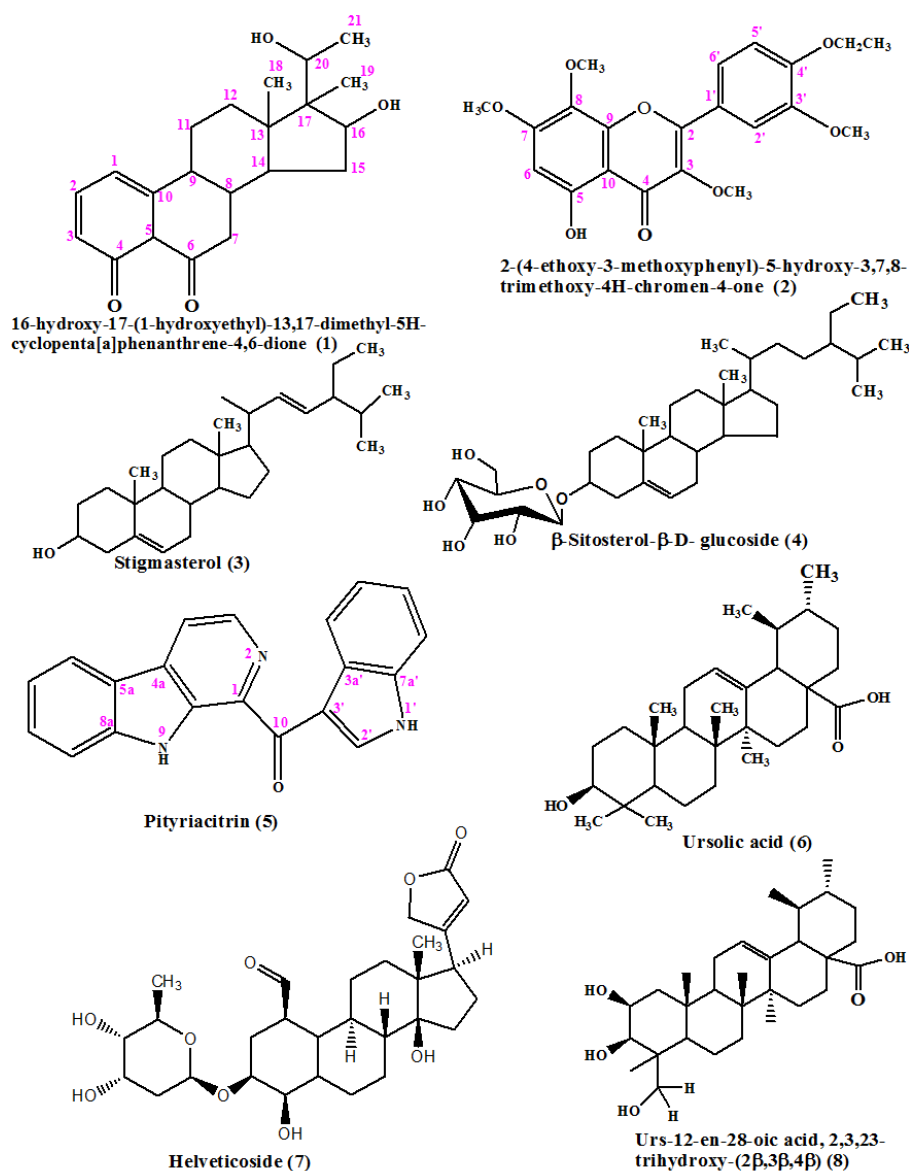


Figure 1. Structures of compounds 1-8 from *Strophanthus hispidus*

showed a correlation of  $\delta_{\text{H}}$  5.60(H-3) with  $\delta_{\text{C}}$  128.8(C-2), which suggests the presence of a double bond at 1 and 2 positions;  $\delta_{\text{H}}$  4.50(H-20) correlates with  $\delta_{\text{C}}$  16.8(C-18), 73.1(C-16) and 65.7(C-20), this suggests the presence of oxo-methine carbon signals at position 20. A correlation was observed between  $\delta_{\text{H}}$  6.10(H-2) with  $\delta_{\text{C}}$  124.1(C-3) and 162.6(C-10) suggesting the presence of olefinic double bonds at positions 2, 3 and 10 respectively. Another proton signal at  $\delta_{\text{H}}$  4.0(H-16) was found to correlate with  $\delta_{\text{C}}$  73.1(C-16) and 65.7(C-20), suggesting the presence of an oxo-methine signal at position 16. Furthermore,  $\delta_{\text{H}}$  3.40(H-16) correlates with  $\delta_{\text{C}}$  29.5(C-15) and 65.7(C-20), suggesting the presence of another oxo-methine signal at position 20. The  $\delta_{\text{H}}$  0.90(H-19) linked with  $\delta_{\text{C}}$  48.5(C-13) and 73.1(C-16) infers a methyl group present at position 19 and a hydroxylated system at position 16. Additionally,  $\delta_{\text{H}}$  1.06(H-21) in association with  $\delta_{\text{C}}$  65.7(C-20) suggests a methyl group at position 21. A correlation between  $\delta_{\text{H}}$  1.07(H-16) and  $\delta_{\text{C}}$  35.9(C-17), 48.5(C-13),

suggests a methyl group at position 18 while proton signal at  $\delta_{\text{H}}$  1.60(H-11) in relation to  $\delta_{\text{C}}$  33.5(C-12) and 57.7(C-9), suggests the presence of an  $\text{sp}^2$ -hybridised methylene signals at positions 11 and 12 respectively (Figures S4, S5).

The  $^1\text{H}$ - $^1\text{H}$  COSY spectrum showed a correlation between  $\delta_{\text{H}}$  4.5(H-20) and  $\delta_{\text{H}}$  1.05(H-21), with another existing between  $\delta_{\text{H}}$  4.0(H-16) and  $\delta_{\text{H}}$  1.50(H-15). Also,  $\delta_{\text{H}}$  2.50(H-7) with  $\delta_{\text{H}}$  2.10(H-8), and then,  $\delta_{\text{H}}$  2.10(H-8) with  $\delta_{\text{H}}$  1.70(H-11) (Figure S6).

The EI-MS spectrum, (Figure S7) showed  $m/z$  344.2 as the molecular ion peak which confirms the molecular mass of the compound. The Fast Atom Bombardment (FAB) mass spectrum (Figure S8) of Compound 1 showed a strong protonated molecular ion at  $m/z$  345.2 as proof of the molecular weight of the compound. The Loss of a molecule of water in the compound gave  $m/z$  326; which is a characteristic of hydroxyl-containing compounds. The  $[\text{C}_2\text{H}_6\text{O}]$  loss gave fragment ion  $[\text{C}_{19}\text{H}_{22}\text{O}_3]^+$  with  $m/z$  298, followed by a loss of  $[\text{OH}]$  group to give  $[\text{C}_{19}\text{H}_{23}\text{O}_2]^+$  with

**Table 2.** NMR spectral assignment of compound 1 (ppm, CDCl<sub>3</sub>, 500 and 125 MHz)

| Position | <sup>1</sup> H-NMR chemical shifts $\delta$ in ppm (coupling constant <i>J</i> in Hz) | <sup>13</sup> C-NMR chemical shifts $\delta$ in ppm / HSQC/carbon type |
|----------|---------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| 1        | 5.95 dd (1.5, 9.5)                                                                    | 138.6, CH                                                              |
| 2        | 6.10 dd (2.5, 9.5)                                                                    | 128.8, CH                                                              |
| 3        | 5.60 s                                                                                | 124.1, CH                                                              |
| 4        | --                                                                                    | 199.3, Qc                                                              |
| 5        | 1.30 m                                                                                | 48.7, CH                                                               |
| 6        | --                                                                                    | 215, Qc                                                                |
| 7        | 2.40 m<br>2.50 m                                                                      | 33.8, CH <sub>2</sub>                                                  |
| 8        | 2.10 m                                                                                | 34.8, CH                                                               |
| 9        | 1.70 m                                                                                | 57.7, CH                                                               |
| 10       | --                                                                                    | 162.6, Qc                                                              |
| 11       | 1.60 s                                                                                | 27.6, CH <sub>2</sub>                                                  |
| 12       | 1.99 m                                                                                | 33.5, CH <sub>2</sub>                                                  |
| 13       | --                                                                                    | 48.5, Qc                                                               |
| 14       | 3.40 d (4.0)                                                                          | 64.2, Qc                                                               |
| 15       | 1.50 m                                                                                | 29.5, CH <sub>2</sub>                                                  |
| 16       | 4.0 m                                                                                 | 73.1, CH                                                               |
| 17       | --                                                                                    | 35.9, Qc                                                               |
| 18       | 1.07 s                                                                                | 16.1, CH <sub>3</sub>                                                  |
| 19       | 0.9 s                                                                                 | 10.4, CH <sub>3</sub>                                                  |
| 20       | 4.50 dd (6.5, 16.0)                                                                   | 65.7, CH                                                               |
| 21       | 1.05 s                                                                                | 16.8, CH <sub>3</sub>                                                  |

Note: s=singlet, t=triplet, J=coupling constant in Hz, q=quartet, d=doublet, dd=double doublet, m=multiplet. Qc=quaternary carbon.

*m/z* 283. A loss of a neutral molecule; [C<sub>10</sub>H<sub>19</sub>O<sub>2</sub>] from the molecular ion gave [C<sub>11</sub>H<sub>9</sub>O<sub>2</sub>]<sup>+</sup> and [C<sub>15</sub>H<sub>25</sub>O<sub>3</sub>] with *m/z* 173 and 91 respectively. Also, loss of [C<sub>8</sub>H<sub>5</sub>O<sub>2</sub>] from the molecular ion gave [C<sub>13</sub>H<sub>21</sub>O<sub>2</sub>]<sup>+</sup> with *m/z* 211. A loss of [C<sub>15</sub>H<sub>17</sub>O<sub>2</sub>] from the molecular ion peak gave fragment ion [C<sub>6</sub>H<sub>11</sub>O<sub>2</sub>]<sup>+</sup> with *m/z* 115, while another loss of [C<sub>5</sub>H<sub>12</sub>O<sub>2</sub>] from the molecular ion gave [C<sub>16</sub>H<sub>16</sub>O<sub>2</sub>]<sup>+</sup> with *m/z* 240; which confirms the base peak ion as illustrated in the proposed fragmentation mechanistic pathways in (Figure S9). This is the first time isolating 16-hydroxy-17-(1-hydroxyethyl)-13,17-dimethyl-5H-cyclopenta[a]phenanthrene-4,6-dione from nature.

A compound, 3 $\beta$ - Acetoxy-17-(3-pyridyl) androsta-5,16-diene (abiraterone acetate) with a similar phenanthrene skeleton as compound **1** has been previously synthesized. This drug was reported to be designed for the treatment of prostatic carcinoma which is hormone- dependent.<sup>34</sup>

Compound-2 was suggested to be a flavone based on its physico-chemical properties as well as its chromatography performance. Its UV absorption at 232, 257 nm is indicative of the presence of chromophoric group in the compound (Figure S10). Infra-red data showed IR (KBr)  $V_{\max}$  cm<sup>-1</sup>: 3727, 3624 (OH group present at C-5), 2932 (C-H<sub>stretch</sub>), typical of the non-aromatic part of the compound, side chain C-1'' and C-2''), 1654 (-C=O,

**Table 3.** NMR spectral assignment for compound 2 in CDCl<sub>3</sub> (500 and 125 MHz)

| Position | <sup>1</sup> H-NMR | <sup>13</sup> C-NMR/ HSQC | HMBC                       | COSY |
|----------|--------------------|---------------------------|----------------------------|------|
| 1        | --                 | --                        | --                         | --   |
| 2        | --                 | 155.9                     | --                         | --   |
| 3        | 3.85 s<br>--       | 60.2<br>138.8             | 138.8                      |      |
| 4        | --                 | 178.8                     | --                         |      |
| 5        | 12.59 s            | 152.7                     | 178.8, 155.9, 132.2, 106.6 |      |
| 6        | 6.48 s             | 90.3                      | 178.8, 152.3, 132.2, 106.6 |      |
| 7        | 3.94 s<br>--       | 56.1<br>158.7             | 158.7                      |      |
| 8        | 3.90 s<br>--       | 60.8<br>132.2             | 132.2                      |      |
| 9        | --                 | 132.3                     | --                         |      |
| 10       | --                 | 106.6                     | --                         |      |
| 1'       | --                 | 122.7                     | --                         |      |
| 2'       | 7.67 dd (2.0, 3.6) | 111.5                     | 150.8, 148.9, 122.1        |      |
| 3'       | --                 | 148.9                     |                            |      |
| 3''      | 3.94 s             | 56.3                      | 148.9                      |      |
| 4'       | --                 | 150.8                     | --                         |      |
| 5'       | 6.97 d (8.4)       | 111.8                     | 150.8, 148.9, 122.7, 111.5 | 7.70 |
| 6'       | 7.70 d (2.0)       | 122.1                     | 150.8, 111.5               | 6.97 |
| 1''      | 4.21 q             | 64.4                      | 150.8, 14.6                | 1.50 |
| 2''      | 1.50 t (7.2)       | 14.6                      | 64.4                       | 4.21 |

Note: s=singlet, t=triplet, J=coupling constant in Hz, q=quartet, d=doublet, dd=double doublet, HMBC=Heteronuclear Multiple Bond Correlation, COSY=Heteronuclear Single Quantum Coherence.

4-keto-3-ene group, presence of  $\alpha,\beta$  unsaturated system which is typical of flavones, 1217 (C-O<sub>stretch</sub>) (Figure S11).

Electron ionization- mass spectroscopy spectrum of the compound (Figure S12) showed *m/z* 402.3; confirming the molecular ion of the compound. The loss of a methyl group on the ethoxy part from the side chain gave *m/z* 371, in addition to the total loss of the ethoxy moiety, 35 resulting in *m/z* 357. The hydroxyl group loss with a methyl group from the molecular ion peak gave *m/z* 355.

The NMR data showed the presence of four methoxyl groups [ $\delta_H$  3.85(3H, s, OCH<sub>3</sub>-3), 3.90(3H, s, OCH<sub>3</sub>-8), 3.94(6H, s, OCH<sub>3</sub>-7 and 3', respectively), with the corresponding  $\delta_C$  60.2, 60.8, 56.1 and 56.3 respectively]. An ethoxy group [ $\delta_H$  4.21 (2H, q, H-1'' and a terminal CH<sub>3</sub>  $\delta_H$  1.50(3H, t, H-2'')], four aromatic protons [ $\delta_H$  6.48(1H, s, H-6), 6.92(1H, d, H-5'), 7.67(1H, dd, H-2') and 7.70(1H, d, H-6'), with the matching  $\delta_C$  90.3 (C-6), 111.8 (C-5'), 111.5 (C-2') and 122.1 (C-6'), respectively] were also observed. A hydroxyl proton  $\delta_H$  12.9 with eleven quaternary carbons (Figures S13 and S14) [158.7(C-7), 155.9(C-2), 152.7(C-5), 150.8(C-4'), 148.9(C-3'), 138.8(C-3), 132.3(C-9), 132.2(C-8), 122.7(C-1') and 106.6(C-10)] including a carbonyl carbon with  $\delta_C$  178.8 (C-4) were detected in the NMR data as shown (Table 3). The above-mentioned data suggested compound **2** to be a tetra- methoxyflavone flavone.

In the HMBC spectrum (Figure S15), correlations were observed between  $\delta_H$  3.85 and  $\delta_C$  138.8(C-3),  $\delta_H$  12.59 and  $\delta_C$  178.8(C-4)/ 155.9(C-2)/ 132.2(C-9), 106.6(C-10). This confirmed the position of the carbonyl carbon. Proton and carbon signals at  $\delta_H$  6.48 and  $\delta_C$  178.8(C-4)/152.3(C-5)/ 132.2(C-9)/106.6(C-10),  $\delta_H$  3.94 and  $\delta_C$  158.7(C-7),  $\delta_H$  3.90 and  $\delta_C$  132.2(C-9),  $\delta_H$  7.67 and  $\delta_C$  150.8(C-4')/148.9(C-3')/122.1(C-6'),  $\delta_H$  6.97 and  $\delta_C$  150.8(C-4')/148.9(C-3')/122.7(C-1')/111.5(C-2'),  $\delta_H$  7.70 and  $\delta_C$  150.8(C-4')/111.5(C-2'), further confirmed the positions of the methoxy and hydroxyl groups on the flavone ring.

The  $^1H$ - $^1H$ -COSY correlations of Compound-2 (Figure S16) showed a strong correlation between two vicinal protons H-5' ( $\delta_H$  6.97) and H-6' ( $\delta_H$  7.70), also H-1'' ( $\delta_H$  4.21) and H-2'' ( $\delta_H$  1.50). This correlation further confirmed the presence of the ethoxy group at C-4', and the other two vicinal protons at C-5' and C-6'.

A previously reported compound, 5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,7,8-trimethoxy-4H-chromen-4-one (2a),  $C_{19}H_{18}O_8$ ,<sup>35</sup> is analogous to our isolated new flavone (2), 2-(4-ethoxy-3-methoxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-Chromen-4-one. The only observable difference in the structure of the two compounds is at position 4', where the OH- group in (2a) is substituted by an ethoxy ( $OCH_2CH_3$ ) group in (2) thus, further confirming an increase of 29 g/mol in molecular mass of the compound-2

All isolated compounds were subjected to cytotoxic assay as shown in Table 4. After 72 hours of treatment, compounds 1, 4, 6, 7, and 8, exhibited significant cytotoxic effects against breast and or skeletal muscle cancer cell lines.

The cytotoxicity of Compound-1 showed a relationship with two compounds with the Phenanthrene skeleton isolated from *Asarum heterotropoides* which displayed a strong cytotoxicity against Human renal proximal tubular epithelial cell lines (HK-2).<sup>36</sup> Also, the new compound-1,

showed  $IC_{50}$  of value of 16.49, 18.81  $\mu M$  against RD and MCF-7.

Ursolic acid exhibited a significant  $IC_{50}$  values of 0.08, 3.17 and 8.07  $\mu M$  against AU565, RD, and MCF-7 cancer cell lines respectively. The isolated Ursolic acid and helveticoside had great and promising cytotoxic properties on breast adenocarcinoma, AU565 with less cytotoxic effect on normal cells as shown in Table 4. Studies have shown that ursolic acid isolated from *Dipterocarpus obtusifolius*<sup>37</sup> and *Juglans regia* L.<sup>38</sup> had a great cytotoxic effect against MCF-7 cells. Ursolic acid has been reported useful in the management of cancer by inhibition of the tumour growth and spread using the B16 mouse melanoma-treated C57BL/6 mice *in-vitro*<sup>39</sup>; the expression of NF- $\kappa$ B activating effect of ursolic acid on melanoma, neuroblastoma cancer cell lines was decreased.<sup>40</sup> The cytotoxic effect of ursolic acid was illustrated by apoptosis, a mechanism of action in Molt4, U937 and K562 leukaemia cell,<sup>41</sup> which is in line with the *in vivo* inhibitory assay of aberrant crypt foci, a precursor of colon cancer showed ursolic acid exhibiting chemotherapeutic effects.<sup>42</sup> Based on findings in existing literature, this is the first time of isolation of ursolic acid from *S. hispidus* though isolation of its methyl ester was reported from *S. speciosus*.<sup>24</sup> Secondary metabolites such as triterpenes has promising cytotoxic properties for the treatment of cancer with different modes of action.<sup>43</sup>

From this study, helveticoside showed strong activity with  $IC_{50}$  of 11.42  $\mu M$  against the AU565 cell line. To the best of our knowledge, this is the first report of helveticoside from *S. hispidus*. Reports have shown cytotoxicity of helveticoside with a strong  $IC_{50}$  range of between 0.034 and 0.596  $\mu M$  on RAW264.7, HepG2, HCT116, SK-OV-3.<sup>29</sup> Helveticoside isolated from *Descurainia sophia* showed induction of gene regulation in A549, human lung cancer cell line.<sup>44</sup>

A previous isolation of pityriacitrin from *S. hispidus* has not been reported in the literature A study on pityriacitrin

**Table 4.** Cytotoxicity of isolated compounds against cancer and normal cell lines

| Compound       | AU565                         | RD                            | MCF-7                         | 3T3                           |
|----------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                | $IC_{50} \pm SEM$ ( $\mu M$ ) | $IC_{50} \pm SEM$ ( $\mu M$ ) | $IC_{50} \pm SEM$ ( $\mu M$ ) | $IC_{50} \pm SEM$ ( $\mu M$ ) |
| 1              | NT                            | 16.49 $\pm$ 0.22              | 18.81 $\pm$ 0.29              | NT                            |
| 2              | NT                            | 69.01 $\pm$ 0.14              | 73.24 $\pm$ 0.03              | NT                            |
| 3              | 25.00 $\pm$ 0.03              | NT                            | NT                            | Not cytotoxic                 |
| 4              | 10.71 $\pm$ 0.12              | 34.28 $\pm$ 0.02              | 42.54 $\pm$ 0.45              | Not cytotoxic                 |
| 5              | 29.29 $\pm$ 2.90              | 25.28 $\pm$ 1.06              | 41.75 $\pm$ 0.43              | Not cytotoxic                 |
| 6              | 8.07 $\pm$ 0.02               | 0.08 $\pm$ 0.0 <sup>a</sup>   | 3.17 $\pm$ 0.26               | Not cytotoxic                 |
| 7              | 11.42 $\pm$ 0.60              | 46.89 $\pm$ 0.05              | 20.57 $\pm$ 1.28              | Not cytotoxic                 |
| 8              | 18.27 $\pm$ 1.73              | 3.15 $\pm$ 0.37               | 38.57 $\pm$ 0.45              | Not cytotoxic                 |
| Doxorubicin*   | 0.08 $\pm$ 0.04               | --                            | --                            | --                            |
| Vincristine    | --                            | 0.41 $\pm$ 0.01               | 0.89 $\pm$ 0.12               | --                            |
| Cycloheximide* | --                            | --                            | --                            | 1.8 $\pm$ 0.1                 |

AU565: Breast adenocarcinoma; RD: Rhabdomyosarcoma; MCF-7: Breast adenocarcinoma; NT: Not tested. \*Standard drug for the study; # The mean difference is significant at  $P < 0.05$  with respect to standard drug.

by Zhang et al showed minimal cytotoxic activity against breast (MDA-231 and MCF-7) and prostate carcinoma (PC3) in agreement with observation in this study.<sup>45</sup>

The inhibition of the cell growth of AU565 breast carcinoma by daucosterol was observed in this study which is correlated with an earlier report on the significant effect of the compound on breast cancer apoptosis and metastasis.<sup>36</sup> Its cytotoxic effects on prostate cancer cells, DU145 and PC3 with IC<sub>50</sub> of 29.25 and 17.45 µg/mL respectively<sup>46</sup> and against lung carcinoma, A549<sup>47</sup> were also reported.

In line with this study, bioactive compounds have pharmacophoric characteristics in common with medications that are now prescribed to treat several illnesses.<sup>48</sup> Natural products offer a wealth of potentially appealing scaffolds and molecular building blocks for synthesis.<sup>49</sup> With this, molecular modification can be used to alter the chemical structure of natural compounds because they have not been developed as medicines. This will accomplish ideal pharmacokinetic and pharmacodynamic qualities or improve efficacy and selectivity for the target, breast cancer, as in this case.

## Conclusion

*Strophanthus hispidus* has shown to be of high potential with hope in the management of cancer. Significant cytotoxicity and selectivity of *S. hispidus* were observed at all stages of isolation of the compounds, therefore, showing little or no toxicity. Hence ursolic, urs-12-en-28-oic acid, 2, 3, 23-trihydroxy-(2β, 3β, 4α) and helveticoside can be developed as a safe and effective pharmaceutical composition against breast adenocarcinoma as a means of inducing cell death, apoptosis. This study, therefore, justifies the claims by traditional medicinal practitioners of the effectiveness of the *S. hispidus* in the management of cancer in South West Nigeria.

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## Competing Interests

The authors declare no conflict of interest.

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## Supplementary Files

The spectral assignments for compounds 3-9 were reported in the supplementary file as Tables S1 and S2 and Figure S1-S16.

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