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**Phytochemical Characterisation of a Cytotoxic Stem Bark Extract  
of *Steganotaenia araliacea* and Identification of a Protoflavanone by LC-SPE-NMR**

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## **Abstract**

A 80% ethanolic stem bark extract of *Steganotaenia araliacea* (Apiaceae) showed cytotoxic properties against MDA-MB-231 (breast), PANC-1 (pancreas) and HT-29 (colon) cancer cell lines. Phytochemical investigations by LC-SPE-NMR led to the identification of spiropreussomerin A, most probably produced by an endophytic fungus, the flavanonol conrauiflavonol / afzelin A, and a protoflavanone that has not been reported before and for which the name steganoprotolflavanone was adopted. Surprisingly none of the previously reported cytotoxic dibenzocyclo-octadiene lactone lignans was obtained as a major constituent of the cytotoxic extract.

## **Keywords**

*Steganotaenia araliacea*

Apiaceae

spiropreussomerin A

conrauiflavonol / afzelin A

protoflavonoid

steganoprotolflavanone

## 1. Introduction

In the course of our work on medicinal plants from Guinea-Conakry (Traoré et al., 2014) the carrot tree or cabbage tree *Steganotaenia araliacea* Hoechst (Apiaceae), which is widely spread in tropical Africa, was selected for a phytochemical investigation into its cytotoxic constituents. *S. araliacea* has been studied before for its biologically active dibenzocyclo-octadiene lactone lignans and their analogues. Steganangin and steganacin were isolated from the stem bark and stem wood and exhibited significant antileukaemic activity in the *in vivo* murine P-388 lymphocytic leukaemia test system (Kupchan et al., 1973). Steganacin was found to inhibit the assembly of tubulin into microtubules by interacting with the colchicine binding site (Wang et al., 1977; Sackett, 1993). Wickramaratne et al. (1993) reported additional lignans from the stem bark, *i.e.* episteganangine, steganoate A, steganoate B and steganolide A. With the exception of steganoate A all of these demonstrated cytotoxic activity against a panel of eleven human tumour cell lines. The organic extract of *S. araliacea* showed cytotoxic activity in the NCI screening program, and bioassay-guided fractionation led to the isolation of 10-demethoxy-stegane together with steganone and prestegane B. Only steganone showed activity against the ovarian OVCAR-3 and colon COLO-205 cancer cell lines (Meragelman et al., 2001). The initial aim of our work was to isolate in a systematic way a series of dibenzocyclo-octadiene lactone lignans of *S. araliacea* stem bark in order to establish structure-activity relationships for this class of compounds, but surprisingly a spirobisanthralene, a flavanone and a protoflavonoid were obtained.

## 2. Results and Discussion

The cytotoxicity of the 80% EtOH *S. araliacea* crude stem bark extract (SA) was assessed *in vitro* on different cell lines. Because of its limited solubility, filtering resulted in a loss of approximately 65% insoluble material. Cytotoxicity on three cancer cell lines, *i.e.* MDA-MB-231, PANC-1 and HT-29 was tested by means of the SRB assay in a concentration range of 3.5 – 350 µg/mL using incubation times of 24 h and 72 h. The survival percentages per concentration per cell line and per incubation time were calculated from the optical density measurements (Supplementary Data, Fig. S1). The calculated IC<sub>50</sub> values are summarised in Table 1. The IC<sub>50</sub> values against the PANC-1 and HT-29 cell lines are considerably lower than

against MDA-MB-231. The latter cells form loosely cohesive grape-like or stellate structures consistent with the more invasive phenotype, exhibiting only an intermediate response to chemotherapy (Holliday and Speirs, 2011). The IC<sub>50</sub> values calculated after an incubation time of 72 h were lower than after 24 h.

Liquid-liquid partition of the dried ethanolic (80%) extract of stem bark, dissolved in MeOH 90%, with petroleum ether, yielded a methanolic fraction (SA1B), which after drying and redissolving in H<sub>2</sub>O was extracted with EtOAc (fraction SA2A). The stem bark extract was found to contain 3 major constituents, which were mainly represented in this EtOAc fraction, and which were subsequently isolated from this fraction by flash chromatography and LC-SPE-NMR by multiple trapping on HySphere resin GP cartridges. Compound **1**, showing UV absorption maxima at 226 and 288 nm and a pseudo-molecular ion [M - H]<sup>-</sup> with *m/z* 333.0779 in HRMS consistent with a molecular formula of C<sub>20</sub>H<sub>14</sub>O<sub>5</sub>, was identified as spiropreussomerin A (Fig. 1). NMR spectra are added as Supplementary Data (Fig. S2 – S6). Library search on the <sup>13</sup>C NMR data suggested this compound to be a deoxypreussomerin analogue. <sup>1</sup>H and <sup>13</sup>C NMR assignments were in agreement with those reported for spiropreussomerin A in CDCl<sub>3</sub> by Chen et al. (2009), although our data were obtained in CD<sub>3</sub>CN (Table 2). The structure of spiropreussomerin A was in complete agreement with the COSY, HSQC and HMBC spectra. Spiropreussomerin A was first obtained from a liquid culture of *Preussia* sp., an endophytic fungus isolated from a mature stem of *Aquilaria sinensis* (Lour.) Gilg. (Thymelaeaceae). It belongs to the spirobisnaphthalenes, a series of fungal secondary metabolites consisting of a 1,8-dihydroxynaphthalene-derived spiroketal unit linked to a second, oxidized naphthalene moiety. The possibility that spiropreussomerin A isolated in this work originates from an endophytic fungus in *Steganotaenia araliacea* can therefore not be excluded. Although deoxypreussomerin analogues possess a wide range of biological properties including antibacterial, antifungal, herbicidal, but also antitumour activity (Ravindranath et al., 2004), spiropreussomerin A did not show cytotoxicity towards human ovarian carcinoma (A2780), human liver carcinoma (BEL-7404), colon carcinoma (HCT-8), gastric carcinoma (BGC-823) and lung adenocarcinoma (A-549) (Chen et al., 2009).

Compound **2**, showing UV absorption maxima at 228, 272, 296 and 310 nm and a pseudo-molecular ion  $[M - H]^-$  with  $m/z$  353.1038 in HRMS consistent with a molecular formula of  $C_{20}H_{18}O_6$ . Library search on the  $^{13}C$  NMR data suggested that compound **2** had a flavanonol skeleton with a 2'', 2''-dimethyl pyran ring.  $^1H$  and  $^{13}C$  NMR assignments were in agreement with those reported for 6,7-(2'',2''-dimethylpyrano)-5,4'-dihydroxy-flavanonol, also known conrauiflavonol or afzelin A (Fig. 1), recorded in acetone- $d_6$  or DMSO- $d_6$ , respectively, although our data were obtained in  $CD_3CN$  (Table 3). Conrauiflavonol was isolated from *Ficus conraui* (Moraceae) by Kengap et al. (2011) and afzelin A from *Hymenostegia afzelii* (Caesalpiniaceae) by Awantu et al. (2011). It can be noted that the name conrauiflavonol is somewhat misleading since it is not a flavonol but a flavanonol (or a dihydroflavonol). The difference between conrauiflavonol and afzelin A is not completely clear. The absolute configuration of conrauiflavonol was established as 2*R*, 3*R* (*trans*) by circular dichroism (CD) spectroscopy, whereas for afzelin A the 2, 3-*trans* relationship was established by X-ray crystallography and NOESY correlations, but not the absolute configuration. For conrauiflavonol a specific optical rotation of +2.55 was reported in chloroform, and for afzelin A +23.5 in methanol. Although both compounds have an established 2, 3-*trans* relationship, they cannot be enantiomers since then they should have opposite specific optical rotations. NMR spectra of compound **2** are added as Supplementary Data (Fig. S7 – S11). The proposed structure was in complete agreement with the COSY, HSQC and HMBC spectra. The specific optical rotation for compound **2** was determined in methanol as  $[\alpha]_D^{20} + 74.8$ . It is hypothesised that the differences in specific optical rotation between compound **2**, conrauiflavonol and afzelin A may be due to differences in enantiomeric purity, implying that compound **2** would have the highest enantiomeric purity.

Compound **3** showed UV absorption maxima at 226, 270, 296 and 310 nm and a pseudo-molecular ion  $[M - H]^-$  with  $m/z$  355.1197 in HRMS consistent with a molecular formula of  $C_{20}H_{20}O_6$ . The specific optical rotation was determined in methanol as  $[\alpha]_D^{20} + 228.8$ . Library search on the  $^{13}C$  NMR data (Table 4) using the NMR Predict database and the  $^1H$  NMR data (Table 4) suggested a great similarity with compound **2**, containing a 2'', 2''-dimethyl pyran ring but with a flavanone skeleton and some modifications on the C-ring. By

analysing the COSY, HSQC and HMBC spectra (Supplementary Data S12 – S16) the structure was elucidated as 6,7-(2'',2''-dimethylpyrano)-5,1'-dihydroxy-4'-oxo-2'-enyl-flavanone, a compound that has not been reported before (Fig. 1). C-2 and C-3 of the flavanone skeleton showed signals at 83.0 and 36.7 ppm, respectively. The quaternary C-5, C-7 and C-9 carbons were assigned to the signals at 159.2, 162.8 and 163.3 ppm, respectively. The quaternary carbons C-6 and C-10 showed signals at 103.9 and 103.7 ppm. The signal at 96.7 ppm was assigned to C-8 and the signal at 197.9 ppm to the carbonyl group of the flavanone. In ring B, C-2' and C-3' were assigned to the signals at 150.2 and 130.5 ppm, respectively. On the same ring C-5' and C-6' were assigned to the signals at 34.3 and 32.4 ppm and C-1' was assigned to the signal at 71.0 ppm. The signal at 199.6 indicated a carbonyl group in position 4'. The signals of the carbons of the dimethyl pyran ring were observed at 115.7 ppm (C-4''), 128.0 ppm (C-3''), 79.4 ppm (C-2'') and the two methyl groups at 28.5 and 28.6 ppm. The HMBC correlations from H-3'' to C-2'' and C-6, and from H-4'' to C-2'', C-6, C-7 and C-5 indicated that the pyran ring was attached to C-7 and C-6 of the flavanone skeleton. C-5 (159.2 ppm) showed a long-range correlation with H-4'' (6.54 ppm) as well as 5-OH (12.33 ppm), clearly defining the orientation of the 2,2-dimethyl pyran ring. Changes in chemical shifts of C-3 and C-1' compared to compound **2** indicated that the hydroxyl group had moved from position 3 to position 1'. The <sup>1</sup>H NMR spectrum showed a doublet of doublets at 4.53 ppm (*J* = 13.2 and 3.0 Hz) assigned to H-2 and a doublet of doublets at 2.66 (*J* = 17.2 and 3.0 Hz) which correlated with a doublet of a doublet at 3.02 ppm (*J* = 17.2 and 13.2 Hz) corresponded to the CH<sub>2</sub> functionality in position 3. The singlet observed at 5.91 ppm was assigned to H-8. The two coupled protons H-2' and H-3' (*J* = 10.3 Hz) corresponded to the signals at 7.08 and 5.97 ppm, respectively. The multiplets at 2.57, 2.43, 2.14 and 2.04 ppm were assigned to the 4 protons in position 5' and 6', respectively. H-4'' and H-3'' corresponded with the two coupled doublets (*J* = 10.1 Hz) at 6.54 and 5.59 ppm, respectively. The protons (H-5'' and H-6'') from the methyl groups at position 2'' were assigned to the singlet at 1.38 ppm. The protons of the hydroxyl groups gave signals at 12.33 ppm (5-OH) and 3.97 ppm (1'-OH). Compound **3** can be considered as a protoflavonoid, since it contains the typical non-aromatic B-ring and a hydroxyl group at C-1'. Therefore the trivial name protosteganoflavanone was adopted.

According to a recent review, protoflavonoids are a rare class of flavonoids that until now mainly has been observed in certain genera of ferns, with the exception of *Apium graveolens*. Both *Steganotaenia araliacea* and *Apium graveolens* belong to the Apiaceae family. Protoflavonoids are considered as a promising class of anticancer compounds (Pouny et al., 2011; Hunyadi et al., 2014). Therefore it can be assumed that from the three compounds isolated in the present work, the flavonoids and more in particular the protoflavonoid protosteganoflavanone may be responsible for the observed cytotoxicity on the cancer cell lines. It is remarkable that the lignans that have been reported previously, were not among the major constituents of the cytotoxic extract of the batch of plant material that was analysed in the present work.

### **3. Materials and methods**

#### *3.1. General experimental procedures.*

All solvents were purchased from Acros Organics (Geel, Belgium) or from Fisher Scientific (Leicestershire, UK) and were at least analytical grade. All reagents were purchased from Acros Organics or Sigma-Aldrich (St. Louis, MO, USA). Solvents used for HPLC, i.e. methanol (MeOH) and acetonitrile were HPLC grade and were purchased from Fisher Scientific. RiOS water was prepared by reverse osmosis and water for HPLC was dispensed by a Milli-Q system from Millipore (Bedford, MA, USA) and passed through a 0.22 µm membrane filter.

Analytical plates for thin layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). Silica gel 60 F<sub>254</sub> plates (20 x 20 cm) were used for normal phase (NP) TLC and silica gel 60 RP-18 F<sub>254</sub> plates (10 x 20 cm) for reverse phase (RP) TLC. The spraying reagent *p*-anisaldehyde was prepared by mixing 0.5 mL *p*-anisaldehyde (Sigma) with 10 mL glacial acetic acid, 85 mL methanol and 5 mL sulphuric acid.

Flash column chromatography was carried out on a Reveleris<sup>®</sup> iES system from Grace (Columbia, MD, USA) using the Reveleris<sup>®</sup> Navigator<sup>™</sup> software, consisting of a binary pump with four solvent selection, an ultraviolet (UV) and evaporating light scattering detector (ELSD)



and a fraction collector. The column used was a pre-packed Flash Grace Reveleris<sup>®</sup> silica cartridge with a particle size of 40 µm. The ELSD carrier solvent was isopropyl alcohol.

An Agilent 1200 series high performance liquid chromatography (HPLC) system with degasser, quaternary pump, automatic injection sampler, thermostatic column compartment and a diode array detector (DAD) (Agilent Technologies, Eindhoven, The Netherlands) was used. The silica-based Apollo C<sub>18</sub> (250 x 4.6 mm, 5 µm) (Grace, Columbia, MD, USA) column was used together with a suitable precolumn to endure the lifetime of the column.

Liquid Chromatography – Solid Phase Extraction – Nuclear Magnetic Resonance spectroscopy (LC-SPE-NMR) was carried out using an Agilent 1200 series with degasser, quaternary pump, automatic injection sampler and an UV/VIS detection (variable wavelength) (Agilent Technologies). The HPLC was connected to a Bruker/Spark solid phase extraction (SPE) system with HySphere Resin General Phase (GP) cartridges (polydivinyl-benzene material with particle size 5-15 µm) to capture and collect the compounds. After the detector, water was added to the eluent stream with a make-up pump (Knauer K 120, Berlin, Germany), to decrease the organic solvent proportion of the elution and to promote better retention of the peaks on the SPE cartridges. By applying multiple trapping the same compounds were repeatedly trapped on the same cartridges. The solvent residues were removed by drying the cartridges with nitrogen gas (N<sub>2</sub>). By means of the Gilson Liquid Handler 125 the compounds were eluted with deuterated solvents into NMR tubes.

NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), operating at 400 MHz for <sup>1</sup>H and at 100 MHz for <sup>13</sup>C, employing a 3-mm broadband inverse (BBI) probe or a 5-mm dual <sup>1</sup>H/<sup>13</sup>C probe using standard Bruker pulse sequences. DEPT-135, DEPT-90 and two-dimensional NMR (COSY, HSQC, HMBC) experiments were recorded. In order to assist structure elucidation a <sup>13</sup>C NMR library was used (NMR Predict version 4.8.57, Modgraph). Deuterated solvents including CDCl<sub>3</sub> (99.8% D), CD<sub>3</sub>OD (99.8% D) and CD<sub>3</sub>CN (99.8% D) were purchased from Sigma-Aldrich.

High resolution mass spectra were obtained with an Agilent 6530 quadrupole-time-of-flight

mass spectrometer (QTOF-MS) equipped with an Agilent Jetstream source that was used with the following parameters: gas temperature 325 °C, gas flow 7 L/min, nebulizer pressure 40 psi, sheath gas temperature 325 °C, sheath gas flow 11 L/min. Capillary, fragmentor, and skimmer voltages were set to 3500 V, 150 V, and 65 V, respectively and the OCT 1 RF V<sub>pp</sub> was set at 750 V. The mass spectrometer was operated in the negative ion mode at 20000 resolution. The Mass Hunter (Agilent Technologies) software was used for data acquisition and processing.

Specific optical rotation was determined on a Jasco P-2000 Polarimeter. The samples were dissolved in methanol and the optical rotation was recorded at 589 nm with a path length of 50 mm.

### 3.2. Plant material

Stem bark of *Steganotaenia araliacea* Hoechst was collected on 15/7/2010 and 25/11/2010 in Tountouroun (prefecture of Labé, Guinea-Conakry) and identified by Prof. Aliou Baldé, A voucher specimen (n° 7HK1) was deposited at the Research and Valorization Center on Medicinal Plants, Dubreka, Guinea. The material was dried in the shade in open air to reduce deterioration of the plant material.

### 3.3. Extraction and isolation

The air-dried stem bark was ground and 2.85 kg was exhaustively extracted with a total of 20 L 80% ethanol. The ethanol was removed under reduced pressure at 40 °C and the aqueous extract was lyophilized, yielding 140 g of dried crude extract. Approximately 2 g of crude extract (SA) was suspended in 100 mL MeOH 90% and subjected to liquid-liquid partition according to Wickramaratne et al. (1993) (Fig. S17). After partition with petroleum ether (PE) (3x 100 mL), both fractions were dried under reduced pressure at 40 °C, yielding 0.16 g PE fraction (SA1A) and 1.28 g methanolic fraction (SA1B). An aliquot of 1.05 g SA1B was then dissolved in 100 mL water and extracted with EtOAc (3x 100 mL). The EtOAc fraction (SA2A) was dried under reduce pressure at 40 °C yielding 0.30 g. The aqueous fraction (SA2B) was lyophilized and yielded 0.61 g.

The different fractions were subjected to TLC (NP and RP) using methylene chloride / *n*-hexane

/ methanol 75 / 20 / 5 as mobile phase, examined under UV at 254 nm and 366 nm, and after spraying with *p*-anisaldehyde reagent (heated at 105 °C until maximal visualization). SA1A contained mostly lipid material. Since the main components of the SA extract were obviously concentrated in fraction SA2A, this fraction was selected for further phytochemical analysis. An aliquot of 0.23 g was dissolved in 2.5 mL methanol, mixed with 0.5 g silica and after drying with N<sub>2</sub> subjected to flash column chromatography on a pre-packed Flash Grace Reveleris<sup>®</sup> silica cartridge of 12 g using a gradient from methylene chloride to ethyl acetate, followed by methanol. Fractions were collected based on UV and ELSD detection, evaluated with NP-TLC and similar TLC-profiles were pooled resulting in 26 subfractions.

HPLC chromatograms were recorded for all obtained subfractions, as well as the SA2A fraction and the crude extract. Samples were prepared with a concentration ranging from 0.4 – 14.6 mg/mL in methanol. Twenty microliter of each sample was injected into the LC-SPE-NMR and with the use of the Apollo column, the compounds were separated. The mobile phases were (A) water + 0.05% TFA and (B) methanol. The gradient was 0 min, 5% B; 5 min, 5% B; 55 min, 100% B; 60 min, 100% B. The flow rate was 1 mL/min and the chromatograms were recorded at 230 nm. The chromatogram from SA2A (Fig. S18) showed three major well-separated peaks with retention times of 32.8, 34.0 and 34.6 min. Subfraction SA2A<sub>10</sub> (Fig. S19) contained two major peaks, SA2A<sub>10A</sub> (compound **1**) and SA2A<sub>10B</sub> (compound **2**) with retention times of 32.8 and 34.6 min, respectively. Subfraction SA2A<sub>17</sub> (Fig. S20) showed one major peak, SA2A<sub>17A</sub> (compound **3**) with a retention time of 34.0 min. These three peaks corresponded with the major peaks in fraction SA2A and therefore these compounds were isolated and identified by means of LC-SPE-NMR.

For the isolation of compounds SA2A<sub>10A</sub> and SA2A<sub>10B</sub> (compounds **1** and **2**), 20 µL of a concentrated solution (5.9 mg/mL) of subfraction SA2A<sub>10</sub> was injected. The flow rate was set at 1.0 mL/min and the detector at 230 nm. Mobile phase A was water containing TFA (0.05%) and B was acetonitrile. The gradient was adjusted to shorten the run time, thus lowering the solvent consumption. The adjusted gradient was 0 min, 45% B; 5 min, 45% B; 25 min, 68 % B; 27.5 min, 100% B and 32.5 min, 100% B. Nineteen multiple trappings were performed and the two compounds were trapped automatically on the HySphere resin GP cartridges by setting a threshold. For the isolation of compound SA2A<sub>17A</sub> (compound **3**), a concentrated solution

(6.0 mg/mL) of subfraction SA2A\_17 was used and 10  $\mu$ L of this solution was injected. The flow rate and detector were the same as above as well as the mobile phases. The gradient was again adjusted to shorten the run time and the used gradient was 0 min, 45% B; 5 min, 45% B; 20 min, 63 % B; 22.5 min, 100% B and 27.5 min, 100% B. Thirty-six multiple trappings were performed and compound SA2A\_17A was repeatedly trapped on a cartridge by setting a threshold. The cartridges were then dried with N<sub>2</sub> to remove all residual solvents and the compounds were eluted with deuterated acetonitrile (99.8% atom D) in 3 mm NMR tubes.

<sup>1</sup>H and <sup>13</sup>C NMR as well as 2D-NMR spectra (COSY, HSQC and HMBC) were then recorded for all compounds.

*Spiropreussomerin A (1)*: <sup>1</sup>H and <sup>13</sup>C NMR: Table 2; HR-ESI-MS:  $m/z = 333.0779$  [M - H]<sup>-</sup> (calcd. for C<sub>20</sub>H<sub>13</sub>O<sub>5</sub>: 333.07629).

*Conrauiflavonol / afzelin A (2)*:  $[\alpha]_D^{20} + 74.8$  (c, 0.15 in MeOH). <sup>1</sup>H and <sup>13</sup>C NMR: Table 3; HR-ESI-MS:  $m/z = 353.1038$  [M - H]<sup>-</sup> (calcd. for C<sub>20</sub>H<sub>17</sub>O<sub>6</sub>: 353.10251).

*Steganoprotolflavanone (3)*:  $[\alpha]_D^{20} + 228.8$  (c, 0.13 in MeOH). <sup>1</sup>H and <sup>13</sup>C NMR: Table 4; HR-ESI-MS:  $m/z = 355.1197$  [M - H]<sup>-</sup> (calcd. for C<sub>20</sub>H<sub>19</sub>O<sub>6</sub>: 355.11816).

### 3.4. Cytotoxic activity

All cell culture reagents and media were purchased from Life Technologies (Ghent, Belgium). All cell lines were maintained at 37 °C and 5% CO<sub>2</sub>/95% air in a humidified incubator. All other reagents, including sulforhodamine B, tris(hydroxymethyl)aminomethane (tris) and trichloroacetic acid were purchased from Sigma-Aldrich or Acros Organics. Malignant human cell lines of different origin were used for the *in vitro* cytotoxicity assessments. Breast adenocarcinoma cells (MDA-MB-231) were cultured in RPMI 1640 medium enriched with 10% foetal bovine serum (FBS), 1% L-glutamine, 1% sodium pyruvate and 1% penicillin / streptomycin. The pancreatic epithelioid carcinoma cells (PANC-1) and the colorectal adenocarcinoma cells (HT-29) were cultured in DMEM also supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. All cell lines were obtained from the American type culture collection (ATCC). Cytotoxicity was evaluated using the spectrophotometric sulforhodamine B (SRB) assay according to Papazisis et al. (1997). Cells were harvested from

exponential phase cultures by trypsinisation and counted with the Scepter™ 2.0 (Merck Millipore, Darmstadt, Germany), which is an automated cell counter based on the Coulter principle. Optimal seeding densities were determined to ensure exponential growth and were incubation time and cell line dependent. Table 5 shows the used cell density per cell line and incubation time. In each experiment 7 different concentrations of plant extract were tested on the different cell lines and 6 replicate wells per concentration were used. Two different incubation times were evaluated, i.e. 24 h and 72 h. After incubation, culture media was removed prior to fixation, which was done by adding 200 µL of 10% cold trichloroacetic acid onto the cells. After one hour at 4 °C, the cells were washed five times with deionised water. The cells were then stained with 200 µL 0.1% SRB in 1% acetic acid for 15 min at room temperature. To remove the unbound stain the cells were washed with 1% acetic acid four times. The plates were left to dry at room temperature. The protein-bound stain was re-dissolved with 200 µL 10 mM unbuffered tris and transferred to 96-well plates for the optical density (OD) reading at 540 nm (Biorad 550 microplate reader Nazareth, Belgium). All experiments were repeated three times.

In order to evaluate its cytotoxicity, SA was suspended in water, filtered through a 0.45 µm membrane and again through a 0.22 µm filter to sterilise the solution. The extract was only poorly soluble; the loss due to filtration was determined by preparing and filtering the solution 6 times. After lyophilisation, the average loss by filtration was calculated as approximately 65%. Nevertheless, HPLC analysis of the *in vitro* stock solution still showed the presence of the three compounds isolated during the phytochemical investigation of the crude extract.

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**Table 1.** IC<sub>50</sub> of the EtOH crude extract of *S. araliacea* on the tested cell lines

IC <sub>50</sub> ± SEM (µg/mL)			
	MDA-MB-231	PANC-1	HT-29
<b>24 h</b>	165.5 ± 8.0	64.0 ± 3.3	68.7 ± 3.9
<b>72 h</b>	95.7 ± 4.8	47.9 ± 1.8	55.1 ± 6.6



**Table 2.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments for compound **1** (spiropreussomerin A) recorded in  $\text{CD}_3\text{CN}$

Position	$\delta_{\text{H}}$ (ppm); multiplicity; $J$ (Hz)	$\delta_{\text{C}}$ (ppm)
<b>1</b>	-	148.6
<b>2</b>	7.07; d; 7.7	110.5
<b>3</b>	7.53; t; 7.7, 8.3	129.0
<b>4</b>	7.61; d; 8.3	122.0
<b>4a</b>	-	135.4
<b>5</b>	7.59; d; 8.3	121.9
<b>6</b>	7.47; t; 7.7, 8.3	128.9
<b>7</b>	6.94; d; 7.7	110.0
<b>8</b>	-	148.6
<b>8a</b>	-	113.8
<b>1'</b>	-	98.9
<b>2'</b>	3.65; d; 4.0	51.4
<b>3'</b>	3.52; dd; 4.0, 2.4	54.3
<b>4'</b>	5.41; d; 5.4	61.5
<b>4a'</b>	-	123.2
<b>5'</b>	7.29; d; 2.7	130.5
<b>6'</b>	6.99; dd; 5.9, 2.7	117.8
<b>7'</b>	-	156.3
<b>8'</b>	7.27; s	119.0
<b>8a'</b>	-	133.6
<b>OH (4)</b>	3.74; d; 6.1	-
<b>OH (7')</b>	8.02; s	-

**Table 3.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments for compound **2** (conrauf flavonol / afzelin A) recorded in  $\text{CD}_3\text{CN}$

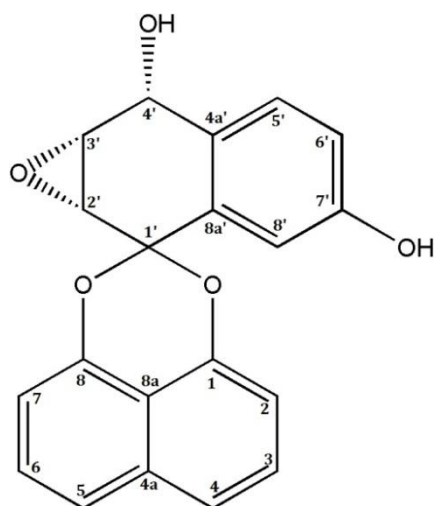
Position	$\delta_{\text{H}}$ (ppm); multiplicity; $J$ (Hz)	$\delta_{\text{C}}$ (ppm)
<b>2</b>	5.04; d; 11.8	84.4
<b>3</b>	4.60; dd; 11.8, 4.1	73.2
<b>4</b>	-	198.7
<b>5</b>	-	158.9
<b>6</b>	-	104.0
<b>7</b>	-	163.2
<b>8</b>	5.88; s	97.0
<b>9</b>	-	163.6
<b>10</b>	-	102.2
<b>1'</b>	-	129.1
<b>2'/6'</b>	7.34; dd; 6.7, 1.9	130.6
<b>3'/5'</b>	6.86; dd; 6.7, 1.9	116.3
<b>4'</b>	-	158.9
<b>2''</b>	-	79.6
<b>3''</b>	5.62; d; 10.1	128.2
<b>4''</b>	6.57; d; 10.1	115.6
<b>5''</b>	1.40; s	28.7
<b>6''</b>	1.40; s	28.6
<b>OH (3)</b>	4.01; d; 4.1	-
<b>OH (5)</b>	11.86; s	-
<b>OH (4')</b>	7.68; s	-

**Table 4.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments for compound **3** (protosteganoflavanone) recorded in  $\text{CD}_3\text{CN}$

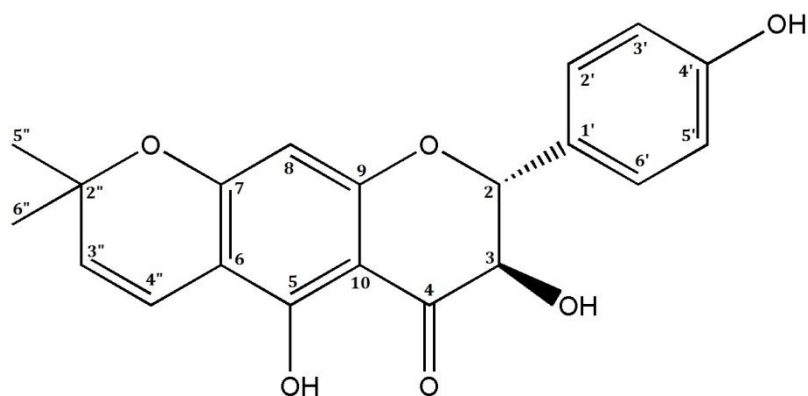
Position	$\delta_{\text{H}}$ (ppm), multiplicity, $J$ (Hz)	$\delta_{\text{C}}$ (ppm)
<b>2</b>	4.53; dd; 13.2; 3.0	83.0
<b>3</b>	2.66; dd; 17.2; 3.0 3.02; dd; 17.2; 13.2	36.7
<b>4</b>	-	197.9
<b>5</b>	-	159.2
<b>6</b>	-	103.9
<b>7</b>	-	162.8
<b>8</b>	5.91; s	96.7
<b>9</b>	-	163.3
<b>10</b>	-	103.7
<b>1'</b>	-	71.0
<b>2'</b>	7.08; d; 10.3	150.2
<b>3'</b>	5.97; d; 10.3	130.5
<b>4'</b>	-	199.6
<b>5'</b>	2.57; m 2.43; m	34.3
<b>6'</b>	2.14; m 2.04; m	32.4
<b>2''</b>	-	79.4
<b>3''</b>	5.59; d; 10.1	128.0
<b>4''</b>	6.54; d; 10.1	115.7
<b>5''</b>	1.38; s	28.6
<b>6''</b>	1.38; s	28.5
<b>OH (5)</b>	12.33; s	-
<b>OH (1')</b>	3.97; s	-

**Table 5.** Cell density per cell line and incubation time.

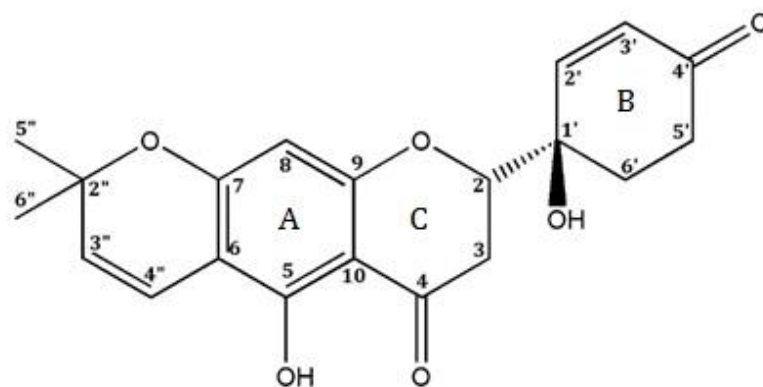
Cell lines	Cell density (cells/well)	
	24 h	72 h
MDA-MB-231	4000	3200
PANC-1	3000	3000
HT-29	4500	3500
PANC02	500	500



1



2



3

**Fig. 1.** Structure of compounds **1** (spiopreussomerin A), **2** (conraui flavonol / afzelin A) and **3** (protosteganoflavanone)

**Phytochemical Characterisation of a Cytotoxic Stem Bark Extract  
of *Steganotaenia araliacea* and Identification of a Protoflavanone by LC-SPE-NMR**

**Supplementary Data**

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**Fig. S1.** Dose-response curves of the three human cancer cell lines treated with SA

**Fig. S2.**  $^{13}\text{C}$  spectra, including DEPT-135 and DEPT-90 of compound **1** (spiopreussomerin A)

**Fig. S3.**  $^1\text{H}$  spectrum of compound **1** (spiopreussomerin A)

**Fig. S4.** COSY spectrum of compound **1** (spiopreussomerin A)

**Fig. S5.** HSQC spectrum of compound **1** (spiopreussomerin A)

**Fig. S6.** HMBC spectrum of compound **1** (spiopreussomerin A)

**Fig. S7.**  $^{13}\text{C}$ -NMR spectra, including DEPT-135 and DEPT-90 of compound **2** (conraui flavonol / afzelin A)

**Fig. S8.**  $^1\text{H}$ -NMR of compound **2** (conraui flavonol / afzelin A)

**Fig. S9.** COSY spectrum of compound **2** (conraui flavonol / afzelin A)

**Fig. S10.** HSQC spectrum of compound **2** (conraui flavonol / afzelin A)

**Fig. S11.** HMBC spectrum of compound **2** (conraui flavonol / afzelin A)

**Fig. S12.**  $^{13}\text{C}$  spectra, DEPT-135 and DEPT-90 of compound **3** (protosteganoflavanone)

**Fig. S13.**  $^1\text{H}$  spectrum of compound **3** (protosteganoflavanone)

**Fig. S14.** COSY spectrum of compound **3** (protosteganoflavanone)

**Fig. S15.** HSQC spectrum of compound **3** (protosteganoflavanone)

**Fig. S16.** HMBC spectrum of compound **3** (protosteganoflavanone)

**Fig. S17.** Liquid-liquid partition

**Fig. S18.** HPLC chromatogram of fraction SA2A

**Fig. S19.** HPLC chromatogram of subfraction SA2A\_10

**Fig. S20.** HPLC chromatogram of subfraction SA2A\_17