Sesquiterpene lactones from *Geigeria aspera* Harv. and their cytotoxicity

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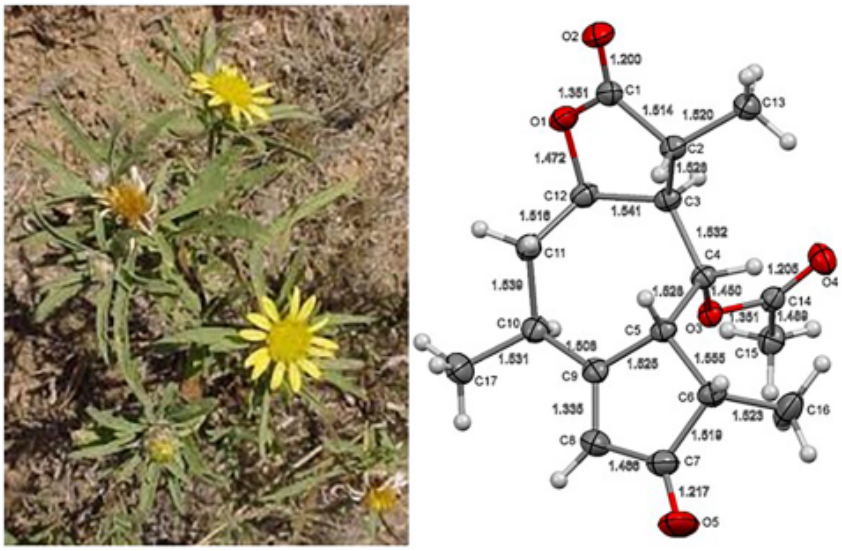
ABSTRACT

Geigeria poisoning, referred to as 'vermeersiekte' is an important plant poisoning in southern Africa. Three sesquiterpene lactones, isogeigerin acetate (**1**) ivalin (**2**) and geigerin (**3**) were isolated and puri ied from *Geigeria aspera* Harv. (Asteraceae). Struc-tures were deduced using 1 and 2D NMR spectroscopy and mass spectrometry, while the absolute con igurations of compounds **1** and **3** were determined for the irst time by X-ray crystal diffraction analyses. Cytotoxicity of isogeigerin acetate, ivalin and gei-gerin were compared by exposing a murine skeletal myoblast (C2C12) cell line to varying concentrations of the three sesquiter-pene lactones isolated. Cell viability was assessed using the methyl-thiazolyl-tetrazolium (MTT) assay. The EC_{50}s were 3.746, 0.0029 and 3.792 mM for isogeigerin acetate (**1**), ivalin (**2**) and geigerin (**3**), respectively. The results indicate that ivalin is much more toxic, approximately 1000 times, *in vitro* compared to isogeigerin acetate and geigerin.



Keywords: Cytotoxicity ; *Geigeria aspera* ; geigerin ; ivalin ; isogeigerin acetate

1. Introduction

The genus, *Geigeria* (family Asteraceae) is a well-defined genus and contains 30 species of which 21 are indigenous to southern Africa (Fadul et al. 2019). *Geigeria* species, commonly referred to as 'vomiting bush' are responsible for vomiting disease, colloquially referred to as 'vermeersiekte', in sheep (Rimington et al. 1936; Grosskopf 1965). *Geigeria aspera* Harv., is a woody, semi-perennial shrub (Bajaj 1989) with bright yellow flowers and grows in savanna areas where frost and fire occur. The plant prefers a moist habitat and is often found near waterways and swampy areas. The genus, *Geigeria*, is regarded as a rich source of sesquiterpene lactones (Grosskopf 1965; Vogelzang et al. 1978; Kellerman et al. 2005; Fadul et al. 2019). Sesquiterpene lactones are relatively stable compounds, colourless and with a bitter taste (Rodriguez et al. 1976). Biogenetically, they are derived from farnesyl pyrophosphate (Zdero and Bohlmann 1989). These compounds are known to poison livestock (Kellerman et al. 2005), to act as insect feeding deterrents and to cause allergic contact dermatitis in humans (Rodriguez et al. 1976). The sesquiterpene lactones

are inhibitors of mitochondrial respiration (Van Aswegen et al. 1982) and studies on their mechanism of action indicated that the α-methylene-γ-lactone and cyclopentenone moieties undergo Michael-type addition with L-cysteine, glutathione, and a number of sulphydryl containing cellular enzymes (Gaspar et al. 1987). This is the first report on the absolute stereochemistry of isogeigerin acetate isolated from *G. aspera*. *In vitro* cytotoxicities of the three pure sesquiterpene lactones are also described.

2. Results and discussion

Phytochemical investigation of the dichloromethane extract from the aerial parts of *G. aspera* led to the isolation of isogeigerin acetate (**1**), obtained as colourless crystalline material. The HR ToF mass spectra of **1** (Supplementary information Figure S5) showed a pseudo molecular ion at *m/z* 307.1519 (calculated for $C_{17}H_{22}O_5$ $[M + H]^+$). HR ESI $^+$ ToF MS data revealed a fragment peak at *m/z* 247.1337 [-HOAc] due to the loss of an acetate group. The base peak in the MS chromatogram at *m/z* 247.1337 corresponds to a molecular formula of $C_{15}H_{19}O_3$. The ^{1}H NMR spectrum of **1** (Supplementary information Figure S1-1) displayed three methyl doublets at δ_H 1.30, 1.32 and 0.98. The presence of an acetyl group was suggested by the ^{1}H NMR signal at δ_H 1.9 and the ^{13}C NMR signal at δ_C 169.6. The downfield signal at δ_H 6.05 was brt, indicating the coupling between H-8 and H-5/H-10 and H-5 was observed at δ_H 3.38 (J = 6.5) as a brd. The ^{13}C NMR spectrum (Supplementary information Figure S1-2) showed seventeen carbon signals, consisting of three carbonyl carbon signals at δ_C 209. 2 (C-7); 176.9 (C-1) and 169.6 (C-14), two oxygenated carbon signals were observed at δ_C 79.0 (C-12) and 68.5 (C-4), three methyl carbon signals at δ_C 22.1 (C-13), 13.3 (C-17) and 11.1 (C-16) and two olefinic carbon signals at δ_C 130.2 (C-8) and 181.1 (C-9). The HMBC correlation of δ_H 6.05/H-8 and 0.98/H-16 with 42.6, 44.1 and 209.2 confirmed the position of the α,β-unsaturated double bond in the ring. The ^{13}C NMR data was not reported before for compound (**1**). From the spectroscopic data, compound (**1**) was identified as isogeigerin acetate. This compound has been previously isolated and the stereochemistry partially defined by Bohlmann et al. (1982). We report for the first time the absolute stereochemistry of isogeigerin acetate (**1**) as deduced by X-ray crystallography. The *MERCURY* (Macrae et al. 2008) view of isogeigerin acetate (**1**) (Supplementary Material) showed that the stereochemistry of the structure differs at C-5 to that reported by Bohlmann et al. (1982), who deduced the stereochemistry from inspection of a model used.

Ivalin (**2**) was isolated from the dichloromethane extract and re-crystallized from ethyl acetate:hexane as a crystalline compound. Spectroscopic data was similar to that published by Kim et al. (2004) and HR ESI$^+$ MS displayed a pseudo molecular ion at *m/z* 249.3260 (calculated for $C_{15}H_{20}O_3$ $[M + H]^+$). The ^{1}H NMR spectrum exhibited two exocyclic methylene groups, one unconjugated and one conjugated with the lactone ring. The two doublets at δ_H 4.82 and 4.49 were assigned to the methylene protons at C-14 and the other two doublets at δ_H 6.08 and 5.55 assigned to the methylene protons at C-13.. Based on the 1 and 2D NMR data observations and a comparison of the data with the literature (Kim et al. 2004), compound (**2**) was identical to that reported by Kim et al. (2004) and determined to be ivalin (2α-hydroxy-5αH-eu-desma-4(15),11(13)-dien-12,8β-olide).

Geigerin (**3**) was isolated as colourless needles. 1 and 2D NMR spectroscopy and mass spectrometry were similar to that reported by Carret and Deprés (2007). This compound has previously been isolated (Rimington et al. 1936; Bohlmann et al. 1982) and the structure confirmed by X-ray analysis of the bromo-geigerin acetate derivative (Hamilton et al. 1962). The relative stereochemistry of compound (**3**) was confirmed by the total synthesis of (±) geigerin (Carret and Deprés 2007). Herewith, we confirm the absolute stereochemistry of the isolated compound, geigerin (**3**) as (3*R*,3a*S*,4*S*,7a*R*,8*S*,9a*R*)-4-hydroxy-3,5,8-trimethyl-3a,7,7a,8,9,9a-hexahydro-3H,4H-azuleno[6,5-b]furan-2,6-dione. The compound crystallized as an enantiomerically pure hydrate with the hydroxyl group acting as a hydrogen-bond donor to the water molecule and the water as a hydrogen-bond donor to lactone O atoms of two further geigerin molecules. It formed hydrogen-bonding chains: -lactone…water…lactone…lactone …water…lactone…etc. with the -OH…water hydrogen-bonds cross-linking neighbouring chains to form hydrogen-bonded layers. X-ray crystallographic analysis confirmed the compound (Supplementary Material).

In vitro biological assays were conducted to assess the cytotoxicity of the three compounds isolated. In the current study, the established murine myoblast cell line (Botha et al. 2017) was used to compare the relative toxicities of the

sesquiterpene lactones. Botha et al. (2017) showed that the commercially available murine skeletal myoblast cell line (C2C12) could be used as a suitable *in vitro* model to evaluate cytotoxicity induced by other and/or combinations of sesquiterpene lactones implicated in 'vermeersiekte' in sheep, with and without metabolic activation. Results from the cytotoxicity assay showed that after 48 h exposure, the median effective concentrations (EC_{50}s) calculated were 3.746 mM, 0.0029 and 3.792 for compounds **1**, **2** and **3**, respectively (Supplementary Material). The results indicated that ivalin (**2**) was much more toxic, approximately 1000 times compared to isogeigerin acetate (**1**) and geigerin (**3**). Apoptosis was the main mechanism through which geigerin (**3**) induced the observed cell death (Botha et al. 2017). Previous studies have also concluded that both mitochondrial respiration and glycolysis are inhibited by these lactones (Van Aswegen et al. 1979; 1982). The irreversible nature of the inhibition may, over an extended period of time, suffice to cause interference with *in vivo* energy production at appreciably lower inhibitor concentrations (Van Aswegen et al. 1982). Ma et al. (2018) showed that ivalin significantly inhibited cell proliferation, migration and invasion in breast cancer cells in a dose-dependent manner *in vitro*. In a study reported by Hussien et al. (2016), ivalin was evaluated for cytotoxicity using human breast cancer (MCF-7) and hepatoma (Hep G2) cell lines. Ivalin exhibited cytotoxic effects against MCF-7 and Hep G2 cells, IC_{50}s of 39.6 and 31.6 µg/ml, respectively. Although compound **2** is a different type of sesquiterpene lactone (eudesmanolide) to that of compounds **1** and **3** (guaianolides), previous studies indicated that the presence of a α-methylene-γ-lactone group play a role in the cytotoxicity of sesqui-terpene lactones (Rodriguez et al. 1976; Arantes et al. 2011; Bruno et al. 2005; Hussien et al. 2016). However, detailed investigations on sesquiterpene lactones of different skeletal types should be undertaken to provide additional information.

3. Experimental section

3.1. Isogeigerin acetate (1)

The DCM crude extract (8.75 g) was flash chromatographed on silica gel using acetone:hexane (1:4) as eluent. From the combined eluents, compound **1** crystallized as a white powder. The compound was re-crystallized from ethyl acetate:hexane to give 522 mg of a colourless crystalline material, isogeigerin acetate (**1**) ($C_{17}H_{22}O_5$; m.p. 145–146 °C). Rf value 0.6 (solvent system; toluene:isopropyl alcohol (90:10, v/v). EI MS *m/z*: 307.152 (M + H); 247.134 ($-CO_2CH_3$; base peak). HREI MS: *m/z* 306.1440 (cal. for $C_{17}H_{22}O_5$; 306.3558**)**. The ^{1}H- and ^{13}C-NMR (400 and 100 MHz, $CDCl_3$) spectra are given in the Supplementary Material.

3.1.1. X-ray crystallographic study of isogeigerin acetate (1)

The structure and absolute stereochemistry of isogeigerin acetate (**1**) was confirmed by X-ray crystallographic analysis to be: (3R,3aR,4R,4aR,5S,8S,9aR)-3,5,8-trimethyl-2,6-dioxo-2,3,3a,4,4a,5,6,8,9,9a-decahydro-azuleno[6,5-b]furan-4-yl acetate. The stereochemistry is similar to that of geigerin (**3**) except for differences necessitated by the change in the position of the ring double bond from between C4a and C-5 in geigerin to between C-7 and C-7a in isogeigerin acetate.

3.2. Ivalin (2)

Flash chromatography of the DCM extract (10.85 g) using an eluent of acetone:toluene (1:12) yielded ivalin (**2**). The compound was re-crystallized from ethyl acetate:hexane to yield a crystalline compound (1.646 g), identified as ivalin ($C_{15}H_{20}O_3$). Spectroscopic data was similar to that published by Kim et al. (2004).

3.3 Geigerin (3)

Flash chromatography of the DCM extract (10.85 g) using an eluent of acetone:toluene (1:12) yielded geigerin (**3**). The compound was re-crystallized from ethyl acetate:hexane to give colourless needles, 865 mg of pure geigerin hydrate. The spectroscopic data is reported in the Supplementary Material and compared to that reported by Carret and Deprés (2007).

3.3.1. X-ray crystallographic study of geigerin (3)

The structure of synthetic geigerin has previously been published as a racemic mixture in space group $P2_1/n$ (Carret and Deprés 2007). The structure of the enantiomerically pure natural product, geigerin (**3**) (Supplementary Materi-

al), corresponds closely to one of the enantiomers of the racemic mixture, except that the orientation of the hydrogen atom of the hydroxyl substituent (H3a) has changed to accommodate the hydrogen bond to the water molecule in the crystal structure of geigerin (**3**). The absolute stereochemistry of geigerin (**3**) was confirmed by the X-ray structure analysis to be (3R,3aS,4S,7aR,8S,9aR)-4-hydroxy-3,5,8-trimethyl-3a,7,7a,8,9,9a-hexahydro-3H,4H-azuleno[6,5-b]furan-2,6-dione.

3.4. In vitro cytotoxicity assay

All chemicals, reagents and cell culture media were soured from Sigma-Aldrich and Merck, Darmstadt, Germany unless otherwise stated. Stock solutions of the sesquiterpene lactones were prepared in acetone and diluted with the incubation medium to the test concentrations. The C2C12 (mouse skeletal myoblast) (CRL-1772™)(American Tissue Culture Collection (ATCC®; Manassas, Virginia, USA) were grown at 37 °C in a humidified atmosphere of 5% CO_2 in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with, 4 mm L-glutamine, 1 mM sodium pyruvate (Pan-Biotech, Aidenbach, Germany), 5% foetal calf serum (FCS) (Gibco), Carlsbad, California, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Lonza, Basel, Switzerland).

The cytotoxic effect of the test sesquiterpenes on the cells was determined using a modified version of the methyl-thiazol-tetrazolium (MTT) viability assay described by Mossman in 1983. C2C12 cells were seeded in 96-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) 24 h prior to commencement of the exposure study at a density of 1500 cells/well; in 200 µl incubation medium. Following a 24 h pre-incubation period, the cells were exposed to incubation medium (negative control) and serial dilutions of sesquiterpenes for 48 h. After termination of the exposure studies, the incubation medium was aspirated, the wells rinsed with 200 µl PBS (phosphate buffered saline), pH 7.4, and 220 µl assay medium (20 µl 0.005 g/ml MTT dissolved in PBS and 200 µl incubation medium) were added to each well. Following a further 2 h incubation period under the same conditions as the exposure studies the MTT medium was replaced with 100 µl dimethyl sulphoxide (DMSO) and the plate shaken for 5 min in the dark to solubilise the MTT formazan crystals. The absorbance of the MTT product was measured at 570 nm and the background at 630 nm using a Synergy HT BioTek microplate reader. Toxicity was expressed in terms of the negative control. Experiments were repeated at least three times in triplicate. Microsoft Excel and GraphPad Prism (Version 6.0) were used to analyse results and determine half maximal effective concentration (EC_{50}) values.

4. Conclusion

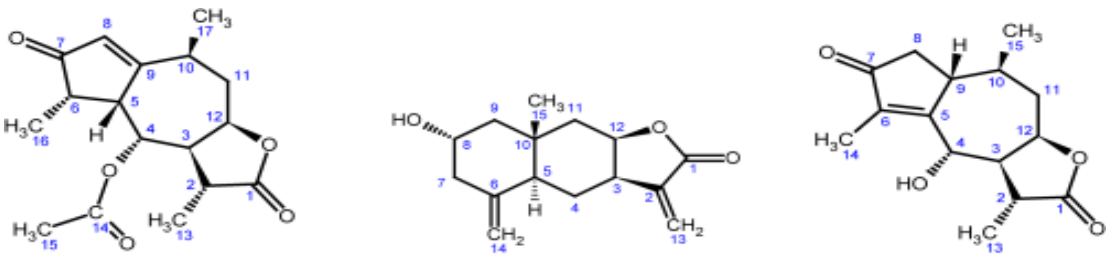
Three sesquiterpene lactones, isogeigerin acetate, ivalin and geigerin were isolated from *Geigeria aspera* and their structures deduced using 1 and 2D NMR spectroscopy and mass spectrometry. The absolute configurations of isogeigerin acetate and geigerin were confirmed for the first time by X-ray crystal diffraction analyses. Cytotoxicity of isogeigerin acetate, ivalin and geigerin were determined by exposing a murine skeletal myoblast (C2C12) cell line to varying concentrations of the three sesquiterpene lactones isolated. Cell viability was then determined using the methyl-thiazolyl-tetrazolium (MTT) assay. The results indicated that ivalin is much more toxic, approximately 1000 times, *in vitro* compared to isogeigerin acetate and geigerin.

Acknowledgements

The authors would like to thank Dr. Daleen Bester who collected the plant material.

Disclosure statement

No conflict of interest reported by authors.



(1) (2) (3)

Figure 1. Structures of isogeigerin acetate (1), ivalin (2) and geigerin (3).

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