



Melatonin Receptors Agonistic Activities of Phenols from *Gastrodia elata*

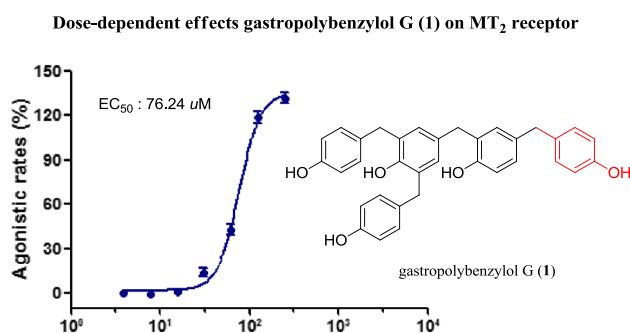
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Abstract

Gastrodia elata is a famous traditional Chinese herb with medicinal and edible application. In this study, three new polybenzyls, gastropolybenzylols G-I (1–3) were isolated from the EtOAc extract of *G. elata*. Their structures were identified by extensive spectroscopic analyses involving HRESIMS, UV, IR, 1D and 2D NMR. Compound 1 showed agonistic effects on MT₁ and MT₂ receptors with agonistic rates of 55.91±4.84% and 165.13±5.65% at the concentration of 0.5 mM, respectively, and an EC₅₀ value of 76.24 μM on MT₂ receptor.

Graphic Abstract



Keywords *Gastrodia elata* · Gastropolybenzylols · Melatonin receptors

1 Introduction

Gastrodia elata, a saprophytic herb from orchid family, mainly distributed in East Asia, Southeast Asia and Oceania. The main constituents of *G. elata* are phenolics, polysaccharides, sterols and organic acids [1]. As a famous traditional Chinese medicine (TCM), the tuber of *G. elata* is commonly used for the treatment of diverse mental diseases, involving insomnia [2, 3], depression [4–7], epilepsy, convulsion [8, 9], and Alzheimer's disease [10, 11]. Plenty of pharmacological investigation suggested that *G. elata* had anti-hypertension [12], anti-tumor [13], anti-virus [14, 15] and anti-inflammation [16, 17] effects. Most of pharmacological components are 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, vanillin, 1,3-bis(4-hydroxybenzyl) citrate, 1-(4-beta-D-glucopyranosyloxybenzyl) citrate and parishin

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B [18]. However few reports about the psychoactive effects of polybenzyls were published [19–22]. In this study, the EtOAc extract of *G. elata* was found to activate MT₂ receptor with an agonistic rate of 105.38% at the concentration of 102.22 $\mu\text{g/mL}$. In order to characterize the active constituents, three new compounds (1–3, Fig. 1) were obtained by various column chromatography. Herein, we report their isolation, structural elucidation and agonistic activities on melatonin (MT₁ and MT₂) receptors.

2 Results and Discussion

2.1 Structure Elucidation

Compound 1 had a molecular formula of C₃₄H₃₀O₅ with 20 degrees of unsaturation, which was deduced from HRESIMS at m/z 517.2040 [M–H][–] (calcd 517.2020). The UV absorptions at 281 and 254 nm, and the IR absorptions at 3426, 1631, 1613, 1511 and 1439 cm^{–1} suggested the presence of hydroxyl and phenyl groups. In the ¹H-NMR spectrum, three sets of protons [δ_{H} 7.01 (4H, d, $J = 8.3$ Hz, H-2, H-6, H-2''', H-6'''), 6.67 (4H, overlap, H-3, H-5, H-3''', H-5'''); 6.93 (2H, d, $J = 8.4$ Hz, H-2'', H-6''), 6.67 (2H, overlap, H-3'', H-5'')], and combining with ¹H-¹H COSY correlations of H-2/H-3, H-6/H-5, H-2'''/H-3''', H-6'''/H-5''', and of H-2''/H-3'', H-6''/H-5'' indicated the existence of 1,4-substituted aromatic rings. One set of protons [δ_{H} 6.85 (1H, brs, H-6''), 6.84 (1H, $J = 8.4, 2.0$ Hz, H-4''), 6.67 (1H, overlap, H-3'')], and combining with ¹H-¹H COSY correlation of H-3''/H-4'' manifested the presence of a 1,2,4-substituted benzene ring. Besides, two *meta*-coupled protons [δ_{H} 6.77 (1H, d, $J = 2.0$ Hz, H-4') and 6.76 (1H, d, $J = 2.0$ Hz, H-6')], four methylenes [δ_{H} 3.79 (4H, s, H-7, H-7'''), 3.75

(2H, s, H-7'), 3.68 (2H, s, H-7'')] were well recognized (Table 1). Compared with 4-[[[2-hydroxy-5-(methoxymethyl) phenyl] methyl]-2,6-bis [(4-hydroxyphenyl) methyl] phenol [19], compound 1 had an extra *para*-hydroxybenzyl [δ_{H} 6.93

Table 1 ¹H-NMR (400 MHz) and ¹³C-NMR (100 MHz) data of compound 1 in CD₃OD

No.	δ_{H} (J in Hz)	δ_{C}	No.	δ_{H} (J in Hz)	δ_{C}
1	–	133.6 (s)	3''	6.67 (1H, overlap)	116.0 (d)
2	7.01 (1H, d, 8.3)	130.9 (d)	4''	6.84 (1H, 8.4, 2.0)	128.5 (d)
3	6.67 (1H, overlap)	116.0 (d)	5''	–	133.8 (s)
4	–	156.3 (s)	6''	6.85 (1H, brs)	132.2 (d)
5	6.67 (1H, overlap)	116.0 (d)	7''	3.68 (2H, s)	41.3 (t)
6	7.01 (1H, 8.3)	130.9 (d)	1'''	–	134.5 (s)
7	3.79 (2H, s)	36.0 (t)	2'''	6.93 (1H, d, 8.4)	130.8 (d)
1'	–	129.6 (s)	3'''	6.67 (1H, overlap)	116.1 (d)
2'	–	154.2 (s)	4'''	–	156.4 (s)
3'	–	129.5 (s)	5'''	6.67 (1H, overlap)	116.1 (d)
4'	6.77 (1H, d, 2.0)	132.0 (d)	6'''	6.93 (1H, d, 8.4)	130.8 (d)
5'	–	134.4 (s)	7'''	3.79 (2H, s)	35.9 (t)
6'	6.76 (1H, d, 2.0)	132.0 (d)	1''''	–	133.9 (s)
7'	3.75 (2H, s)	35.9 (t)	2''''	7.01 (1H, d, 8.3)	130.9 (s)
1''	–	129.4 (s)	3''''	6.67 (1H, overlap)	116.0 (d)
2''	–	154.1 (s)	4''''	–	154.2 (s)
			5''''	6.67 (1H, overlap)	116.0 (d)
			6''''	7.01 (1H, d, 8.3)	130.9 (d)

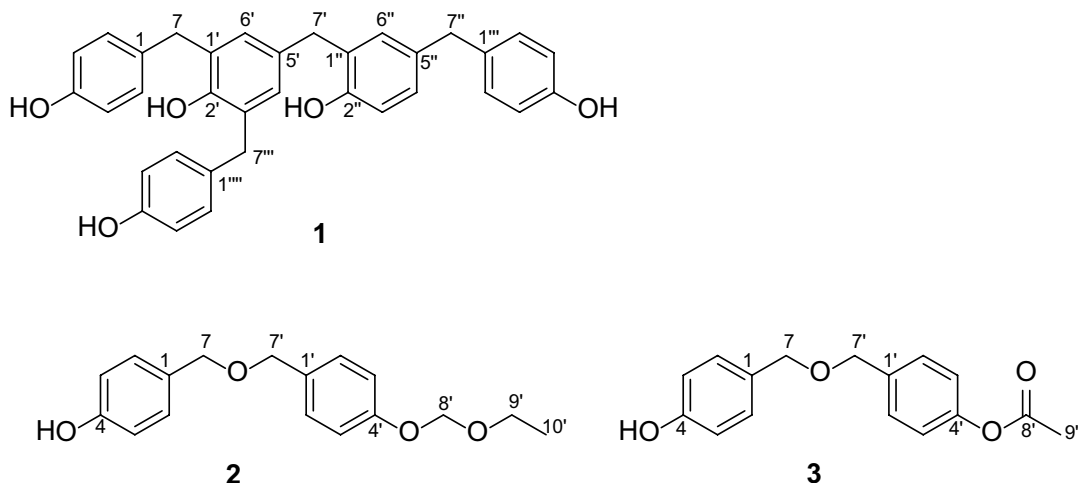


Fig. 1 Structures of compounds 1–3

(2H, d, $J = 8.4$ Hz, H-2''', H-6'''), 6.67 (2H, overlap, H-3''', H-5'''), 3.68 (2H, s, H-7''); δ_C 156.4 (C-4''', s), 134.5 (C-1''', s), 130.8 (C-2''', C-6''', d), 116.1 (C-3''', C-5''', d), 41.3 (C-7'', t)], but with the absence of a methoxymethyl group. Based on the HMBC correlations from H-7'' (δ_H 3.68, s) to C-2''', C-6'' (δ_C 132.1, d), C-4'' (128.5, d), C-6'' (δ_C 132.2, d), the linkages of C-7'' to C-5'' and C-1''' were established (Fig. 2). Thus, compound **1** was elucidated and named as gastropolybenzylol G.

Compound **2** was assigned a molecular formula of $C_{17}H_{20}O_4$ with 8 degrees of unsaturation according to the HRESIMS at m/z 333.1344 [$M+HCOO$]⁻ (calcd for 333.1344). The UV (274, 247 and 226 nm) and the IR (3423, 1613 and 1513 cm^{-1}) absorptions showed the presence of hydroxyl and phenyl groups. Compared with 4,4'-hydroxybenzyl ether [23], compound **2** had an additional ethoxymethoxy group [δ_H 5.21 (2H, s, H-8'), 3.70 (2H, q, H-9'), 1.18 (3H, t, H-10'); δ_C 94.2 (C-8', t), 65.2 (C-9', t), 15.5 (C-10', q)] in accordance with the HMBC correlation from H-8' to C-9'. The HMBC correlation from H-8' (δ_H 5.21, 2H, s) to C-4' (δ_C 158.5, s) indicated the ethoxymethoxy group at C-4'. Therefore, compound **2** was identified and named as gastropolybenzylol H.

Compound **3** showed a molecular formula of $C_{16}H_{16}O_4$ with 9 degrees of unsaturation according to the [$M+H$]⁺ ion at m/z 273.1122 (calcd for 273.1121). The UV absorptions at 275, 250 and 229 nm suggested the existence of phenolic structure and the IR absorptions at 3430, 1703, 1614 and 1518 cm^{-1} manifested the presence of hydroxyl, carbonyl and phenyl groups. Compared with 4,4'-hydroxybenzyl ether [23], compound **3** had an extra acetyl group [δ_C 172.9 (s, C-8'), 21.0 (q, C-9')]. Thus, compound **3** was determined as the acetylated derivative of 4,4'-hydroxybenzyl ether and named as gastropolybenzylol I.

The known compounds were identified as 4, 4'-dimethoxy-dibenzyl ether [24] (**4**), 4,4'-diethoxybenzyl ether [25] (**5**), 4-[(4-(methoxymethyl) phenoxy) methyl] phenol [23] (**6**) and 4-[[4-[(4-(ethoxymethyl) phenoxy) methyl] phenoxy] methyl] phenol [26] (**7**).

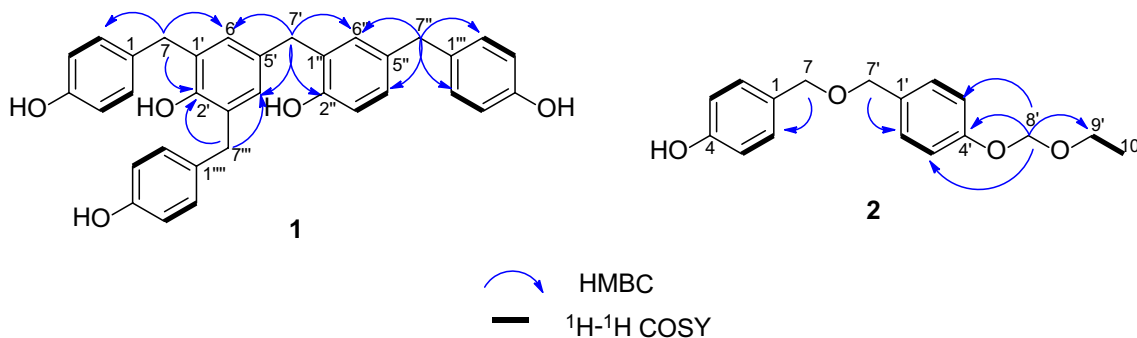


Fig. 2 The key ¹H-¹H COSY and the HMBC correlations of compounds **1** and **2**

2.2 MT₁ and MT₂ Receptors Agonistic Activities

Compounds **1** and **2** were evaluated for their agonistic activities on MT₁ and MT₂ receptors *in vitro*. Melatonin was used as the positive control. As showed in Fig. 3, compound **1** could activate MT₁ and MT₂ receptors with agonistic rates of 55.91 ± 4.84 and $165.13 \pm 5.65\%$ at the concentration of 0.5 mM. Due to **1** with better agonistic activities on MT₂ receptor, further study provided an EC₅₀ value of 76.24 μ M on MT₂ receptor (Fig. 4).

3 Experimental Section

3.1 General Procedures

NMR spectra were undertaken by Avance III-400/III-600 spectrometers (Bruker, Bremerhaven, Germany). UV spectra were recorded on a UV-2401 equipment (Shimadzu, Kyoto, Japan). IR spectra were obtained on Bio-Rad FTS-135 (Hercules, California, USA) spectrometers using KBr

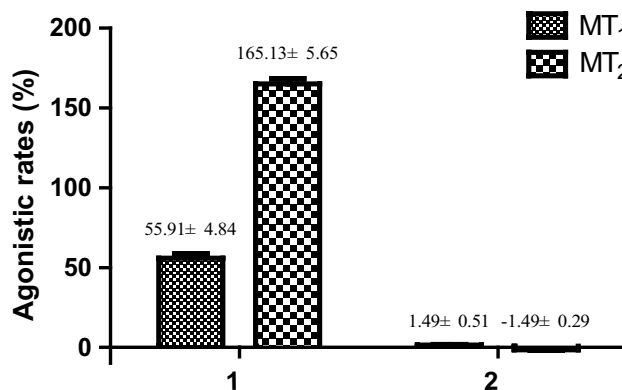


Fig. 3 MT₁ and MT₂ receptors agonistic activities of compounds **1** and **2** (0.5 mM). Data are expressed as mean \pm S.D., $n=3$. Melatonin was used as the positive control with EC₅₀ values of 1.0 nM (MT₁) and 25.0 nM (MT₂)

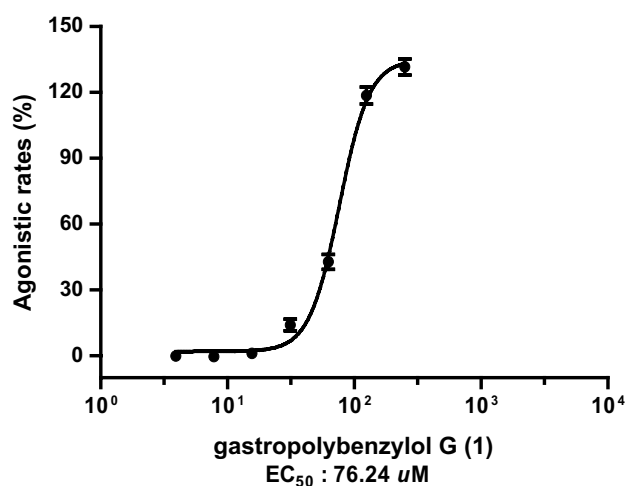


Fig. 4 Dose-dependent effects of gastropolybenzylol G (**1**) on MT₂ receptor

pellets. LCMS-IT-TOF (Shimadzu, Kyoto, Japan) was used for detecting MS spectra. Thin-layer chromatography (TLC) analyses were performed on silica gel GF254 plates (Jiangyou, Chemical Co. Ltd., Yantai, China). Compounds were purified by silica gel (200–300 mesh, Qingdao Makall group Co. Ltd., Qingdao, China), Sephadex LH-20 (Amersham Biosciences, Sweden) column chromatography and HPLC using Shimadzu LC-CBM-20 system (Shimadzu, Kyoto, Japan) with an Agilent XDB-C₁₈ column (9.4 × 250 mm, Agilent, California, USA). 10% H₂SO₄ in EtOH was applied for detecting spots by heating after sprayed. Melatonin as positive control was obtained from Damas-beta Co. Ltd. (Shanghai, China).

3.2 Plant Material

Gastrodia elata Bl. was bought from Zhaotong in Yunnan Province of China, in September 2014, and was identified by Prof. Li-Gong Lei, Kunming Institute of Botany, CAS. A voucher specimen (No. 20141107) was deposited at the Laboratory of Anti-virus and Natural Medicinal Chemistry, Kunming Institute of Botany, CAS.

3.3 Extraction and Isolation

Fresh rhizomes of *G. elata* (45.0 kg) were cut into slices and extracted with 90% aqueous ethanol (45 L × 3) at room temperature. The combined extract was concentrated under reduced pressure and partitioned between H₂O and EtOAc. The EtOAc extract (88 g) was separated by silica gel column chromatography (CC) using gradient elution with EtOAc-CHCl₃ (0:100, 5:95, 10:90, 20:80, 40:60, 100:0, v/v) as the mobile phase to yield five fractions (Fr. 1–Fr. 5). Fr. 2 (22 g) was separated by silica gel CC with EtOAc-petroleum ether

(5:95, 10:90, 20:80, 40:60, 100:0) to yield five fractions (Fr. 2.1–Fr. 2.5). Then, Fr. 2.2 (3.4 g) was further separated by silica gel CC (Me₂CO-petroleum ether, 3:97, 5:95, 10:90, 20:80, 50:50) to give five fractions (Fr. 2.2.1–Fr. 2.2.5). Compounds **2** (10 mg) and **5** (30 mg) were purified from Fr. 2.2.1 by HPLC on an Agilent XDB-C₁₈ column with the elution of MeCN–H₂O (85:15, t_R = 30 min) and MeCN–H₂O (90:10, t_R = 27 min), respectively. Compound **4** (24 mg) was isolated from Fr. 2.3 by Sephadex LH-20 (MeOH–CHCl₃, 50:50) and finally purified by HPLC with the mobile phase of MeCN–H₂O (60:40, t_R = 23 min). Fr. 2.4 was purified from by Sephadex LH-20 (MeOH–CHCl₃, 50:50) and HPLC on an Agilent XDB-C₁₈ column with the elution of MeCN–H₂O (60:40, t_R = 20 min) to yield compound **3** (3.5 mg). Fr. 3 was subjected to silica gel CC (Me₂CO-petroleum ether, 5:95, 10:90, 20:80, 40:60, 100:0) to yield five fractions (Fr. 3.1–Fr. 3.5). Fr. 3.1 was isolated by silica gel CC (Me₂CO–CHCl₃, 5:95, 10:90, 20:80, 40:60, 100:0), Sephadex LH-20 CC (MeOH–CHCl₃, 50:50), from which compounds **6** (2 mg, MeCN–H₂O, 60:40, t_R = 25 min) and **7** (37 mg, MeCN–H₂O, 45:55, t_R = 20 min) were obtained by HPLC purification. Further purification on Fr. 3.5 yielded compound **1** (10 mg) by silica gel CC, Sephadex LH-20 CC (MeOH–CHCl₃, 50:50) and semi-preparative HPLC (MeCN–H₂O, 30:70, t_R = 40 min).

Gastropolybenzylol G (1), pale yellow powder, UV (MeOH) λ_{max} (log ε): 281 (4.03), 254 (3.25) nm; IR (KBr) ν_{max}: 3426, 1631, 1613, 1511, 1439, 1247, 1229 cm⁻¹; HRESIMS m/z 517.2040 [M–H]⁻ (calcd 517.2020). ¹H-NMR and ¹³C-NMR data showed in Table 1.

Gastropolybenzylol H (2), yellow colloidal solid, UV (MeOH) λ_{max} (log ε): 275 (3.38), 248 (2.61), 226 (4.30) nm; IR (KBr) ν_{max}: 3423, 1613, 1513, 1223, 1072, 1003 cm⁻¹; HRESIMS m/z 333.1344 [M+COOH]⁻ (calcd 333.1344). ¹H-NMR and ¹³C-NMR data showed in Table 2.

Gastropolybenzylol I (3), white powder, UV (MeOH) λ_{max} (log ε): 275 (3.48), 250 (2.89), 229 (4.42) nm; IR (KBr) ν_{max}: 3430, 1703, 1614, 1518, 1268, 1245, 1029, 830 cm⁻¹; HRESIMS m/z 273.1117 [M+H]⁺ (calcd 273.1121). ¹H-NMR and ¹³C-NMR data showed in Table 2.

3.4 Bioassay of Agonistic Activities on MT₁ and MT₂ Receptors

Agonistic activities of compounds **1** and **2** were evaluated on HEK293 cell lines stably expressing the human melatonin MT₁ and MT₂ receptors. Referring to the previous study [27, 28], cells were cultivated by adding Dulbecco's modified eagle medium, G418 (400 μg/mL) and fetal bovine serum (10%) in 5% CO₂ incubator at 37 °C. Then, cells were put in a Matrigel[®] coated 96-well black plate at a density of 4 × 10⁴/well and proliferated in CO₂ for 24 h. Wash Free Fluo-8 Calcium Assay Kit (HD Biosciences Co.

Table 2 ^1H -NMR and ^{13}C -NMR data of compounds **2** and **3** in CD_3OD

No.	2		3	
	δ_{H} (500 MHz, <i>J</i> in Hz)	δ_{C} (125 MHz)	δ_{H} (600 MHz, <i>J</i> in Hz)	δ_{C} (150 MHz)
1	–	130.2 (s)	–	129.9 (s)
2	7.15 (1H, d, 8.6)	130.9 (d)	7.17(1H, d, 8.7)	131.2 (d)
3	6.75 (1H, d, 8.6)	116.1 (d)	6.85(1H, d, 8.7)	116.0 (d)
4	–	158.3 (s)	–	160.5 (s)
5	6.75 (1H, d, 8.6)	116.1 (d)	6.85(1H, d, 8.7)	116.0 (d)
6	7.15 (1H, d, 8.6)	130.9 (d)	7.17 (1H, d, 8.7)	131.2 (d)
7	4.40 (2H, s)	72.8 (t)	4.92 (2H, s)	71.2 (t)
1'	–	132.7 (s)	–	129.4 (s)
2'	7.24 (1H, d, 8.7)	130.6 (d)	7.15 (1H, d, 8.5)	130.6 (d)
3'	7.00 (1H, d, 8.7)	117.2 (d)	6.68 (1H, d, 8.5)	116.3 (d)
4'	–	158.5 (s)	–	158.6 (s)
5'	7.00 (1H, d, 8.7)	117.2 (d)	6.68 (1H, d, 8.5)	116.3 (d)
6'	7.24 (1H, d, 8.7)	130.6 (d)	7.15 (1H, d, 8.5)	130.6 (d)
7'	4.42 (2H, s)	72.4 (t)	4.85 (2H, s)	67.3 (t)
8'	5.21 (2H, s)	94.2 (t)	–	172.9 (s)
9'	3.70 (2H, q)	65.2 (t)	1.94 (3H, s)	21.0 (q)
10'	1.18 (3H, t)	15.5 (q)	–	–

Ltd., Hd03-0010) was used for detecting calcium flow assay. Flex Station 3 Benchtop Multi-Mode Micro Plate Reader was applied for recording and reading data at room temperature using specified settings (excitation wave length, 485 nm, emission wave length, 525 nm, emission cut-off, 515 nm). EC_{50} values were calculated by GraphPad Prism 5 software.

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Compliance with Ethical Standards

Conflict of interest These authors have no conflict of interest to declare.

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