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A new β-hydroxydihydrochalcone from *Tephrosia uniflora*, and the revision of three β-hydroxydihydrochalcones to flavanones

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ABSTRACT

The CH_2Cl_2/MeOH (1:1) extract of the stems of *Tephrosia uniflora* yielded the new β-hydroxydihydrochalcone (*S*)-elatadihydrochalcone-2'-methyl ether (**1**) along with the three known compounds elongatin (**2**), (*S*)-elatadihydrochalcone (**3**), and tephrosin (**4**). The structures were elucidated by NMR spectroscopic and mass spectrometric data analyses. Elongatin (**2**) showed moderate antibacterial activity (EC_{50} of 25.3 μM and EC_{90} of 32.8 μM) against the Gram-positive bacterium *Bacillus subtilis*, and comparable toxicity against the MCF-7 human breast cancer cell line (EC_{50} of 41.3 μM). Based on the comparison of literature and predicted NMR data with that obtained experimentally, we propose the revision of the structures of three β-hydroxydihydrochalcones to flavanones.

1. Introduction

Tephrosia species (Leguminosae) are widely distributed and utilized in herbal medicine for the treatment of a variety of disorders including stomach ache [1], diarrhoea [2], asthma [3,4], inflammation and respiratory problems [1,5]. They have also been used to treat snake bites [6]. The genus is known to generate prenylated flavonoids [5] and isoflavonoids [7] that possess antimicrobial [8], anticancer [9], anti-inflammatory [10], antiplasmodial and cytotoxic [11] effects. Some flavonoids, especially isoflavonoids, exert their antimicrobial effect [12] by inhibiting DNA synthesis, metabolism, or membrane formation of bacteria [13]. Motivated by our interest in the identification of bioactive metabolites from Kenyan plants, we have investigated the stems of *T. uniflora* for its constituents. It is a perennial herb with axillary flowers and small seeds, and is found, for example, in the Amboseli ecosystem, Kenya [14,15]. Previous phytochemical investigation of this plant provided an isoflavone, a rotenoid and phytosterols [6]. Herein, we report the isolation of a new β-hydroxydihydrochalcone (**1**) and of three known compounds (**2**–**4**) from its stem, and the evaluation of the antibacterial activity and the cytotoxicity of elongatin (**2**), one of the isolated constituents.

2. Results and discussion

Chromatographic separation of the CH_2Cl_2/MeOH (1:1) extract of the stems of *Tephrosia uniflora* led to the isolation of the new β-hydroxydihydrochalcone (*S*)-elatadihydrochalcone-2′-methyl ether (**1**) along with the known compounds elongatin (**2**) [6,16], (*S*)-elatadihydrochalcone (**3**) [17], and tephrosin (**4**) [18] (Fig. 1). The compounds were identified by NMR spectroscopic and mass spectrometric data analyses, and by comparison of their spectroscopic data with that in the literature.

Compound **1** was isolated as a colourless solid, and was assigned the molecular formula $C_{22}H_{24}O_5$ based on ESIMS ($[M+H]^+$ *m/z* 369.3) and NMR data (Table 1) analyses. The UV (λ_{max} = 214, 263, 310 nm), ^1H NMR [δ_H 3.25 (*dd, J* = 17.4, 2.8 Hz) and 3.15 (*dd, J* = 17.4, 9.5 Hz) for CH_2-α, and 5.28 (*dd, J* = 9.5, 2.8 Hz), for H-β] and ^{13}C NMR [δ_C 204.4 (C=O), 53.7 (C-α), and 70.6 (C-β)] data suggested the compound to have a β-hydroxydihydrochalcone skeleton [17]. The ^1H NMR signals at δ_H 7.40 (H-2/6), 7.34 (H-3/5), and 7.26 (H-4) with the corresponding

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carbon resonances at δ_C 126.0 (C-2/6), 128.5 (C-3/5), 127.6 (C-4) suggest that ring A is unsubstituted. Ring B, on the other hand, is tetra-substituted with a 2,2-dimethylpyran and two methoxy groups. Its C-2′, C-4′ and C-6′ oxygenation is in line with the biogenesis of chalcones [19], and is further supported by the observed ESI-MS fragment ion at *m/z* 247.3 (**1a**, Fig. 1) formed through α-cleavage [20]. The HMBC correlations of H-5' (δ_H 6.19) to C-3' (δ_C 108.3), and C-4' (δ_C 156.6), of H-4" (δ_H 6.46) to C-4' (δ_C 156.6), and of H-3" (δ_H 5.54) to C-3' (δ_C 108.3) allowed the placement of the 2,2-dimethylpyran residue at C-3′/C-4′, with the oxygen attached to C-4′ (δ_C 156.6). The HMBC correlations of 2'-OMe (δ_H 3.75) to C-2′ (δ_C 154.6), and 6'-OMe (δ_H 3.76) to C-6′ (δ_C 157.9), and the NOESY cross-peaks of 2'-OMe (δ_H 3.75) to H-4" (δ_H 6.46), and 6'-OMe (δ_H 3.76) to H-5' (δ_H 6.19), allowed the placement of two methoxy groups at C-2′ (δ_C 154.6) and C-6′ (δ_C 157.9). As H-5' (δ_H 6.19) did not provide strong, ^3J cross peaks to any oxygenated aromatic carbons, these groups have to be two- and four-bonds away from it. The negative Cotton effect at 275 nm in the ECD spectrum of **1** suggested it to be *S*-configured at its β carbon, similar to elatadihydrochalcone (**3**), a co-metabolite that has earlier been reported from *T. elata* with a negative Cotton effect at 290 nm [17], and to a synthetic β-hydroxydihydrochalcone that has been reported by Ferreira and co-workers to show a negative Cotton effect at 240 nm [21]. Due to the low concentration of the studied sample, the broad and less intense Cotton effect >300 nm reported for some related compounds was not detected. Based on the above spectroscopic evidence, the new compound **1** was characterized as (*S*)-2',6'-dimethoxy-3'/4'(2",2"-dimethyl-2*H*-chromen-6-yl)-β-hydroxydihydrochalcone, and was given the trivial name elatadihydrochalcone-2'-methyl ether.

β-Hydroxydihydrochalcones are a rare subclass of chalcones [17]. Some compounds that have originally been reported as β-hydroxydihydrochalcones were later revised to flavanones [17]. A review of the recent literature suggests that further flavanones may have mistakenly been reported as β-hydroxydihydrochalcones [22–24]. To clarify the spectroscopic difference between these two compound classes, herein we compare the ^{1}H and ^{13}C NMR data of the β-hydroxydihydrochalcones (*S*)-elatadihydrochalcone-2'-methyl ether (**1**), (*S*)-elatadihydrochalcone (**3**) [17], ziganin (**5**) [23], 3-(*S*)-hydroxy-3-phenyl-1-(2',4',6'-trihydroxyphenyl)propan-1-one (**6**) [22], and balanochalcone (**7**) [24] with those of the corresponding flavanones (**5a**, **6a** and **7a**). We further give the predicted chemical shifts of the β-hydroxydihydrochalcone and flavanone skeletons, and note that the chemical shifts of the α- and β-protons (Table 2), and of the corresponding carbon atoms (Table 3) provide a helpful tool for differentiation between these compound classes.

In β-hydroxydihydrochalcone **3** (Table 2), H-β resonates at δ_H 5.28, and CH_2-α at δ_H 3.45 and δ_H 3.34, whereas the H-2 of flavanone **5a** resonates at δ_H 5.40, its CH_2–3_{ax} at δ_H 3.14 and its CH_2–3_{eq} at δ_H 2.73. A systematic comparison of the chemical shifts (Table 2) suggest that the values reported for the H-β and H-2α of **5** [23], **6** [22] and **7** [24] that were originally suggested to be β-hydroxydihydrochalcones better fit to the H-2 and CH_2–3 chemical shifts of flavanones **5a** [25], **6a** [26] and **7a** [27], respectively. The C-α, C-β and C═O of β-hydroxydihydrochalcone **3** resonate at δ_C 52.7, 70.2 and 204.2, respectively (Table 3), whereas the C-2, C-3 and C-4 of flavanone **5a** resonate at δ_C 42.9, 79.3 and 196.5, respectively. The chemical shifts of the corresponding carbons of compounds **5**, **6** and **7** resemble those of C-2, C-3 and C═O of flavanones **5a**, **6a** and **7a** rather than the chemical shifts of C-α, C-β and C═O of β-hydroxydihydrochalcones. Based on this spectroscopic evidence (Tables 2 and 3), we propose the revision of **5**, **6**, and **7** from β-hydroxydihydrochalcones to flavanones **5a**, **6a**, and **7a**, respectively (Fig. 1).

We also note that the oxygenation pattern of ring A in compound **7** [24] and (ring B in compound **7a**) do not correspond to the reported NMR data (Table 2 and Table 3). Ring A in **7** (ring B in 7a) is symmetrical and hence C-2 and C-6 (C-2′ and 6′ in **7a**) are chemically equivalent and should resonate at the same frequency. In contrast, different chemical shifts have been reported [24] for these positions (Tables 2 and 3) suggesting that the originally assigned substitution pattern of this ring in this compound is erroneous. The reported NMR data is not in agreement

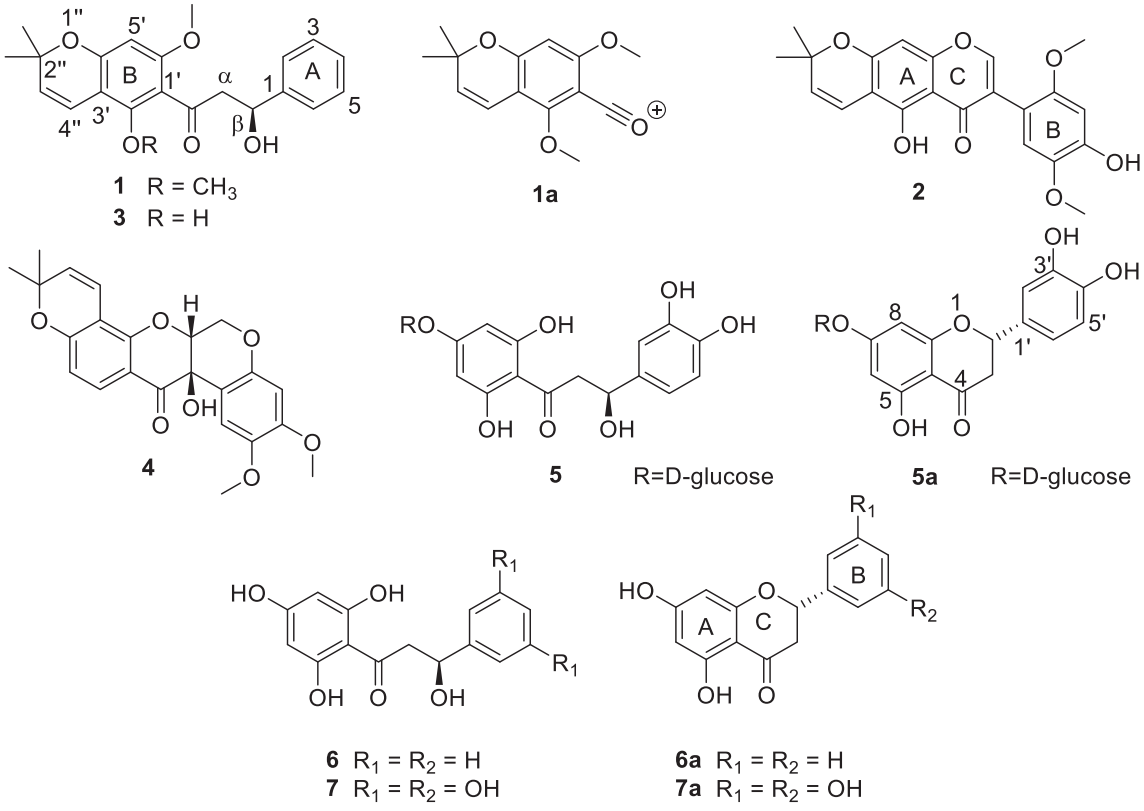

Fig. 1. The structures of the compounds isolated from the stem of *Tephrosia uniflora*, and of the compounds revised herein.

with oxygenation at C-2′ and C-6′ but that of C-2′ and C-5′. As we only have access to pdf copies of the NMR data of this compound, we recommend the re-analysis of **7a** to ensure the accuracy of our suggestion for correction.

Having a β-hydroxydihydrochalcone skeleton as a working structure, the interpretation of the mass spectrometric data may easily lead to the misassignment of flavanones to β-hydroxydihydrochalcones, despite the molecular weight of flavanones being 18 amu lower. For compound **5**, the HRESIMS data showing the *m/z* 469.26742 signal corresponding to the protonated molecular ion has allegedly been used to establish its molecular formula; however, the spectrum has not been attached as supplementary material [23] and hence this information cannot be confirmed. For compound **6** [22], the HRESIMS signal *m/z* 297.07603 $[M + Na]^+$ was used to establish the molecular formula, corresponding to the β-hydroxydihydrochalcone structure **7**. However, the spectrum shown in the supplementary material of this report does not show this signal [24], but instead m/z 289.0696 that was (erroneously) interpreted as "a quasi-molecular ion peak $[M+H-H_2O]^+$" of β-hydroxydihydrochalcone **7**. This mass signal corresponds to the protonated molecular ion of flavanone **7a**, supporting the NMR-based revision of **7** to **7a**. This MS-based misassignment is analogous to that of 4,2′,4′,β-tetrahydroxy-6′-methoxy-α,β-dihydrochalone [31], which showed m/z 286.0823 (HREIMS) that was reported as $[M - H_2O]^+$ instead of recognizing it as the molecular ion peak of the corresponding flavanone, 4′,7-dihydroxy-5-methoxyflavanone [17].

Elongatin (**2**) isolated from *T. uniflora* was evaluated for activity against the model bacteria *B. subtilis* and *E. coli* as well as for cytotoxicity against the MCF-7 human breast cancer cell line. It showed moderate antibacterial activity (EC_{50} of 25.3 μM, EC_{90} of 32.8 μM) against *B. subtilis* and cytotoxicity (EC_{50} 41.3 μM), which is in agreement with a previous report on related flavonoids [12].

In conclusion, we report the isolation of (*S*)-elatadihydrochalcone-2′-methyl ether (**1**), a new β-hydroxydihydrochalcone, along with three known compounds from the stem of *Tephrosia uniflora*. In addition, we suggest the revision of the structures of the previously reported β-hydroxydihydrochalcones ziganin (**5**) [23], 3-(*S*)-hydroxy-3-phenyl-1-(2′,4′,6′-trihydroxyphenyl)propan-1-one (**6**) [22], and balanochalcone (**7**) [24] to the flavanones eriodictyol-7-*O*-β-D-glucopyranoside (**5a**) [25], pinocembrin (**6a**) [26], and 5,7,3′,5′-tetrahydroxyflavanone (**7a**) [27], respectively. To avoid future misidentification of flavanones as β-hydroxydihydrochalcones, we recommend the comparison of the ^1H and ^{13}C NMR data of compounds to be discovered with the data given in Tables 2 and 3. Thus, ^1H NMR chemical shift of H-3/α < 2.9 ppm along with ^{13}C NMR chemical shifts C-2/β ~80 ppm and C-3/α > 50 ppm are indicators for a flavanone skeleton, whereas H-3/α > 3.1 ppm, C-2/β ~70 ppm and C-3/α < 45 ppm suggest a β-hydroxydihydrochalcone skeleton. Moreover, the ^{13}C NMR chemical shift of C-4 (C=O) of flavanones is typically <200 ppm, whereas those of β-hydroxydihydrochalcones is >200 ppm.

3. Materials and methods

3.1. Plant material

The stem of *Tephrosia uniflora* was collected along the Emali-Loitokitok road, Makueni County, in July 2016. The plant was authenticated by Mr. Pactrick C. Mutiso of the University Herbarium, Department of Biology, University of Nairobi, where a voucher specimen (PCM-2016/010) was deposited.

3.2. General experimental procedure

ECD experiments were run on a JASCO J-810 spectropolarimeter. UV spectroscopy was performed on a Shimadzu UV-1650 spectrophotometer. NMR spectra were acquired on a Bruker Avance NEO 500 MHz spectrometer equipped with a TXO cryogenic probe. MestreNova (v14.0.0) software was used to process the spectra. TLC analyses was performed on Merck pre-coated silica gel 60 F_{254} aluminum plates. Preparative reversed-phase HPLC chromatography was carried out using a Waters 600E HPLC system using the Chromulan software (v.0.88, Pikron Ltd) and an RP-C8 Kromasil® column (250 mm × 25 mm, 5 μm). Column chromatography was done using silica gel 60 (mesh 230–400) and Sephadex LH-20 (GE Healthcare).

3.3. Extraction and isolation from the stem of Tephrosia uniflora

The air-dried stems of *Tephrosia uniflora* (500 g) were extracted (4 × 1 L) with CH_2Cl_2/MeOH (1:1) at room temperature. The crude extract (60 g) was partitioned between EtOAc and H_2O. The EtOAc layer was concentrated on a rotary evaporator to yield 30 g of crude extract. The crude EtOAc extract was adsorbed on silica gel, loaded onto a 300 g silica gel column, and eluted with isohexane containing increasing amounts of EtOAc (1% to 99% *v*/v). Based on their TLC profiles, the eluates were then pooled into 15 fractions. The fraction that was eluted with 3% EtOAc in isohexane was purified by column chromatography over Sephadex LH-20 (CH_2Cl_2/MeOH, 1:1), followed by further purification on Preparative TLC (isohexane/EtOAc, 7:3) to yield compound **2** (20 mg) as a white amorphous solid. The fraction that was eluted with

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data for compound **1**, in $CDCl_3$.

No	δ_H (*J* in Hz)	(δ_C)	HMBC	COSY	NOESY	TOCSY
CH_2-α	3.25 *dd* (17.4, 2.8)	53.7	C-7, C-β	H-β	H-β, H-2/6	H-β
	3.15 *dd* (17.4, 9.5)		C-7, C-β	H-β	H-β	H-β
β	5.28 *dd* (9.5, 2.8)	70.6	C-1, C-2/6	H-α	H-α, H-2/6	H-α
1	–	143.2	–	–	–	–
2/6	7.40 *m*	126.0	C-4, C-β	–	H-βc, H-α	H-3/5, H-4
3/5	7.34 *m*	128.5	C-1, C-3/5	H-4	–	H-2/6, H-4
4	7.26 *m*	127.6	C-2/6	H-3/5	–	H-2/6, H-3/5
7	–	204.4	–	–	–	–
1’	–	117.6	–	–	–	–
2’	–	154.6	–	–	–	–
3’	–	108.3	–	–	–	–
4’	–	156.6	–	–	–	–
5’	6.19 *s*	96.3	C-1’, C-3’, C-4’, C-6’	–	6-OMe	–
6’	–	157.9	–	–	–	–
2”	–	76.9	–	–	–	–
3”	5.54 *d* (9.9)	128.1	C-3′, C-2″, C-2″-Me	H-4”	2″-Me, H-4″	H-4″
4”	6.46 *d* (9.9)	116.4	C-2′, C-4′, C-2”	H-3”	2-OMe, H-3″	H-3″
2″-Me_2	1.43 *s*	28.1	C-2”, C-2″-Me	–	H-3″	–
2’-OMe	3.75 *s*	56.0	C-2’	–	H-4″	–
6’-OMe	3.76 *s*	63.9	C-6’	–	H-5’	–

Table 2
^{1}H NMR spectral data of the skeletons of β-hydroxydihydrochalcones and flavanones[a].

Position	**1** ($CDCl_3$)	**3** ($CDCl_3$)	**5** ($CDCl_3$)	**5a** (acetone-d_6)	**6** ($CDCl_3$)	**6a** (DMSO-d_6)	**7**** (CD_3OD)	**7a** (CD_3OD)
2 (β)[b]	5.27 dd (2.8, 9.5)	5.28 dd (3.0, 9.0)	5.32, dd (3.4, 12.8)	5.40 *dd* (3.1, 12.6)	5.43 dd (3.0, 13.0)	5.44 dd (3.2, 12.8)	5.30 dd (3.0, 13.0)	5.26 dd (3.0, 14.4
3 (α)	3.25 dd (2.8, 17.4)	3.45 dd (3.0, 18.0)	3.12 dd (12.8, 17.2)	3.14 dd (12.6, 17.1)	3.09 dd (3.0, 13.0)	3.06 dd (12.8, 17.2)	3.09 dd (12.5, 17.3)	3.05 dd (12.6, 16.8)
	3.15 dd (9.5, 17.4)	3.34 dd (9.0, 18.0)	2.74 dd (3.4, 17.2)	2.73 dd (3.1, 17.1)	2.83 dd (3.0, 17.0)	2.77 dd (3.2, 17.2)	2.72 dd (3.0, 17.0)	2.68 dd (3.0, 17.4)
6 (3′)			6.20 d (2.2)	5.94 (2.2)	6.01 s	5.52 d (2.2)	5.90 d (2.0)	5.85 d (2.4)
8 (5′)	6.19 s	5.87 s	6.17 d (2.2)	5.95 (2.2)	6.01 s	6.01 d (2.2)	5.92 d (2.0)	5.87 *d* (2.4)
2′ (2)	7.40 m	7.26–7.44 m	6.91, bs	7.04 (1.7)	7.45–7.39 m	7.41 m	6.81 s	6.77 s
3′ (3)	7.34 m	7.26–7.44 m			7.45–7.39 m	7.41 m		
4′ (4)	7.26 m	7.26–7.44 m			7.45–7.39 m	7.41 m	6.94 s	6.78 s
5′ (5)	7.34 m	7.26–7.44 m	6.77 d (8.3)	6.87 (8)	7.45–7.39 m	7.41 m		
6′ (6)	7.40 m	7.26–7.44 m	6.79 dd (8.3, 1.8)	6.88 (8, 1.7)	7.45–7.39 m	7.41 m	6.81 s	6.90 d (1.2)

[a] β-Hydroxydihydrochalcones: (*S*)-elatadihydrochalcone-2′-methyl ether (**1**) and elatadihydrochalcone (**3**), ziganin (**5**) [23], 3-(*S*)-hydroxy-3-phenyl-1- (2′,4′,6′-trihydroxyphenyl)propan-1-one (**6**) [22], balanochalcone (**7**) [24]. Flavanones: eriodictyol 7-*O*-β-D-glucopyranosid (**5a**) [25], pinocembrin (**6a**) [26] and 5,7,3′,5′-tetrahydroxyflavanone (**7a**) [27].
[b] Numbering in flavanones (numbering in β-hydroxydihydrochalcones); ** NMR data was extracted from the ^{1}H NMR spectrum given in the Supplementary Material of [24].

Table 3
^{13}C NMR spectral data of β-hydroxydihydrochalcones and flavanones[a].

Position	**1**	**1****	**3**	**3****	**5**	**5a**	**5a****	**6**	**6a**	**6a****	**7**	**7a**	**7a****
2 (β)[b]	70.6	70.9	70.2	70.9	79.3	79.3	78.9	79.2	80.2	79.5	80.5	80.5	80.2
3 (α)	53.7	47.1	52.7	47.1	42.6,	42.9	42.7	43.3	40.5	43.1	44.1	44.1	43.4
4 (C=O)	204.4	202.3	204.2	203.0	197.2	196.5	196.5	195.8	196.8	196.8	197.8	197.6	194.2
4a (1′)	117.6	110.9	105.6	106.0	103.5	102.0	102.7	103.2	102.7	102.7	103.4	103.2	102.3
5 (2′)	154.6	160.3	161.9	161.7	163.2	164.6	164.4	164.3	164.4	164.4	164.8	165.5	164.4
6 (3′)	108.3	111.1	102.9	104.3	95.3	95.2	95.6	96.8	96.8	96.7	97.0	97.1	96.7
7 (4′)	156.6	160.6	160.7	161.1	165.6	166.6	166.9	164.8	167.6	166.9	168.4	168.5	166.9
8 (5′)	96.3	93.1	91.4	92.6	96.5	96.1	96.7	95.5	95.9	95.6	96.2	96.2	95.6
8a (6′)	157.9	160.4	163.0	161.0	163.3	163.7	163.8	163.2	163.6	164.2	165.5	164.8	163.4
1′ (1)	143.2	143.3	143.4	143.3	130.1	129.7	130.1	138.3	139.6	139.1	131.8	131.7	142.6
2′ (2)	126.0	126.5	125.9	126.5	113.3	115.4	115.7	126.2	127.5	126.3	116.3	116.2	116.8
3′ (3)	128.5	128.3	128.4	128.3	145.6	145.7	145.8	128.9	129.5	128.6	146.9	146.9	146.6
4′ (4)	127.6	128.1	127.4	128.1	145.1	145.4	145.8	128.9	129.4	128.1	114.7	119.2	114.4
5′ (5)	128.5	128.3	128.4	128.3	114.8	114.1	114.3	128.9	129.5	128.6	146.5	146.5	146.6
6′ (6)	126.0	126.5	125.9	126.5	117.9	118.6	118.5	126.2	127.5	126.3	119.3	114.6	116.8

[a] β-Hydroxydihydrochalcones: (*S*)-elatadihydrochalcone-2′-methyl ether (**1**) and elatadihydrochalcone (**3**), of ziganin (**5**) [23], 3-(*S*)-hydroxy-3-phenyl-1- (2′,4′,6′-trihydroxyphenyl)propan-1-one (**6**) [22], and balanochalcone (**7**) [24]. Flavanones: eriodictyol 7-*O*-β-D-glucopyranosid (**5a**) [25], pinocembrin (**6a**) [26] and 5,7,3′,5′-tetrahydroxyflavanone (**7a**) [27].
[b] Numbering in flavanones (numbering in β-hydroxydihydrochalcones); ** calculated using CSEARCH-NMR-Server. [28–30].

4% EtOAc in isohexane was further purified by preparative HPLC (MeOH-H_2O, gradient elution 5%–90% H_2O) to give compound **1** (8 mg) as a colourless solid. The fraction that was eluted with 5% EtOAc was further purified on preparative HPLC (MeOH-H_2O, gradient elution 5%–90% H_2O) to give compound **3** (10 mg) and compound **4** (12 mg) as colourless solids.

3.4. (S)-Elatadihydrochalcone-2′-methyl ether (1)

Colourless solid. UV (λ_{max}, MeOH): 214, 263, 310 nm. ECD (MeOH, c 0.01): $[\Theta]_{304}$ –9.1, $[\Theta]_{275}$ –31, $[\Theta]_{266}$ –22. ^{1}H and ^{13}C NMR (Table 1). ESI-MS (*m/z*): 369.3 (13, $[M + H]^+$), 263.3 (8), 258 (9), 247 (100, $[C_{14}H_{15}O_4]^+$), 102 (5).

3.5. Antibacterial assays

Antibacterial activity of the isolated compound **2** was determined against the Gram-positive *B. subtilis* and Gram-negative *E. coli* bacteria following standard procedures [32]. In summary, compound **2** was dissolved in 100% DMSO to 10 mg/mL concentration and stored at −20 °C. Bacterial cultures of *B. subtilis* and *E. coli* were grown in Mueller-Hinton (HiMedia Laboratories Pvt. Limited, Mumbai, India) broth for 24 h until reaching the optical density (O·D = 0.5) [33], then diluted 10 times in pre-warmed medium and the compound added to a final concentration of 35 μg/mL into a 384-well microtiter plate and incubated for 24 h at 37 °C without agitation. The resazurin assay for assessing viability was performed as described [34], by adding 10 μL of AlamarBlue solution to each well, followed by a 1 h incubation at 37 °C without agitation. Viability was determined using POLARstar Omega (BMG Labtech, Cape Town, S.A.) set at excitation λ = 540 nm and emission filter λ = 590 nm, where the fluorescence bleed-through between the wells was controlled through a chest-like plate design. In all assays, the known antibiotic ampicillin was used as a positive control and DMSO as negative control, following the same 2-fold dilution concentration range as the compounds under testing. Effective concentrations (EC_{50} and EC_{90}) values were calculated from three independent replicate experiments using 2-fold dilution intervals. Non-linear regression dose-response inhibition following a log(agonist) vs. response was performed using GraphPad Prism version 9.2.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3.6. Cytotoxicity assays

MCF-7 cells were used to assess the cytotoxic effect of the isolated compounds, as published elsewhere [35]. Briefly, the cells were cultured

and kept in exponential growth in Dulbecco's Eagle's medium (Modified Medium) supplemented with 10% foetal calf serum and reseeded into 96-well microtiter plates to settle for 24 h as pre-assay preparation. The stock solution of the compound was added to a final concentration of 0.35% *v/v* of DMSO in the culture medium. The cell viability was determined using PrestoBlue (ThermoFisher) cell viability reagent following the manufacturer's instructions and for a 24 h incubation period. The fluorescence from resorufin was measured using POLARstar Omega (BMG Labtech, Cape Town, S.A) set at excitation $\lambda = 540$ nm and emission filter $\lambda = 590$ nm. Each assay contained a DMSO control at the equivalent starting concentration, positive control (uninhibited cell growth) and negative control (cell medium only). Cell viability was expressed as a percentage of solvent-only control with half maximal effective concentration (EC_{50}) values, associated standard deviation (SD) and standard error (SE) range, calculated from three independent replicate experiments using 2-fold dilution intervals. Non-linear regression dose-response inhibition following a log(agonist) vs. response – Find ECanything was performed using GraphPad Prism version 9.2.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2022.105166.

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