

## Structure characterization and biological activity of 2-arylbenzofurans from an Indonesian plant, *Sesbania grandiflora* (L.) Pers

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### ABSTRACT

A new 2-arylbenzofuran, sesbagrandiflorain C (1), together with four known compounds, 2-(3,4-dihydroxy-2-methoxyphenyl)-4-hydroxy-6-methoxybenzofuran-3-carbaldehyde (2), 2-(4-hydroxy-2-methoxyphenyl)-5,6-dimethoxybenzofuran-3-carboxaldehyde (3), sesbagrandiflorain A (4) and sesbagrandiflorain B (5), have been isolated from the stem bark of an Indonesian plant, *Sesbania grandiflora* (L.) Pers. The chemical structure of compound 1 was elucidated by UV, IR, MS, and NMR spectroscopic techniques. The proton and carbon NMR resonances of 1 were also compared with the predicted chemical shifts obtained from DFT quantum mechanical calculations with Gaussian. None of the compounds showed antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in an agar diffusion assay. However, sesbagrandiflorains A (4) and B (5) exhibited moderate activity against *Mycobacterium tuberculosis* H37Rv. In addition, compounds 1–5 have moderate cytotoxicity against HeLa, HepG2, and MCF-7 cancer cell lines.

### 1. Introduction

The Fabaceae is the third largest and one of the most economically important families of flowering plants. Many fabaceous plants, particularly those from the Papilionoideae subfamily, are frequently used as traditional medicines to treat illnesses such as diabetes, cough, urinary problems, eye diseases, lung diseases, toothache, fever, dysentery and other infections (Neto et al., 2008; Roosita et al., 2008; Vitor et al., 2004; Watjen et al., 2007). Members of this family of plants have been known to produce alkaloids, non-proteinogenic amino acids, anthraquinones, coumarins, cyanogenic glycosides, flavonoids, isoflavonoids, phenylpropanoids, and terpenoids (Wink and Mohamed, 2003; Kobayashi et al., 1996, 1997; Kitagawa et al., 1996). Among them, isoflavonoids are mainly identified in plants from the Papilionoideae subfamily (Kirmizibekmez et al., 2015). They demonstrate a wide-range

of bioactivities, such as anti-microbial, anti-insecticidal, and allelopathic activities (Dixon and Sumner, 2003).

Among members of the Papilionoideae subfamily is *Sesbania grandiflora*, a flowering plant that is native to tropical Asia including Indonesia. Different parts of this plant have been used as herbal medicines to treat anemia, bronchitis, fever, and tumors (Wagh et al., 2009; Laladhas et al., 2010; Powell et al., 1984). Earlier studies on the leaves, seeds, and roots of *S. grandiflora* showed the presence of various secondary metabolites, e.g.,  $\alpha$ -5-methyl-5-pentacosanol, galactomannan, and flavonoids, some of which exhibited antituberculosis activity against *Mycobacterium tuberculosis* H37Rv (Tiwari and Bajpai, 1964; Pollard et al., 2011; Hasan et al., 2012; Noviany et al., 2012). Recently, we reported the isolation of two phenolic compounds, sesbagrandiflorains A (4) and B (5), from the stem bark of *S. grandiflora* (Noviany et al., 2018). However, their biological properties were unknown. Here,

**Abbreviations:** ESI-MS, electrospray ionization mass spectrometry; EtOAc, ethyl acetate; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HR-TOF-MS, high resolution time-of-flight mass spectrometry; HSQC, heteronuclear single quantum coherence; IR, infrared; *M. tuberculosis*, *Mycobacterium tuberculosis*; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; RP HPLC, reverse phase high performance liquid chromatography; TLC, thin layer chromatography; UV, ultraviolet

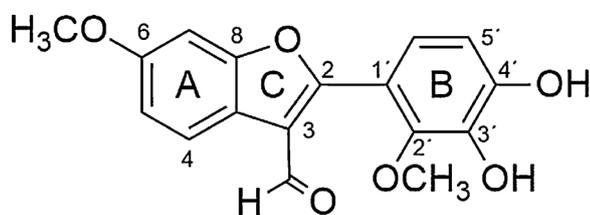
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## Sesbagrandiflorain C (1)

Fig. 1. Chemical structure of compound 1.

we report the isolation and structural characterization of other bioactive constituents in the EtOAc extract of the stem bark of *S. grandiflora* including a new 2-arylbenzofuran, sesbagrandiflorain C (1) (Fig. 1), and two known compounds, 2-(3,4-dihydroxy-2-methoxyphenyl)-4-hydroxy-6-methoxybenzofuran-3-carbaldehyde (2) and 2-(4-hydroxy-2-methoxyphenyl)-5,6-dimethoxybenzofuran-3-carboxaldehyde (3) (Fig. 1S, supplementary data). Compounds 4 and 5 were also isolated from this extract as major metabolites. Furthermore, we determined the antibacterial activity and the cytotoxicity of compounds 1–5, as well as the antituberculosis activity of compounds 4 and 5.

## 2. Results and discussion

The stem bark of *S. grandiflora*, collected from Sumberdadi Village, Pringsewu, Bandar Lampung, Indonesia, was macerated sequentially using *n*-hexane, EtOAc, and 90 % aqueous MeOH at room temperature. Repeated silica gel column chromatography of the EtOAc extract afforded a single spot on TLC, which gave high quality 1D and 2D NMR spectra (Figs. 2S–20S, supplementary data). Analysis of the NMR data showed two sets of resonances similar to those reported previously for sesbagrandiflorain A with equal intensities, suggesting a possibility of a dimer compound. However, high-resolution (+)-ESI-MS analysis of the compound showed signals corresponding to sesbagrandiflorain “monomers”, indicating that the sample contained two equal amounts of closely related sesbagrandiflorain analogs. Further purification using high-performance liquid chromatography (HPLC) provided a new 2-arylbenzofuran, sesbagrandiflorain C (1), and a previously reported related compound 2, which was isolated from the leaves of *Andira inermis* (Kraft et al., 2001).

Sesbagrandiflorain C (1) was isolated as a yellowish solid. Its molecular formula was established to be  $C_{17}H_{14}O_6$  ( $m/z$  315.08765 [ $M+H$ ]<sup>+</sup>) by HR-TOF-MS analysis. The <sup>1</sup>H NMR spectrum of 1 is similar to that of sesbagrandiflorain A (4) including a unique aldehyde proton at 10.05 ppm (Table 1). However, compound 1 lacks the two aromatic proton resonances with *J* values of ~2 Hz (typical for meta aromatic protons) found in 4. Instead, compound 1 has two additional aromatic proton resonances ( $\delta_H$  6.87; 7.10 ppm) with *J* values of 8.0 Hz, indicating different positions of aromatic substitutions in 1. The <sup>13</sup>C NMR spectrum of 1 exhibited the existence of two methyl carbons, five sp<sup>2</sup> aromatic ring carbons, nine oxygenated/non-oxygenated quaternary carbons and a carbonyl carbon ( $\delta_C$  186.8) (Table 1). Detailed analysis of 2D NMR data [<sup>1</sup>H-<sup>1</sup>H COSY, HSQC, HMBC and NOESY (Fig. 2) and (Figs. 8S–11S, supplementary data)] for 1 revealed that the A and C rings of 1 are identical to those in 4, whereas the B ring is different. While no HMBC correlations were observed to connect the C-2 and C-3 fragments in the C-ring of 1, direct comparisons of the NMR data with those for 2–5 revealed that 1 also has a benzofuran-3-carbaldehyde skeleton. HMBC correlations between the OCH<sub>3</sub> protons ( $\delta_H$  3.73) and C-2' ( $\delta_C$  147.1) as well as between H-6' ( $\delta_H$  7.10) and C-2' ( $\delta_C$  147.1) indicate that the OCH<sub>3</sub> group is located at C-2' (Fig. 2). All together the data support the chemical structure of 1 to be 2-(3,4-dihydroxy-2-methoxyphenyl)-6-methoxybenzofuran-3-carbaldehyde (Fig. 1).

Table 1  
<sup>1</sup>H and <sup>13</sup>C NMR data for compounds 1–3.

Position	1		2		3	
	$\delta_C^a$	$\delta_H^c$	$\delta_C^a$	$\delta_H^c$	$\delta_C^b$	$\delta_H^c$
2	162.9	–	163.8	–	162.5	–
3	117.1	–	118.4	–	117.1	–
4	122.0	8.04 (d, <i>J</i> = 8.5)	156.6	–	103.3	7.65 (s)
5	113.2	7.03 (dd, <i>J</i> = 8.5, 2.0)	98.1	6.37 (d, <i>J</i> = 2.0)	148.0	–
6	159.0	–	161.3	–	149.0	–
7	95.7	7.23 (d, <i>J</i> = 2.0)	87.7	6.73 (d, <i>J</i> = 2.0)	95.4	7.25 (s)
8	155.5	–	152.1	–	149.1	–
9	118.3	–	106.9	–	117.3	–
1'	113.6	–	113.2	–	108.9	–
2'	147.1	–	146.9	–	159.0	–
3'	138.8	–	138.8	–	99.7	6.78 (d, <i>J</i> = 2.0)
4'	149.5	–	149.9	–	161.8	–
5'	111.6	6.87 (d, <i>J</i> = 8.0)	111.7	6.88 (d, <i>J</i> = 7.0)	108.0	6.68 (dd, <i>J</i> = 8.5, 2.0)
6'	122.1	7.10 (d, <i>J</i> = 8.0)	122.2	7.13 (d, <i>J</i> = 7.0)	132.5	7.50 (d, <i>J</i> = 8.5)
MeO-C(6)	55.2	3.91 (s)	55.3	3.86 (s)	55.2	3.87 (s)
MeO-C(2')	60.4	3.73 (s)	60.6	3.76 (s)	61.4	3.90 (s)
MeO-C(5)	–	–	–	–	55.6	3.91 (s)
HO-4	–	–	–	10.18 (s)	–	–
CHO	186.8	10.05 (s)	190.3	9.83 (s)	186.9	10.03 (s)

<sup>a</sup> measured at 125 MHz.

<sup>b</sup> measured at 175 MHz.

<sup>c</sup> measured at 500 MHz.

To confirm the <sup>1</sup>H and <sup>13</sup>C NMR assignments of compound 1, we performed computational NMR chemical shifts prediction using DFT quantum mechanical calculations (Willoughby et al., 2014). Conformational analyses of 1 were performed in Gaussian 16 using DFT/B3LYP functional with the 6-31+G(d,p) basis set for geometry optimization and frequency calculations for the candidate structure. All conformers with a relative energy difference < 5 kcal/mol from the most stable conformer (Fig. 2d) were kept and carried forward for NMR analysis. NMR shielding tensors were computed with the GIAO (gauge-independent (or including) atomic orbitals) method in Gaussian 16 using the DFT/MPW1PW91 functional with the 6-311+G(2d,p) basis set, with acetone as the solvent, and the obtained tensor values were then converted to the predicted chemical shifts using the equation described in (Willoughby et al., 2014) (Table 1S, supplementary data). The mean absolute error (MAE) between the computed and the experimental data sets was 0.0825, which is within the “correct fit” cut off of 0.10 ppm (Willoughby et al., 2014).

The 2-arylbenzofuran-3-carbaldehyde 2 was isolated as a yellowish solid and the molecular formula was established to be  $C_{17}H_{14}O_7$  ( $m/z$  331.08261 [ $M+H$ ]<sup>+</sup>), suggesting the presence of an additional hydroxyl substitution in 2. This is consistent with the <sup>1</sup>H NMR spectrum of 2, in which only four aromatic protons are present as opposed to five protons in 1 (Table 1, Fig. 12S, supplementary data). The <sup>13</sup>C NMR spectrum of 2 showed resonances of two methyl carbons, four sp<sup>2</sup> aromatic ring carbons, ten oxygenated/non-oxygenated quaternary carbons and a carbonyl ( $\delta_C$  190.3 ppm) (Table 1, Fig. 13S, supplementary data). Further detailed assessment of the 2D NMR data [HSQC and HMBC (Figs. 14S and 15S, supplementary data)] for 2 as well as comparisons of its <sup>1</sup>H and <sup>13</sup>C NMR data with those reported in the literature (Kraft et al., 2001) revealed the chemical structure of 2 as depicted in Fig. 1S.

From another fraction of the EtOAc extract, we also isolated a known 2-arylbenzofuran compound, 2-(4-hydroxy-2-methoxyphenyl)-5,6-dimethoxybenzofuran-3-carboxaldehyde (3) (Fig. 1S, supplementary data), using a combination of SiO<sub>2</sub> column chromatography and

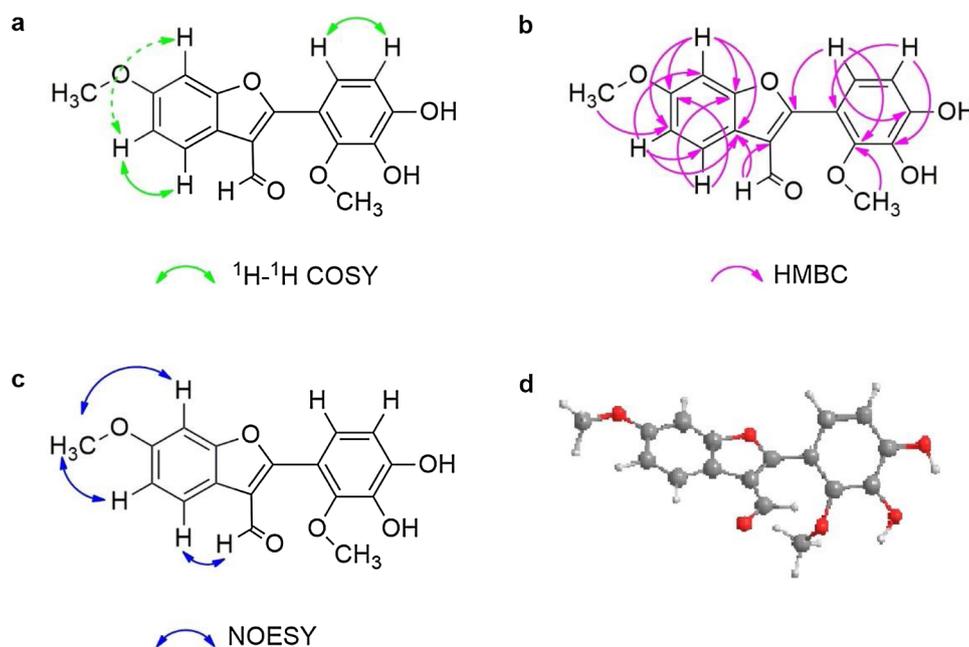


Fig. 2. 2D NMR and conformational analyses of compound 1. (a)  $^1\text{H}$ - $^1\text{H}$  COSY correlations; (b) HMBC correlations; (c) NOESY correlations; (d) the lowest energy conformation of compound 1 obtained from quantum mechanical calculations with Gaussian 16.

reversed-phase HPLC. This compound was previously isolated from the roots of *Ononis vaginalis* plants (Abdel-Kader, 2001). Compound 3 was acquired as a colorless solid. HR-TOF-MS analysis of 3 revealed a molecular formula of  $\text{C}_{18}\text{H}_{16}\text{O}_6$  ( $m/z$  329.10330  $[\text{M}+\text{H}]^+$ ), one carbon atom more than 1 or 2.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3 showed the existence of an additional methoxy group in 3. The complete chemical structure of 3 was determined based on its 1D and 2D NMR (HSQC, HMBC, and NOESY) data (Figs. 16S–20S, supplementary data) as well comparisons of its  $^1\text{H}$  and  $^{13}\text{C}$  NMR data with those reported in the literature (Abdel-Kader, 2001).

In addition, we have isolated the previously reported major metabolites sesbagrandiflorains A (4) and B (5) (Fig. 1S, supplementary data) (Noviany et al., 2018) from a different fraction of the EtOAc extract. Together, compounds 1–5 were tested for their microbial property against *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, and *Escherichia coli* using an agar diffusion assay. However, none of them showed any activity in this assay. The relatively abundant compounds 4 and 5 were then evaluated for their antituberculosis activity against *Mycobacterium tuberculosis* H37Rv by tetrazolium micro-plate assay (TEMA) (Hasan et al., 2012). The results showed that compounds 4 and 5 have moderate activity against *M. tuberculosis* with the minimum inhibition concentration (MIC) values of 200 and 12.5  $\mu\text{g}/\text{mL}$ , respectively. The greater activity of compound 5 compared to compound 4 suggests that a free hydroxy group at C-6 is important for their activity. However, a more detailed structure-activity relationship study is required to provide better understanding of their anti-TB activity. Additionally, compounds 1–5 were tested for their cytotoxicity against cancer cell lines HeLa (cervical adenocarcinoma), HepG2 (liver carcinoma), and MCF-7 (mammary adenocarcinoma). The results showed that 1–5 have moderate cytotoxicity against two or more of the tested cancer cell lines (Table 2).

### 3. Experimental

#### 3.1. General experimental procedures

TLC was done on silica gel 60 GF<sub>254</sub> plates (Merck; 0.25 mm) and sprayed with the staining reagent  $\text{Ce}(\text{SO}_4)_2$ . Column chromatography (CC) was performed using silica gel (Kieselgel 60, 70–230 mesh ASTM;

Table 2

Cytotoxicity of compounds 1–5 on a number of cancer cell lines.

Compound	CC <sub>50</sub> ( $\mu\text{M}$ )		
	HeLa	HepG2	MCF-7
1	> 100	8.25	35.1
2	31.5	25.4	10.2
3	31.5	> 100	9.0
4	31.3	30.8	0.65
5	30.5	31.3	21.3

Merck). Preparative TLC was conducted on square glass plates (Kieselgel F<sub>254</sub>; Merck). HPLC was carried out using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were measured in acetone- $d_6$  (TMS as an internal standard), on an Agilent 500 MHz spectrophotometer (Agilent Technologies) or Bruker Avance III 700 MHz spectrometer equipped with a 5 mm  $^{13}\text{C}$  NMR cryogenic probe or a Bruker 500 MHz spectrometer. HR-ESI-MS was carried out in positive ion mode on a 6230 TOF mass spectrometer (Agilent Technologies). IR spectra were produced using a Nicolet IR100 FT-IR spectrophotometer (Thermo Fisher Scientific). UV spectra were produced using an Eppendorf BioSpectrometer® kinetic instrument.

#### 3.2. Bacteria strains, cell lines and biochemicals

Middlebrook 7H9 broth and the albumin-dextrose-catalase (ADC) and oleic acid-albumin-dextrose-catalase (OADC) growth supplements were purchased from Difco. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] for biochemistry was purchased from Sigma-Aldrich. Tween-80 was purchased from Merck. Isoniazid was purchased from Duchefa Biochemie. *Mycobacterium tuberculosis* H37Rv ATCC 27294 was purchased from the American Type Culture Collection.

#### 3.3. Plant material

Samples of *S. grandiflora* stem bark were collected on 27 November

2016 from Sumberdadi Village, Pringsewu, Lampung Province, Indonesia. The plant specimen (NV5/NRGD/2016) was identified at the Herbarium Bogoriense, LIPI Bogor, Indonesia.

### 3.4. Extraction and isolation

The extraction and purification of the active metabolites were done according to the methods described in our previous paper (Noviany et al., 2018). Briefly, the air-dried plant material (3.0 kg) was macerated consecutively with *n*-hexane, EtOAc, and 90 % aqueous MeOH. The EtOAc extract (40 g) was fractionated using SiO<sub>2</sub> vacuum liquid chromatography (VLC) (35–70 Mesh), eluted with *n*-hexane–EtOAc gradient from 100 % *n*-hexane to 100 % EtOAc, to give fractions E1–E5. Fraction E4 (9.5 g) was then fractionated using VLC (silica gel, 100 % *n*-hexane to 100 % EtOAc) to yield sub-fractions E4.1–E4.9. Fraction E4.6 was subsequently subjected to VLC and SiO<sub>2</sub> column chromatography (70–230 mesh), eluted with *n*-hexane–acetone (23:2 v/v), to afford compound 4 (230 mg). Fraction E4.7 (1.0 g) was also subjected to SiO<sub>2</sub> column chromatography using *n*-hexane–EtOAc (19:1–1:1 v/v) as the mobile phase to yield fractions E4.7.1–E4.7.6. Fractions E4.7.3 and E4.7.4 were further chromatographed individually using SiO<sub>2</sub> column eluted with *n*-hexane–acetone (19:1–1:1 v/v) to give a mixture of compounds 1 and 2. The mixture was then separated by reverse-phase HPLC (YMC ODS, 250 × 10 mm, gradient 5–100 % MeOH in H<sub>2</sub>O for 70 min, 3 mL/min) to yield compounds 1 (2.0 mg) and 2 (5.5 mg). Fractions E4.7.5 was also chromatographed using SiO<sub>2</sub> column eluted with *n*-hexane–acetone (19:1–1:1 v/v) to give subfractions, from which compound 5 was obtained as yellow crystals (after the fractions were kept at room temperature for several days). Fraction E4.8 (1.4 g) was subjected to SiO<sub>2</sub> column chromatography and eluted with *n*-hexane–EtOAc (19:1–1:1 v/v) as the mobile phase to give 32 sub-fractions (E4.8.1–E4.8.32). Sub-fractions E4.8.19 – E4.8.21 were pooled and further purified by reverse-phase HPLC (YMC ODS, 250 × 10 mm, isocratic 60 % MeOH in H<sub>2</sub>O for 75 min; 3 mL/min) to afford compound 3 (4.5 mg).

#### 3.4.1. Sesbagrandiflorain C (1)

Compound 1 was obtained as a yellowish solid; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup> 3397, 2927, 1654, 1498, 1193, 1142; UV (MeOH)  $\lambda_{\max}$  (nm) ( $\epsilon$ ) 206 (9370), 240 (4970), 341 (2720); ESI-TOF-MS  $m/z$  315.08765 [M + H]<sup>+</sup>, calculated for C<sub>17</sub>H<sub>15</sub>O<sub>6</sub> for 315.08631; <sup>1</sup>H and <sup>13</sup>C NMR spectral data (acetone-*d*<sub>6</sub>): see Table 1.

#### 3.4.2. 2-(3,4-Dihydroxy-2-methoxyphenyl)-4-hydroxy-6-methoxybenzofuran-3-carbaldehyde (2)

Compound 2 was obtained as a yellowish solid; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup> 3397, 3218, 2943, 1601, 1499, 1192, 1145; UV (MeOH)  $\lambda_{\max}$  (nm) ( $\epsilon$ ) 216 (7190), 266 (3320), 359 (1610); ESI-TOF-MS  $m/z$  331.08261 [M + H]<sup>+</sup>, calculated for C<sub>17</sub>H<sub>15</sub>O<sub>7</sub> for 331.08233; <sup>1</sup>H and <sup>13</sup>C NMR spectral data (acetone-*d*<sub>6</sub>): see Table 1.

#### 3.4.3. 2-(4-Hydroxy-2-methoxyphenyl)-5,6-dimethoxybenzofuran-3-carboxaldehyde (3)

Compound 3 was obtained as a colorless solid; IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup> 3489, 2922, 1684, 1204; UV (MeOH)  $\lambda_{\max}$  (nm) ( $\epsilon$ ) 211 (7470), 248 (4120), 288 (2080), 350 (2470). ESI-TOF-MS  $m/z$  329.10330 [M + H]<sup>+</sup>, calculated for C<sub>18</sub>H<sub>17</sub>O<sub>6</sub> for 329.10196; <sup>1</sup>H and <sup>13</sup>C NMR spectral data (acetone-*d*<sub>6</sub>): see Table 1.

### 3.5. Computational calculation

Conformational analysis of sesbagrandiflorain C (1) was performed in Gaussian 16 using DFT/B3LYP functional with the 6-31+G(d,p) basis set for geometry optimization and frequency calculations for the candidate structure. All conformers with energy difference < 5 kcal/mol from the most stable conformer were kept and carried forward for

NMR analysis. NMR shielding tensors were computed with the GIAO (gauge-independent (or including) atomic orbitals) method in Gaussian using the DFT/MPW1PW91 functional with the 6-311+G(2d,p) basis set, with acetone as the solvent. The obtained tensor values were then converted to predicted chemical shifts using the equation described in Willoughby et al. (2014) with appropriate scaling and referencing factors (slope and intercept, respectively) obtained from <http://cheshirenmr.info/ScalingFactors.htm>. Assessment of goodness of fit was done by calculating the mean absolute error (MAE) between the computed and the experimental data sets. MAEs of ≤ 0.10 ppm are considered to be ‘correct’ fits and those of ≥ 0.20 ppm are considered to be ‘incorrect’ structure matches (Willoughby et al., 2014).

### 3.6. Antibacterial activity assay

Agar disc diffusion assay was used to evaluate the antibacterial activity of the EtOAc extract. Five different bacteria, *S. aureus*, *B. subtilis*, *P. aeruginosa*, *M. smegmatis*, and *E. coli* were used. The EtOAc extract was dissolved in MeOH to a concentration of 10 mg/mL. The positive control for this experiment was either ampicillin (for *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli*) or apramycin (for *M. smegmatis*). Both the extract and the positive control (10  $\mu$ L each) were loaded onto sterile diffusion discs and left to dry for 20 min. For *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *M. smegmatis*, the agar plates were prepared by adding a layer of bacterial infused YMG soft agar to an YMG plate and left to solidify. The bacterial infused YMG soft agar was prepared by growing each of the bacteria in separate 15 mL falcon tubes with liquid YMG medium for two days and mixed it with warm YMG agar. The paper discs, impregnated with the extract or the positive control, were placed onto each plates using aseptic techniques. For *E. coli*, all procedures mentioned above were done using Luria-Bertani (LB) medium instead of YMG. In addition, the *E. coli* plates and liquid cultures were incubated at 37 °C. After 24 h of incubation, the plates were stained with MTT (1 mg/mL in de-ionized water) to enhance the contrast of the inhibition zones to the bacterial growth.

### 3.7. Antituberculosis assay

The antituberculosis activity was performed by a colorimetric tetrazolium micro-plate assay (TEMA) with minor modifications as described in our previous paper (Hasan et al., 2012).

### 3.8. Cytotoxicity assay

Cytotoxicity assay was performed based on a method described by O’Brien et al. (2000). HepG2, MCF-7, and HeLa cells were maintained in culture in Eagle’s Minimum Essential Medium (EMEM, ATCC, cat # 30–2003) supplemented with 10 % fetal bovine serum (FBS, Heat Inactivated, Gibco, cat # 10082–147) at 37 °C and 5 % CO<sub>2</sub>. Cells were dispensed into black, clear bottom, 384-well plates 24 h prior to compound treatment. One column in each plate did not receive cells to serve as a low-signal controls. Compounds were dissolved in DMSO and added with the D300 digital dispenser (HP) as a 12-point, half-log titration series in triplicate. Sixteen wells in each plate were left untreated to serve as high-signal controls. DMSO was normalized to 0.5 % in every well. After 48 h at 37 °C and 5 % CO<sub>2</sub>, a solution of resazurin (Acros, cat # 189900050) in PBS was added to every well to a final concentration of 44  $\mu$ M. After 4–6 h at 37 °C and 5 % CO<sub>2</sub>, fluorescence was measured with the microplate reader (Synergy 4, Biotek).

### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phytol.2019.12.008>.

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