*Article*

# Crystal Structures and Cytotoxicity of *ent*-Kaurane-Type Diterpenoids from Two *Aspilia* Species

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**Abstract:** A phytochemical investigation of the roots of *Aspilia pluriseta* led to the isolation of *ent*-kaurane-type diterpenoids and additional phytochemicals (**1–23**). The structures of the isolated compounds were elucidated based on Nuclear Magnetic Resonance (NMR) spectroscopic and mass spectrometric analyses. The absolute configurations of seven of the *ent*-kaurane-type diterpenoids (**3–6**, **6b, 7** and **8**) were determined by single crystal X-ray diffraction studies. Eleven of the compounds were also isolated from the roots and the aerial parts of *Aspilia mossambicensis*. The literature NMR assignments for compounds **1** and **5** were revised. In a cytotoxicity assay, 12$\alpha$-methoxy-*ent*-kaur-9(11),16-dien-19-oic acid (**1**) (IC$_{50}$ = 27.3 $\pm$ 1.9 μM) and 9$\beta$-hydroxy-15$\alpha$-angeloyloxy-*ent*-kaur-16-en-19-oic acid (**3**) (IC$_{50}$ = 24.7 $\pm$ 2.8 μM) were the most cytotoxic against the hepatocellular carcinoma (Hep-G2) cell line, while 15$\alpha$-angeloyloxy-16$\beta$,17-epoxy-*ent*-kauran-19-oic acid (**5**) (IC$_{50}$ = 30.7 $\pm$ 1.7 μM) was the most cytotoxic against adenocarcinomic human alveolar basal epithelial (A549) cells.

**Keywords:** Asteraceae; *Aspilia pluriseta*; *Aspilia mossambicensis*; *ent*-kaurane diterpenoid; X-ray crystal structure; cytotoxicity

## 1. Introduction

The genus *Aspilia* belongs to the family Asteraceae. The majority of plants in this family are herbaceous, while trees and shrubs are rare [1]. Plants belonging to the Asteraceae family are found worldwide, except Antarctica [2]. They are found in cooler montane habitats or temperate areas in tropical regions, and are not common dwellers of hot lowland tropical rain forests [1,2]. The family of Asteraceae is one of the largest plant families and the richest in vascular plants in the world. The family has about 1,600–1,700 genera and 24,000–30,000 species [1,3,4]. Plants from the genus *Aspilia* (Asteraceae) occur widely in South, South-West, and West Kenya, from the coast to Lake

Victoria. The genus *Aspilia* exhibits biological activities, including antibacterial and antifungal effects, mainly attributed to the presence of kaurane-type diterpenoids [3,5] and sesquiterpene lactones [5,6].

*Aspilia pluriseta* Schweinf has been used in traditional medicine to treat lacerations, bruises and burns, and it is reputed to aid in the healing of cutaneous lesions [7]. The plant is found in Kenya and is commonly known as 'Dwarf Aspilia' [7]. The presence of diterpenoids from aerial parts of *A. pluriseta* has been reported previously [8], and four of these diterpenoids exhibited moderate activity against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum*. The aqueous extract of the plant was also reported by the same authors to exhibit hypoglycemic properties in alloxanized mice. *A. pluriseta* is locally known in Kenya as *Muuti* (Kikuyu), *Wuti* (Kamba), *Ol-oiyabase* (Maasai), and *Shilambila* (Luhya). Many communities in Kenya, as well as some in the rest of Eastern and Southern Africa, use the plant ethnomedically to treat wounds [7].

*Aspilia mossambicensis* (Oliv.) Wild is a shrub native to central and Eastern tropical Africa. The plant is found in the Democratic Republic of Congo, Ethiopia, Kenya, Malawi, Mozambique, Tanzania, Uganda, Zambia, and Zimbabwe [9]. In Eastern Africa, the plant is well known for the treatment of cystitis, gonorrhea, abdominal pain, intestinal worms, and skin infections [9–12]. The thiophene derivatives, thiarubrines A and B, have previously been isolated from *Aspilia mossambicensis* [9]. The roots of this plant exhibited antibacterial activity, which was suggested to explain its use by wild chimpanzees [9,11]. Herein, we report the phytochemical investigation and the cytotoxicity study of the constituents of *Aspilia pluriseta* Schweinf and *Aspilia mossambicensis* (Oliv.) Wild (Asteraceae).

## 2. Results and Discussion

Compound **1**, $[\alpha]_D^{20} -88°$, was isolated as colorless crystals (m.p. 184–186 °C) from the CH$_2$Cl$_2$/MeOH (1:1) extract of the roots of *Aspilia pluriseta*. HRMS (Figure S7, Supplementary Material) showed a [M–H]$^-$ ion peak at $m/z$ = 329.2191, which is in agreement with the molecular formula C$_{21}$H$_{30}$O$_3$. The NMR spectra (Table 1) indicated that this compound is a kaurene diterpenoid (Figure 1). The $^1$H–NMR spectrum further revealed the presence of three olefinic protons, namely H-11 ($\delta_H$ 5.30), H-17a ($\delta_H$ 4.84) and H-17b ($\delta_H$ 4.94), suggesting two double bonds. The $^{13}$C–NMR chemical shifts of C-16 ($\delta_C$ 152.9) and C-17 ($\delta_C$ 108.1) are typical of a terminal double bond in an *ent*-kaurene skeleton.
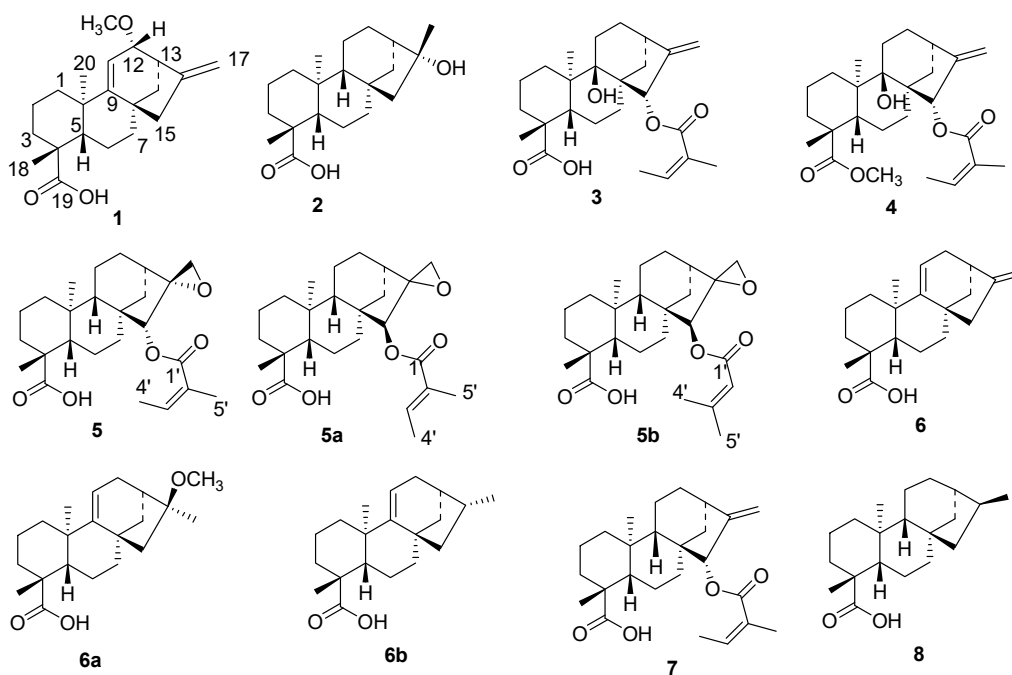


**Figure 1.** Structures of compounds **1**–**8**.

**Table 1.** The $^1$H (800 MHz) and $^{13}$C-NMR (200 MHz) data for compound **1** acquired in CDCl$_3$.

| Position | $\delta_C$ Lit. [13] * | $\delta_C$ | $\delta_H$, mult. (*J* in Hz) | HMBC ($^2J$, $^3J$) |
|---|---|---|---|---|
| 1 | 38.17 | 40.6 | 1.14 *ddd* (13.5, 9.5, 4.2) | C-2, C-3, C-10, C-20 |
| | | | 1.90 *ddd* (13.5, 3.5, 1.4) | C-2, C-3, C-10, C-20 |
| 2 | 18.35 | 20.0 | 1.43 *dddd* (14.2, 9.5, 3.9, 3.5) | C-1, C-3, C-4, C-5, C-10 |
| | | | 1.79 *ddddd* (14.2, 11.1, 4.2, 3.5, 1.4) | C-1, C-4, C-5 |
| 3 | 29.03 | 38.1 | 0.93 *ddd* (13.4, 11.1, 3.9) | C-1, C-2, C-4, C-18, C-19 |
| | | | 2.08 *ddd* (13.4, 3.5, 3.5) | C-1, C-4, C-5, C-7 |
| 4 | 43.43 | 44.6 | | |
| 5 | 43.81 | 46.1 | 1.56 *dd* (11.1, 8.5) | C-4, C-7, C-9, C-10, C-18, C-19, C-20 |
| 6 | 20.07 | 18.3 | 1.82 *dddd* (14.2, 10.0, 8.5, 2.5) | C-3, C-4, C-5, C-7, C-10 |
| | | | 2.43 *dddd* (14.2, 11.1, 9.5, 3.5) | C-4, C-5, C-8 |
| 7 | 40.60 | 28.9 | 1.42 *ddd* (13.8, 3.5, 2.5) | C-5, C-6, C-8, C-9, C-15 |
| | | | 1.95 *ddd* (13.8, 10.0, 9.5) | C-6, C-8, C-9, C-14, C-15 |
| 8 | 44.66 | 43.4 | | |
| 9 | 160.28 | 160.2 | | |
| 10 | 38.94 | 38.9 | | |
| 11 | 115.42 | 115.3 | 5.30 *dd* (4.3, 1.4) | C-8, C-9, C-10, C-12, C-13, C-15, C-20 |
| 12 | 81.79 | 81.7 | 3.38 *dd* (4.3, 2.9) | C-9, C-11, C-13, C-16, C-20, OCH$_3$-12 |
| 13 | 46.17 | 43.7 | 2.89 *dd* (2.9, 1.4) | C-10, C-11, C-12, C-15, C-16 |
| 14 | 40.60 | 40.5 | 1.31 *dd* (10.8, 4.3) | C-7, C-8, C-9, C-12, C-13, C-15 |
| | | | 1.58 *dd* (10.8, 2.5) | C-9, C-12, C-13, C-15, C-16 |
| 15 | 47.17 | 47.1 | 2.08 *dd* (15.4, 4.3) | C-7, C-8, C-9, C-16, C-17 |
| | | | 2.35 *dd* (15.4, 2.5) | C-7, C-9, C-13, C-14, C-16, C-17 |
| 16 | 153.00 | 152.9 | | |
| 17 | 108.12 | 108.1 | 4.84 *dd* (3.0, 1.6) | C-12, C-13, C-15, C-16 |
| | | | 4.94 *dd* (3.0, 1.6) | C-12, C-13, C-15, C-16 |
| 18 | 28.22 | 28.2 | 1.17 *s* | C-3, C-4, C-5, C-8, C-19 |
| 19 | 182.98 | 183.2 | | |
| 20 | 23.41 | 23.4 | 1.01 *s* | C-1, C-5, C-9, C-10 |
| OCH$_3$-12 | 56.53 | 56.5 | 3.34 *s* | C-12 |

* CDCl$_3$ at 100 MHz [13].

The second double bond was placed between C-9 ($\delta_C$ 160.2) and C-11 ($\delta_H$ 5.30; $\delta_C$ 115.3) by comparison of the NMR data with that found in the literature [13–15]. Signals indicating the presence of a methoxy ($\delta_H$ 3.34, $\delta_C$ 56.5) and a carboxylic acid ($\delta_C$ 183.2) substituent were observed. The Heteronuclear Multiple Bond Correlations (HMBCs) of CH$_3$-18 ($\delta_H$ 1.17), H-3 ($\delta_H$ 0.93), and H-5 ($\delta_H$ 1.56) with the carboxy resonance C-19 ($\delta_C$ 183.2) suggested the location of the carboxy group (C-19) at C-4. Out of the three methyl groups expected in kaurene diterpenoid, only two, i.e., CH$_3$-18 ($\delta_H$ 1.17, $\delta_C$ 28.2) and CH$_3$-20 ($\delta_H$ 1.01, $\delta_C$ 23.4), were observed. This corroborated the suggestion of the third methyl group being oxidized to a carboxylic acid (C-19, $\delta_C$ 183.2). The methoxy group OCH$_3$-12 ($\delta_H$ 3.34) showed HMBC correlation with C-12 ($\delta_C$ 81.7), whereas H-12 ($\delta_H$ 3.38) showed HMBC correlation with C-9 ($\delta_C$ 160.2), C-11 ($\delta_C$ 115.3), C-13 ($\delta_C$ 43.7), C-16 ($\delta_C$ 152.9), and OCH$_3$-12 ($\delta_C$ 56.5). Furthermore, CH$_3$-20 ($\delta_H$ 1.01) showed HMBC correlation with C-1 ($\delta_C$ 40.6), C-5 ($\delta_C$ 46.1), the olefinic carbon C-9 ($\delta_C$ 160.2), and C-10 ($\delta_C$ 38.9). This confirmed that the second double bond in the molecule is located at C-9. Moreover, the HMBC correlation of CH$_2$-14 ($\delta_H$ 1.31, 1.58) with a deshielded carbon C-12 ($\delta_C$ 81.7) is in agreement with OCH$_3$ being connected to C-12. The above findings confirmed the identity of compound **1** as a C-12-methoxy substituted *ent*-kaur-9(11),16-dienoic acid derivative. The relative configuration at C-12 was deduced from the Nuclear Overhauser Effect (NOE) of OCH$_3$-12 ($\delta_H$ 3.34) to H-13 ($\delta_H$ 2.89) (Figure 2), indicating them to be syn-oriented, and hence OCH$_3$-12 to be α-oriented. It should be noted that H-12 ($\delta_H$ 3.38) also showed a weak NOE to H-13 ($\delta_H$ 2.89), which is expected in a strained ring system. The proposed configuration at C-12 is further corroborated by the NOE of H-12 ($\delta_H$ 3.38) with H-14b ($\delta_H$ 1.58). The NOE of H-12 ($\delta_H$ 3.38 ppm) with H-17b ($\delta_H$ 4.94 ppm) supported H-12 to be β-oriented, and hence OCH$_3$-12 to be α-oriented. Based on the above spectroscopic evidence, compound **1**, 12α-methoxy-*ent*-kaur-9(11),16-dien-19-oic acid, (Figure 1) was identified as (4*R*,4a*S*,6a*S*,9*R*,10*S*,11b*R*)-10-methoxy-4,11b-dimethyl-8-methylene-1,2,3,4,4a,5,6,7,8,9,10,11b-dodecahydro-6a,9-methanocyclohepta[*a*]naphthalene-4-carboxylic acid. This compound has previously been reported both as synthetic derivative [13] and as a natural product [14,15]. However,

our NMR data assignment differs from that reported in the literature [13] for its C-3 and C-7. The accuracy of the corrected assignment, given in Table 1, is corroborated by the HMBC correlations of H-3 and H-7 (Table 1), by the HSQC crosspeaks of $CH_2$-3 ($\delta_H$ 0.93/2.08) to C-3 ($\delta_C$ 38.1) along with the TOCSY(Total Correlation Spectroscopy) correlations of $CH_2$-3 to $CH_2$-1 ($\delta_H$ 1.14/1.90) and $CH_2$-2 ($\delta_H$ 1.43/1.79), and by the HSQC (Heteronuclear Single Quantum Correlation) crosspeaks of $CH_2$-7 ($\delta_H$ 1.42/1.95) to C-7 ($\delta_C$ 28.9) along with the TOCSY correlations of $CH_2$-7 to $CH_2$-6 ($\delta_H$ 1.82/2.43) and H-5 ($\delta_H$ 1.56) (Figures S4–S6, Supplementary Material). Besides the compound having been reported earlier, it is unlikely to be an extraction artifact as the extraction (with $CH_2Cl_2$/MeOH, 1:1) has been performed at low temperature at neutral pH that does not promote formation of methyl ethers. The compound has been detected in the crude extract indicating that the compound is a natural product and has not formed during the chromatographic isolation.
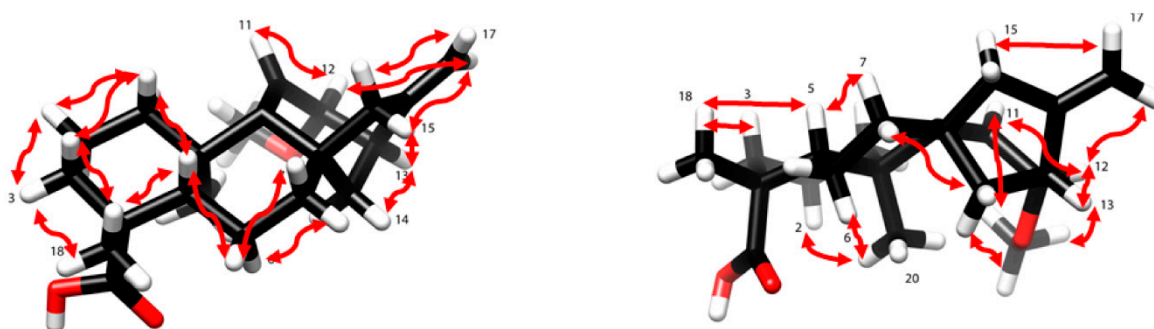


**Figure 2.** Some of the key NOE correlations observed for compound **1**. The NOESY (Nuclear Overhauser Effect Spectroscopy) spectrum (800 MHz, CDCl$_3$, 25 °C, 700 ms mixing time) is shown in the Supplementary Materials.

Additional compounds (Figures 1 and 3) isolated from the roots of *Aspilia pluriseta* include (16*R*)-hydroxy-*ent*-kauran-19-oic acid (**2**) [16,17], 9β-hydroxy-15α-angeloyloxy-*ent*-kaur-16-en-19-oic acid (**3**) [18], methyl-9β-hydroxy-15α-angeloyloxy-*ent*-kaur-16-en-19-oate (**4**) [19], 15α-angeloyloxy-16β,17-epoxy-*ent*-kauran-19-oic acid (**5**) [20], *ent*-kaur-9(11),16-dien-19-oic acid (**6**) [21], 15α-angeloyloxy-*ent*-kaur-16-en-19-oic acid (**7**) [22], *ent*-kaur-9(11),16-dien-12-one (**9**) [23] and methyl-*ent*-kaur-16-en-19-oate (**10**) [24]. The aerial part of *Aspilia pluriseta* contained *ent*-kaur-16-en-19-oic acid (**11**) [21,25], *ent*-kaur-16-en-19-ol (**12**) [26], lanosterol (**13**) [27], stigmasta-5,22(E)-dien-3β-ol (**14**) [28], 3β-hydroxy-olean-12-en-29-oic acid (**15**) [29], and carissone (**16**) [30].

Similar phytochemical investigation of the roots of *Aspilia mossambicensis* resulted in the isolation of methyl-15α-angeloyloxy-*ent*-kaur-16-en-19-oate (**17**) [24,31], 12-oxo-*ent*-kaur-9(11),16-dien-19-oic acid (**18**) [32], (16S)-*ent*-kauran-19-oic acid (**8**) [33], oleanolic acid (**19**) [34] (Figure 3), and compounds **3**–**5**. The aerial part of *A. mossambicensis* afforded compound **6** [21,26], 3β-acetyloxy-olean-12-ene (**20**) [35,36] *ent*-kaur-9(11),16-diene (21) [37], 15a-hydroxy-kaur-9(11),16-diene (**22**) [38], and methyl cinnamate (**23**) [39].

The crystal structures for compounds **3**–**8** (Figure 4) are also reported here, where the identities of the compounds were confirmed, and the absolute configurations established. In the crystal state, compound **3** exhibited a continuous network, involving intermolecular O(9)-H···O(19a) and O(19b)-H···O(21) hydrogen bonds. Similar O(9)-H···O(19a) hydrogen bonding motif was found in compound **4**, which also showed static disorder exhibiting two different spatial orientations of (Z)-2-methylbut-2-enoyl group in an approximately 1:1 ratio. Compounds **5**–**8** showed common double hydrogen bonding motifs for carboxylic acids leading to the formation of hydrogen-bonded pairs.
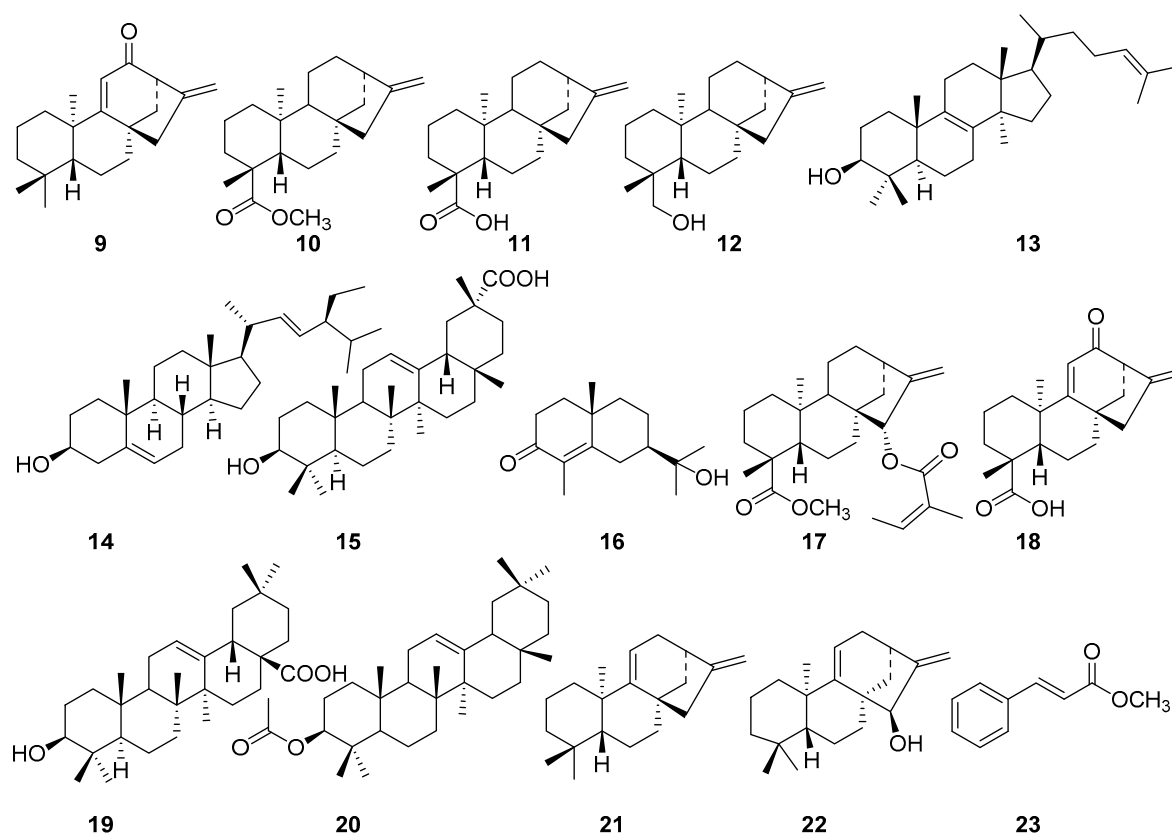
**Figure 3.** Structures of compounds **9–23**.

Crystal structure of compound **6** was determined at 120 K, which corresponds to the previously reported structure at room temperature [40]. Crystal structure analysis of a synthetic derivative of compound **6** led to the identification of *ent*-kaur-9(11)-en-19-oic acid (**6b**). In the structure **6b**, there is a slight (~10%) disorder in the main ring system, where carbons C7–C17 have different positions giving a shade of different conformations for the corresponding aliphatic rings. The data quality for compound **7** is slightly deficient and the absolute structure of it could not be justified on the basis of data. The X-ray diffraction data parameters, thermal ellipsoid diagrams, and hydrogen bonding geometries are presented in the Supplementary Information section of this article.

In most of the *ent*-kaurane-type diterpenoids (except for compounds **9**, **21**, and **22**) isolated from the two *Aspilia* species, *A. pluriseta* and *A. mossambicensis*, the α-methyl group at C-4 (C-19) is oxidized into carboxylic acid or methyl ester groups, which could be a characteristic feature of the genus *Aspilia* [3,8]. There are also examples where oxidation has occurred at C-12 (compounds **1**, **9** and **18**), C-16 (compound **2**), C-9 (compounds **3** and **4**), and C-15 (compounds **4**, **5**, **6**, and **18**). In agreement with the literature [36], oxidation has not been observed at other carbon atoms in *ent*-kaurane-type diterpenoids of *Aspilia* species.

Some *ent*-kaurane-type diterpenoids, including 16,17-epoxy-15β-tigloyloxy-*ent*-kauran-18-oic acid (**5a**) and 16,17-epoxy-15β-senecioyloxy-*ent*-kauran-18-oic acid (**5b**), were reported earlier from *Aspilia pluriseta* [8]. These compounds were reported to have the 15β-tigloyloxy and 15β-senecioyloxy groups, respectively, occupying the less favorable orientation [8]; however, the authors have not provided evidence for these proposals. Our single crystal X-ray analyses have shown that the C-15 substituent of compounds **3**, **4**, **5**, and **7** is an angeloyloxy group occupying the more favourable-15α-position (Figure 4). In fact, the proposed stereochemical assignment of *ent*-kaurane-type diterpenoids reported from this genus, particularly in highly functionalized compounds, lacks evidence. We have filled this knowledge gap by determining the absolute configuration of seven *ent*-kaurane-type diterpenoids, as shown in Figure 4, using single crystal X-ray analyses.
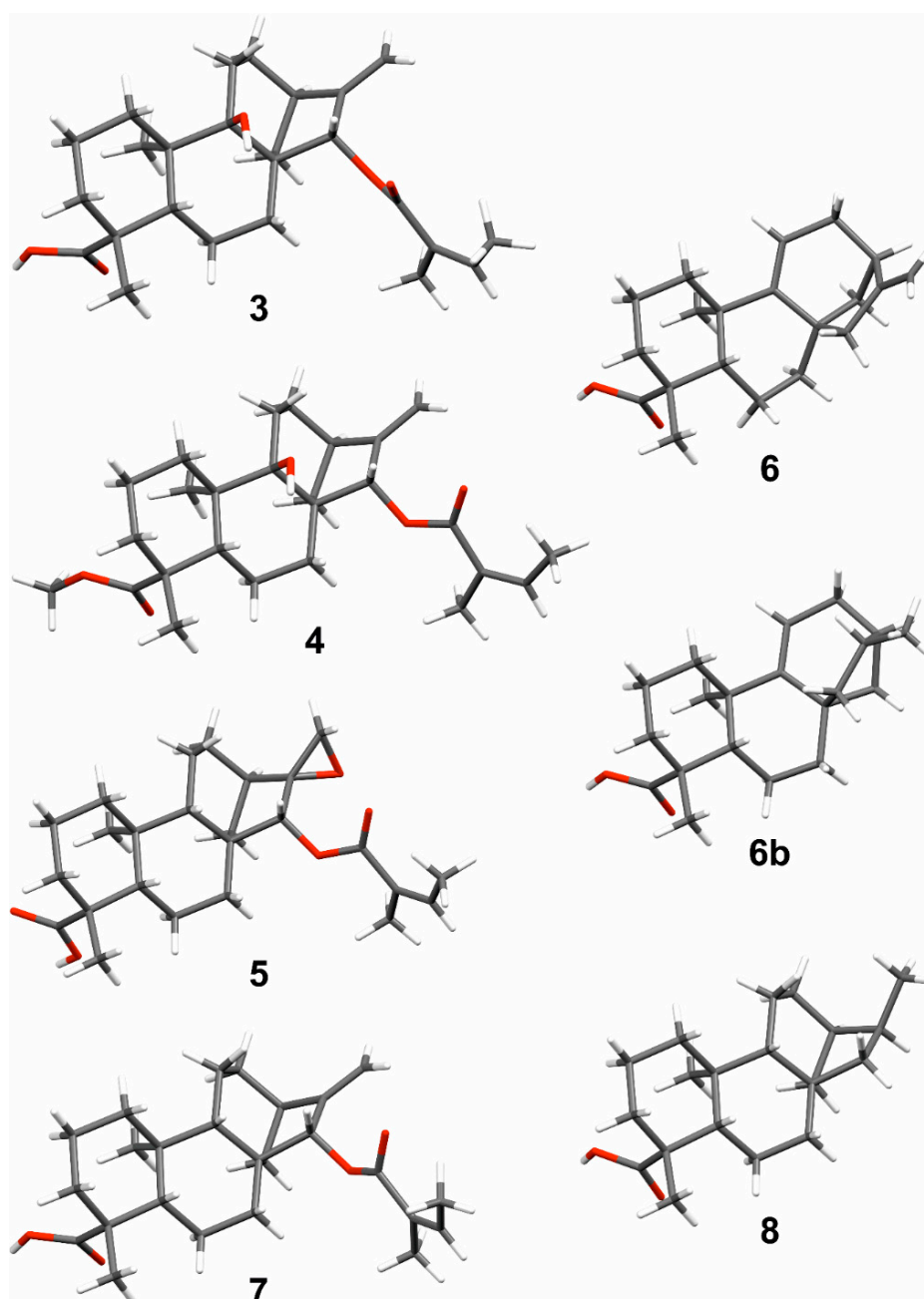
**Figure 4.** Crystal structure representations of compounds **3–8**. The structures were deposited with the following CCDC (Cambridge Crystallographic Data Centre) codes: **3** (1868318), **4** (1868319), **5** (1868321), **6** (1868320), **6b** (1868324), **7** (1868323), and **8** (1868322).

The $^{13}$C-NMR data of compound **5** (Table 2) is in close agreement to that previously reported in the literature [20], except for the chemical shift assignment of C-2 and C-12 (Table 2). Related structures, **5a** and **5b**, have been proposed for two compounds earlier reported [8] from *Aspilia pluriseta.* The $^{13}$C-NMR assignment (Table 2) for these compounds differs from our assignment, which is based on 2D NMR correlations, despite the common 16,17-epoxy-15-oxy-*ent*-kauran-18-oic acid skeleton (Figure 1). The $^{13}$C-NMR chemical shifts of C-4' ($\delta_C$ 27.4) and C-5' ($\delta_C$ 20.8) in compound **5a** do not support a tigloyloxy group at C-15, as proposed in Reference [8]; methyl carbon atoms in such group are expected to resonate at ~14 ppm (for C-4') and at ~11 ppm (for C-5'), based on chemical shift prediction [41] and previous literature [14]. The NMR spectra of compound **5b** that are given in the supporting information of [8] are of low quality and do not allow confirmation of the proposed

assignment. It should be noted that the numbering used in this paper does not follow the literature convention [3]. Hence, the carboxylic group of **5b** and of its structural analogues should not be assigned as C-18, but rather as C-19, following reference [3]. Overall, several details reported [8] for these compounds appear debatable, and consequently so are the proposed structures. To avoid such uncertainties, the NMR assignments of all compounds discussed in this paper are presented in Tables S1 and S2 in the Supplementary Material.

**Table 2.** The literature reported NMR data for **5**, **5a,** and **5b** and the $^1$H (800 MHz) and $^{13}$C-NMR (200 MHz) data for compound **5** acquired in CDCl$_3$.

| Position | 5 [20] | 5a [8] | 5b [8] | 5 | 5 |
|---|---|---|---|---|---|
| | $\delta_C$ | $\delta_C$ | $\delta_C$ | $\delta_C$ | $\delta_H$, mult. (*J* in Hz) |
| 1 | 41.2 | 40.6 | 40.6 | 40.6 | 0.80 *ddd* (7.2, 7.1, 1.3)<br>1.86* *dd* (2.9, 1.4) |
| 2 | 28.9 | 19.8 | 19.0 | 19.7 | 1.55 *ddd* (7.3, 3.6, 2.4)<br>1.75 *dd* (3.7, 3.6) |
| 3 | 37.7 | 36.7 | 36.4 | 37.6 | 0.96 *ddd* (13.7, 13.6, 4.3)<br>2.11 *dd* (13.7, 3.1) |
| 4 | 43.6 | 46.9 | 47.8 | 43.5 | |
| 5 | 56.7 | 20.3 | 56.6 | 56.5 | 1.16 *dd* (9.1, 7.1) |
| 6 | 19.0 | 41.2 | 20.3 | 20.8 | 1.76 *ddd* (5.7, 3.4, 2.1)<br>1.86* *ddd* (3.4, 3.4, 2.7) |
| 7 | 35.4 | 47.8 | 41.2 | 35.3 | 1.25 *ddd* (14.4, 13.9, 4.4)<br>1.79 *ddd* (13.8, 13.2, 4.3) |
| 8 | 47.9 | 52.9 | 43.6 | 47.8 | |
| 9 | 52.9 | 43.6 | 53.0 | 52.8 | 1.28 *dd* (13.8, 3.8) |
| 10 | 39.8 | 56.6 | 39.8 | 39.7 | |
| 11 | 19.8 | 20.8 | 19.8 | 18.9 | 1.40 *ddd* (13.8, 3.4, 3.4, 3.1)<br>1.81 *dd* (13.8, 4.3) |
| 12 | 20.8 | 28.9 | 28.9 | 28.8 | 1.50 *ddd* (13.5, 7.8, 7.2) |
| 13 | 41.2 | 36.4 | 35.1 | 41.1 | 1.82 *dd* (13.8, 4.4) |
| 14 | 36.5 | 37.7 | 37.7 | 36.4 | 1.68 *dd* (14.5, 3.3)<br>1.97 *dd* (13.1, 3.4) |
| 15 | 81.9 | 81.2 | 81.2 | 81.9 | 4.73 *br s* |
| 16 | 66.3 | 66.4 | 66.4 | 66.3 | |
| 17 | 49.6 | 49.6 | 49.6 | 49.6 | 2.78 *dd* (5.6, 1.3)<br>3.09 *dd* (5.8, 1.3) |
| 18 | 28.8 | 28.9 | 28.9 | 28.7 | 1.28 *s* |
| 19 | 182.3 | 182.6 | 182.6 | 182.7 | |
| 20 | 15.7 | 15.8 | 16.0 | 15.9 | 1.03 *s* |
| 1' | 167.9 | 166.5 | 166.5 | 167.8 | |
| 2' | 128.1 | 129.0 | 115.9 | 128.0 | |
| 3' | 137.3 | 137.1 | 156.8 | 137.3 | 5.96 *q* (7.1) |
| 4' | 15.9 | 27.4 | 20.8 | 15.7 | 1.96 *d* (1.9) |
| 5' | 20.6 | 20.8 | 27.4 | 20.6 | *s* |

Most of the compounds isolated in this study were assayed against two normal (BEAS-2B and LO$_2$) and two cancer (A549 and Hep-G2, Table 3) cell lines. Of the fifteen compounds tested, **1**, **3**, **5**, **9,** and **18** showed cytotoxicity towards some of the cell lines. Compound **1** showed selective activity against the human hepatoma (HepG2) cancer cell line without significant toxicity to other cell lines (IC$_{50}$ above 100 µM). Compound **3** was moderately cytotoxic against the Hep-G2 cancer cell line (IC$_{50}$ = 24.7 ± 2.8), but also showed weak cytotoxicity towards the normal cell lines LO$_2$ (IC$_{50}$ = 57.2 ± 1.2) and BEAS-2B (IC$_{50}$ = 89.9 ± 2.0 µM), indicating low selectivity. Compound **5** (IC$_{50}$ = 30.7 ± 1.7 µM) was the most active against the A549 cell line, followed by compound **18** (IC$_{50}$ = 80.5 ± 1.8 µM). The other compounds tested, i.e., **2**, **4**, **6**, **7**, **10**, **11**, **14,** or **17**, did not exhibit significant cytotoxicity (IC$_{50}$ above 100 µM). The lack of cytotoxicity against the two normal cell lines of these diterpenoids is valuable information, considering that some kaurane-type diterpenoids from *Aspilia* species have showed antibacterial and antifungal effects [5].

**Table 3.** Cytotoxicity (IC$_{50}$, μM) of compounds isolated from *Aspilia* species against various cell lines.

| Compound | Normal Cell Lines | | Cancer Cell Lines | |
| --- | --- | --- | --- | --- |
| | **BEAS-2B** | **LO$_2$** | **A549** | **Hep-G2** |
| **1** | >100 | >100 | >100 | 27.3 ± 1.9 |
| **2** | >100 | >100 | >100 | >100 |
| **3** | 89.9 ± 2.0 | 57.2 ± 1.2 | >100 | 24.7 ± 2.8 |
| **4** | >100 | >100 | >100 | >100 |
| **5** | >100 | >100 | 30.7 ± 1.7 | >100 |
| **6** | >100 | >100 | >100 | >100 |
| **6a** | >100 | >100 | >100 | >100 |
| **6b** | >100 | >100 | >100 | >100 |
| **7** | >100 | >100 | >100 | >100 |
| **9** | >100 | 75.3 ± 2.8 | >100 | >100 |
| **10** | >100 | >100 | >100 | >100 |
| **11** | >100 | >100 | >100 | >100 |
| **14** | >100 | >100 | >100 | >100 |
| **17** | >100 | >100 | >100 | >100 |
| **18** | 38.6 ± 2.5 | 30.0 ± 1.7 | 80.5 ± 1.8 | 81.3 ± 0.3 |
| Paclitaxel | <0.1 | <0.1 | 0.0033 | 0.19 |

## 3. Materials and Methods

### 3.1. General Experimental Procedures

NMR spectra were acquired on a Bruker Avance II 600 MHz, a Bruker Avance III HD 800 MHz (Bruker BioSpin AG, Fällanden, Switzerland) or a Varian Unity 500 MHz (Varian Inc, Palo Alto, CA, USA) NMR spectrometer, using the residual solvent peaks as a reference. The spectra were processed using the software MestReNova (version 10.0, Mestrelab Research S.L., Santiago de Compostela, Spain) Coupling constants (*J*) are given in Hz. EI-MS and LC-MS were carried out using 70 eV ionization electron voltage on a Micromass GC-TOF spectrometer (Micromass, Wythenshawe, Waters Inc., UK). TLC (Thin Layer Chromatography) was carried out on Merck pre-coated silica gel 60 F$_{254}$ plates (Merck, Darmstadt, Germany). Preparative TLC was performed on 20 × 20 cm glass plates, pre-coated with silica gel 60F$_{254}$ with thicknesses of 0.25 to 1 mm. Column chromatography was run on silica gel 60 Å (70–230 mesh). Gel filtration was performed on Sephadex LH-20(Merck, Darmstadt, Germany).

### 3.2. X-ray Diffraction Analyses

The single crystal X-ray diffraction data were collected using Agilent Super-Nova (Agilent Technologies, Wrocław, Poland) dual wavelength diffractometer with a micro-focus X-ray source and multilayer optics monochromatized Cu-Kα (λ = 1.54184 Å) radiation. Program *CrysAlisPro* [42] was used for the data collection and reduction. The intensities were corrected for absorption using analytical face index absorption correction method. The structures were solved with intrinsic phasing method (*SHELXT* [43]) and refined by full-matrix least squares on $F^2$ with *SHELXL-2018/3* [44]. Anisotropic displacement parameters were assigned to non-H atoms. All C-H hydrogen atoms were refined using riding models. Hydroxy hydrogens were found from electron density maps and restrained to the proper distance from oxygen atom (0.84 Å). All hydrogen atoms were refined with $U_{eq}$(H) of 1.5 × $U_{eq}$(C,O) for hydroxy and terminal methyl groups or 1.2 × $U_{eq}$(C) for other C-H groups. Further geometric least-squares restraints (s = 0.02) were applied to structures **4**, **6b,** and **7** to obtain more chemically reasonable bond distances between disordered atoms. Anisotropic displacement parameters of few disordered or terminal atoms were restrained (s = 0.01, st = 0.02) to be more equal in structures **3**, **4**, **6b,** and **7**. CCDC 1868318-1868324 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk).

### 3.3. Plant Materials

The roots and aerial parts of *Aspilia pluriseta* and *Aspilia mossambicensis* (Asteraceae) were collected from Ngon'g Forest, Kenya. The plants were identified by Mr. Patrick B. Chalo Mutiso, of the Herbarium, School of Biological Sciences, University of Nairobi, Kenya, where voucher specimens (SY2015/04 for *Aspilia pluriseta,* and SY2015/05 for *Aspilia mossambicensis*) were deposited.

### 3.4. Extraction, Isolation and Derivatization

The air-dried and ground roots of *A. pluriseta* (0.8 kg) were extracted with $CH_2Cl_2$/MeOH (1:1) for 24 h affording 47 g of extract. A portion of the extract (35 g) was subjected to column chromatography on silica gel (700 g) and eluted with *hexane* containing increasing amounts of EtOAc. The fraction eluted with 10% EtOAc in *hexane* was purified by crystallization from acetone affording compound **1** (36 mg) [9]. Crystallization (from acetone) of the combined fractions eluted with 5–10% EtOAc in *hexane* afforded compound **2** (47 mg) [12,13]. Preparative TLC separation of the fraction eluted with 15% EtOAc in *hexane* yielded 22 mg of compound **3** [14] and 31 mg of compound **4** [15]. Compound **5** (97 mg) [16] was obtained from the fraction eluted with 30% EtOAc in *hexane*, after purification over Sephadex LH-20 ($CH_2Cl_2$/MeOH; 1:1). Fractional crystallization (from acetone) of the combined fractions eluted with 35–40% EtOAc in *hexane* afforded 218 mg of compound **6** [17] and 89 mg of compound **7** [18]. Purification of the fraction obtained with 60% EtOAc in *hexane* on Sephadex LH-20 (eluted with $CH_2Cl_2$/MeOH; 1:1) led to the isolation of *ent*-kaur-9(11),16-dien-12-one (**9**, 27 mg) [19] and methyl-*ent*-kaur-16-en-19-oate (**10**, 36 mg) [20]. The isolated compounds have been observed in the crude extract, ahead of chromatographic separation, by TLC. This confirmed their presence in the crude extracts, and thus, these are natural products and were not formed during the chromatographic separation.

The air-dried and ground aerial part of *A. pluriseta* (0.8 kg) was extracted, as described above, giving 53 g of crude extract. A portion of the extract (40 g) was subjected to column chromatography on silica gel (800 g) and eluted with *hexane* containing increasing amounts of EtOAc. The fractions eluted with 5–15% EtOAc in *hexane* were combined and purified on Sephadex LH-20 (eluted with $CH_2Cl_2$/MeOH; 1:1) affording lanosterol (**13**, 58 mg) [27] and stigmasta-5,22(E)-dien-3β-ol (**14**, 71 mg) [28]. Elution of the main silica gel column with 20% EtOAc in *hexane* afforded 42 mg of 3β-hydroxy-olean-12-en-29-oic acid (**15**) [29]. The fraction eluted with 30% EtOAc in *hexane* led to the isolation of *ent*-kaur-16-en-19-oic acid (**11**, 367 mg) [17,21] and *ent*-kaur-16-en-19-ol (**12**, 32 mg) [8,26]. Purification of the fraction eluted with 40% EtOAc in *hexane* over Sephadex LH-20 ($CH_2Cl_2$/MeOH; 1:1) yielded carissone (**16**, 26 mg) [30].

The air-dried and ground roots (0.7 kg) of *Aspilia mossambicensis* were extracted with $CH_2Cl_2$/MeOH (1:1) by cold percolation (4 × 24 h) to give gummy brown extract (47 g). A portion of the extract (40 g) was subjected to column chromatography on silica gel (800 g) and eluted with *hexane* containing increasing amounts of EtOAc. Fractional crystallization of the eluent with 25% EtOAc in *hexane* led to the isolation of 64 mg of methyl-15α-angeloyloxy-*ent*-kaur-16-en-19-oate (**17**) [24,31] and 23 mg of 12-oxo-*ent*-kaur-9(11), and 16-dien-19-oic acid (**18**) [32]. Purification of the mother liquor on Sephadex LH-20 resulted in the separation of (16S)-*ent*-kauran-19-oic acid (**8**, 17 mg) 33] and oleanolic acid (**19**, 28 mg) [34]. Elution of the column with 40% EtOAc in *hexane* led to the isolation of additional amounts of compounds **3** (14 mg), **4** (7 mg), and **5** (10 mg).

The aerial part (1.0 kg) of *A. mossambicensis* was extracted as above, yielding 82 g of crude extract. A portion of this extract (40 g) was subjected to column chromatography over silica gel (800 g) and eluted with *hexane* containing increasing amounts of EtOAc. Elution of the column with 20% EtOAc in *hexane* led to the isolation of 24 mg of 3β-acetyloxy-olean-12-ene acetate (**20**) [35,36]; elution with 25% EtOAc in *hexane* afforded 73 mg of *ent*-kaur-9(11),16-diene (**21**) [39]. Elution with 30–40% EtOAc in *hexane* resulted in a mixture of two compounds. These were separated by column chromatography on Sephadex LH-20 (eluted with $CH_2Cl_2$/MeOH; 1:1), yielding 21 mg of 15α-hydroxy-kaur-9(11),16-diene (**22**) [38], and 15 mg of methyl cinnamate (**23**) [40].

Derivatization of compound **6**. A concentrated $H_2SO_4$ (2 drops) was added to MeOH (2 mL) solution of compound **6** (0.05 g, 0.165 mmol). The reaction mixture was stirred at 25 °C for 18 h, after which the mixture was poured onto water (20 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic layer was dried over $MgSO_4$, and was then filtered and concentrated in a rotary evaporator, giving 37 mg of **6a** [45].

To hydrogenate *ent*-kaur-9(11),16-dien-19-oic acid (**6**), a solution was obtained by dissolving 100 mg in 20 mL ethanol, in a 50 mL round-bottomed flask, and 5% palladium on charcoal (30 mg) was then added to the solution. The flask was sealed with an airtight rubber stopper. Nitrogen gas was bubbled through the solution to eliminate traces of air using a small syringe inserted through the rubber stopper. Hydrogen gas was then bubbled into the system for 2 days at room temperature. The reaction mixture was filtered and the solvent evaporated yielding compound **6b** (87 mg).

*12α-Methoxy-ent-kaur-9(11),16-dien-19-oic Acid* (**1**) colorless crystals, m.p. 158%–160 °C. $[\alpha]_D^{20}$ −88 (*c* 0.25, acetone), $^1H$ and $^{13}C$-NMR ($CD_2Cl_2$) data (Table 1). ESIMS, *m/z* (rel. int.) 329 (12, [M–H]$^-$), 315 (11), 299 (100), 253 (71), 281 (10), 171 (18), 182 (3). HRMS [M–H]$^-$ *m/z* 329.2191 $C_{21}H_{29}O_3$ (Calculated: 329.2117).

*15α-Angeloyloxy-ent-kaur-16α,17-epoxy-ent-kauran-19-oic Acid* (**5**). Colorless crystals, m.p. 242–243 °C. $^1H$ and $^{13}C$-NMR ($CDCl_3$) data, see Table 2. ESIMS, *m/z* (rel. int): 417 (100, [M+H]$^+$), 317 (80), 299 (71), 271 (64), 253 (23).

### 3.5. Cell Culture

Reagent and cells. Adenocarcinomic human alveolar basal epithelial (A549) and human hepatoma (HepG2) cancer cell lines, immortalized normal human liver (LO$_2$), human bronchial epithelial (BEAS-2B), and fibroblast-like CCD19Lu cells were purchased from ATCC (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics: Penicillin (50 U/Ml) and streptomycin (50 μg/mL; Invitrogen, Paisley, Scotland, UK). All cells were incubated at 37 °C in a 5% humidified $CO_2$ incubator). All test compounds were dissolved in DMSO at a final concentration of 50 mM and stored at −20 °C before use.

### 3.6. Cytotoxicity Assay

Cytotoxicity was assessed using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) assay, as described previously [46]. Briefly, $5 \times 10^3$ cells per well were seeded in 96-well plates before drug treatments. After overnight cell culture, the cells were exposed to different concentrations of selected compounds (0.19–100 μM) for 72 h. Cells without drug treatment were used as controls. Subsequently, 10 μL of 5 mg/mL MTT solution was added to each well and incubated at 37 °C for 4 h, followed by the addition of 100 μL solubilization buffer (12 mM HCl in a solution of 10% SDS) and overnight incubation. The absorbance, $A_{570}$ nm, was then determined in each well on the next day. The percentage cell viability was calculated using the expression: % Viability = $A_{treated}$/ $A_{control}$ × 100, and was given as cytotoxicity in Table 3.

## 4. Conclusions

Twenty-three compounds, mostly *ent*-kaurane-type diterpenoids, were isolated from *Aspilia pluriseta* and *A. mossambicensis*. Besides giving a full NMR assignment, the absolute configuration of seven of the isolated compounds was established by single crystal X-ray diffraction analyses. The isolated compounds were tested for their cytotoxicity against four cell lines. Compounds **1**, **3**, **5**, **9**, and **18** showed moderate to weak cytotoxicity against the cell lines. Compound **3** was the most cytotoxic (IC$_{50}$=24.7 ± 2.8 μM) against the human hepatoma (Hep-G2) cancer cell line without toxicity against the tested normal cell lines.

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**Sample Availability:** Samples of the compounds are available from the authors.