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Polymethoxyflavones as agents that prevent formation of cataract: Nobiletin congeners show potent growth inhibitory effects in human lens epithelial cells

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ABSTRACT

Posterior capsular opacification (PCO) is the most frequent complication and the primary reason for visual decrease after extracapsular cataract surgery. The proliferation and migration of leftover lens epithelial cells (LECs) after surgery may contribute to the development of PCO. To prevent PCO, a rational approach would be to inhibit both the proliferation and the migration of LECs using nontoxic xenobiotics. Nobiletin, one of the most abundant polymethoxyflavones (PMFs) in citrus peel, and its synthetic congeners displayed a potent inhibition of LEC proliferation. Structural features which enhance anti-proliferative activity have also been discussed.

Cataract is the most common cause of vision impairment in the world today. It is curable with highly effective surgery, which involves the extracapsular extraction of the natural opaque lens fibers and the implantation of an intraocular lens (IOL).[1] However, many patients gradually develop posterior capsular opacification (PCO), also known as after-cataract (Fig. 1).[2–7] PCO progresses through three stages, which can be roughly described as: (1) the improper proliferation of lens epithelial cells (LECs) left behind after-cataract surgery; (2) the migration of LECs onto the posterior capsule underlying the intraocular lens and into the light path; (3) an epithelial–mesenchymal transition (EMT) resulting in the formation of fibroblasts and spindle-like myofibroblasts.[8–13] The incidence of PCO within 2 months to 5 years after initial surgery is as high as 50% in adults and 100% in children.[14]

Flavonoids are a group of polyphenolic compounds ubiquitously distributed throughout the plant kingdom. Several studies report that some flavonoids have protective effects against lens opacification in both in vivo and in vitro models of cataract.[15–18] However, the detailed mechanism of action has not been addressed so far. Recently, we reported that nobiletin (**1**) (Fig. 2), one of the most abundant polymethoxyflavones (PMFs) in *Citrus* species, and its three metabolites inhibit the production of the pro-matrix metalloproteinase (proMMP)-9.[19] This enzyme plays an important role in the migration of LECs, which occurs during the second stage of PCO development. Therefore, these flavones could be potent lead compounds for developing chemotherapeutic agents against PCO. Moreover, the study of divergent nobiletin congeners has unveiled a relationship between their structures and their biological activities.[20]

On the other hand, the effects of flavones on LEC proliferation, occurring at the first stage of PCO development, have thus far been left almost unexplored. With the exception of studies on baicalein[21] and quercetin,[22] no systematic study of the growth inhibitory effects of flavones has been reported. Herein, we examined the effects of nobiletin (**1**) and 16 synthetic congeners on the proliferation of LECs, one of the major processes leading to PCO.

We first examined the effects of nobiletin (**1**) on the proliferation of the human lens epithelial cell line SRA01/04.[23,24] Cell growth curve assays were performed using Alamar Blue stain[25] to monitor the changes in growth over 4 days. As demonstrated in Figure 3A, the proliferation of SRA01/04 cells in the nobiletin-treated group was significantly suppressed in a dose-dependent manner when compared to the control group. This tendency became more apparent over time. On day 4, the inhibitory rates of **1** at doses of 32 and 64 μM were 47% and 80%, respectively. These findings strongly suggesting that nobiletin (**1**) inhibits the proliferation of LECs were substantiated by western blot analysis of expression of proliferating cell nuclear antigen (PCNA), a marker

Abbreviations: IOL, intraocular lens; PCO, posterior capsule opacification; LEC, lens epithelial cell; EMT, epithelial–mesenchymal transition; PMF, polymethoxyflavone; proMMP, pro-matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase.

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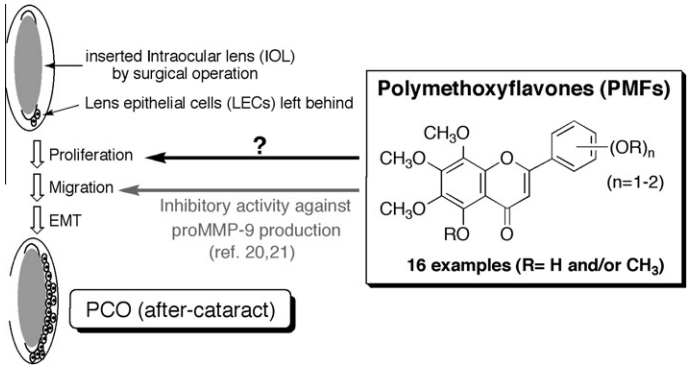


Figure 1. Pathogenesis of posterior capsule opacification (PCO) and proposed therapeutic roles of polymethoxyflavones (PMFs).

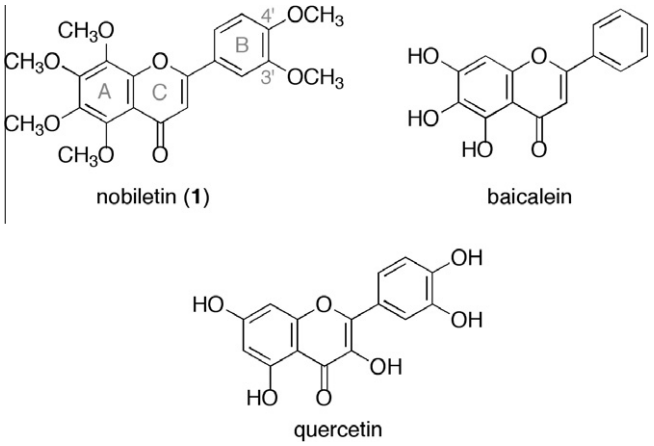


Figure 2. Structure of nobiletin, baicalein, and quercetin.

for cell proliferation.[26] As shown in Figure 3B, nobiletin (**1**) significantly decreased PCNA expression (p <0.05),[27] without significantly reducing β-actin expression.

Taking these observations into account, we next investigated the effects of other nobiletin congeners (Fig. 4) on the proliferation of SRA01/04 cells. In addition to the 14 PMFs reported previously,[19,20] compounds **7a**,[28] **7b**,[29] and **11**[30] were synthesized as per our previously reported protocol[19,20] as depicted in Scheme 1. Compound **7a** proved to be identical to the non-natural flavone synthesized previously.[31] Compound **11** was a flavone obtained from *Scutellaria baicalensis*.[32] The Alamar Blue assay[25] was performed using these compounds.[33,34] The results are shown as growth inhibitory rates compared to the control group on day 4. As demonstrated in Table 1, tangeretin (**6a**),[35] **7a**, **8a**, and **10**[35] were found to possess significantly stronger anti-proliferative activities, unlike that of nobiletin (**1**). Furthermore, in comparing the functional groups on the B ring, intriguing patterns emerged. Compounds **6b**, **7b**, and **8b**, which possess hydroxyl group(s) on the B ring, showed significantly lower inhibitory activities against LEC proliferation than compounds **6a** (p <0.001), **7a** (p <0.001), and **8a** (p <0.001), respectively. Similar results can be seen in comparisons between nobiletin (**1**) and **3** (p <0.01), between compounds **4a** and **4b** (p <0.001), and between compounds **5a** and **5b** (p <0.01) as well.[27] These observations suggest that a demethylation at the methoxy group(s) on the B-ring is associated with significant suppressive effects on the inhibition of LEC proliferation.

We also examined the effects of the 5-demethylated congeners **9**–**12** on the proliferation of SRA01/04 cells. Among these, compound **10** exhibited considerable inhibitory activity on cell growth. It is worth noting that compound **10** also exerted marked inhibitory effects (IC_{50}: 0.7 μM) on proMMP-9 production in PMA-treated cells.[20] Similarly, the growth inhibitory rates of compounds

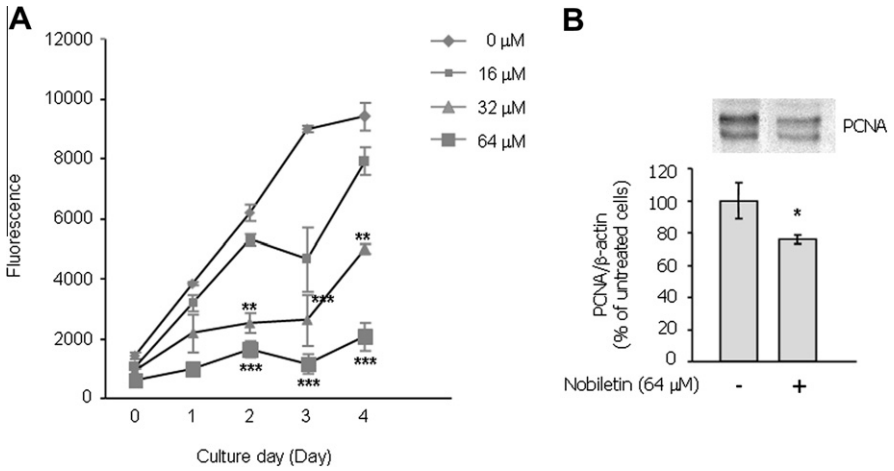


Figure 3. The antiproliferative effects of nobiletin on human lens epithelial cell line SRA01/04. (a) [A]: Cell growth curve assay by means of Alamar Blue stain. Data (n = 4) are shown as means ± SD. Asterisks indicate results that were significantly different from untreated control cells at each time (**: p <0.01; ***: p <0.001). [B]: Western blot analysis of proliferating cell nuclear antigen (PCNA) expression. Data (n = 4) are shown as means ± SD (*: p <0.05, significantly different from untreated group).

11 and **12** tended to augment compared to those of the corresponding 5-methoxy congeners **4b** and **6b**. On the other hand, the anti-proliferative activity of compound **9** was significantly lower (p <0.01) than that of nobiletin (**1**). This finding seems to be consistent with the report by Akao et al.[36] that the methoxy group at C5 in nobiletin (**1**) contributed to its anti-proliferative effect on human neuroblastoma SH-SY5Y cells.

In summary, we analyzed the growth inhibitory activity of nobiletin (**1**) and its 16 congeners on SRA01/04 cells. Compounds **6a**, **7a**, **8a**, and **10** showed potent growth inhibitory activities. Compound **10** exhibited both potent anti-proliferative effects on LEC cells and a strong inhibitory activity against proMMP-9 production. This compound would be a suitable lead for targeting multiple stages of PCO development. Our findings indicate the potential of such PMFs not only for medical treatment but also for a reasonable preventive measure against PCO. We hope that their effective use will replace laser capsulotomy in the near future.

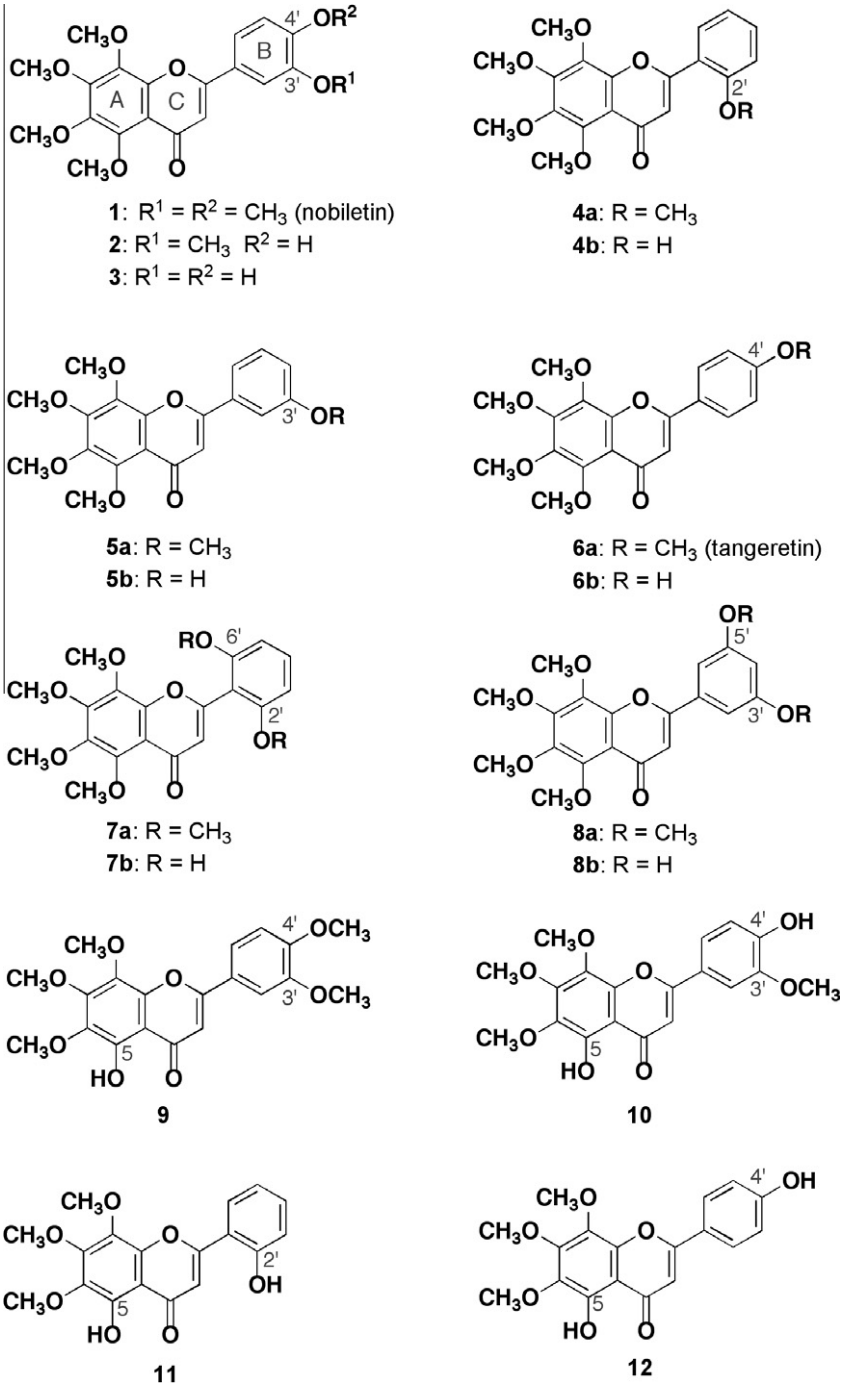
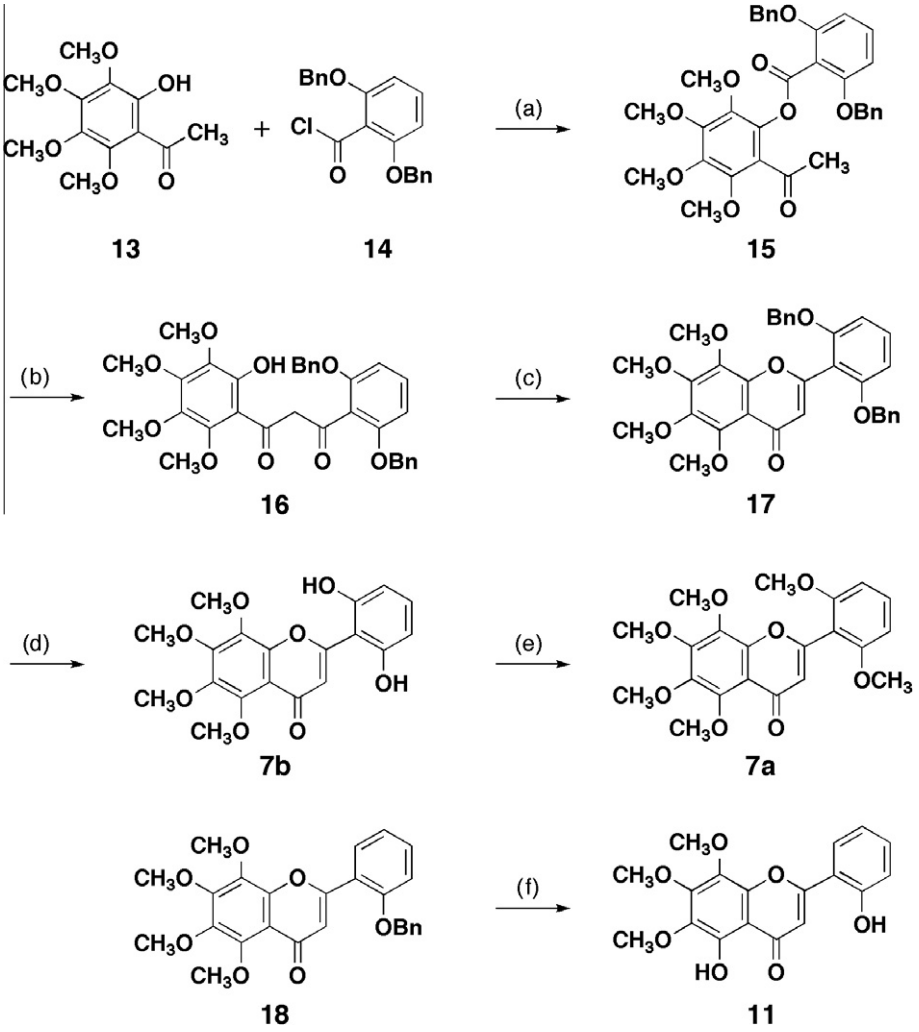


Figure 4. Structures of the nobiletin congeners.



Scheme 1. Representative synthetic procedure of compounds **7a**, **7b** and **11**: Reagents and conditions: (a) Et_3N (1.2 equiv), DMAP (0.1 equiv), CH_2Cl_2; 46% (82% based on recovered **13**); (b) *t*-BuOK (1.1 equiv), THF, reflux, 1 h; (c) *p*-TsOH·H_2O (0.25 equiv), benzene, reflux (Dean–Stark), 10 h; 81% in two steps; (d) H_2 (1 atm), 20% $Pd(OH)_2/C$ (cat.), EtOAc/EtOH (1:1), 1 h; 86%; (e) CH_3I (5 equiv), K_2CO_3 (3 equiv), DMF; 63%; (f) (i) BCl_3 (2 equiv.), CH_2Cl_2, −78 °C to rt; (ii) H_2 (1 atm), 20% $Pd(OH)_2/C$ (cat.), EtOAc/EtOH (1:1), 1 h; 82% in two steps.

Table 1
Growth inhibitory rates[a] and IC_{50} values for proMMP-9 production on PMA-treated SRA01/04 cells[b]

Compound	Substituent(s) on B ring	Growth inhibitory rate (%) at 32 μM[a]	IC_{50} (μM) for proMMP-9 production[b]
1: Nobiletin	3′,4′-Dimethoxy	47.4 ± 5.3	20.9 ± 6.5
2	3′-Methoxy-4′-Hydroxy	30.7 ± 4.1	3.7 ± 0.3
3	3′,4′-Dihydroxy	26.5 ± 5.5	12.3 ± 1.2
4a	2′-Methoxy	23.3 ± 4.2	13.7 ± 5.8
4b	2′-hydroxy	4.2 ± 3.8	0.4 ± 0.1
5a	3′-Methoxy	42.0 ± 5.5	5.5 ± 4.0
5b	3′-Hydroxy	18.3 ± 7.1	2.5 ± 3.4
6a: Tangeretin	4′-Methoxy	75.9 ± 6.5	6.8 ± 3.9
6b	4′-Hydroxy	32.0 ± 8.4	2.6 ± 1.4
7a	2′,6′-Dimethoxy	75.9 ± 4.8	> 64
7b	2′,6′-Dihydroxy	5.1 ± 4.3	55.0 ± 2.0
8a	3′,5′-Dimethoxy	59.9 ± 7.8	12.0 ± 6.6
8b	3′,5′-Dihydroxy	11.5 ± 6.8	8.1 ± 4.4
9	3′,4′-Dimethoxy	17.2 ± 8.0	3.0 ± 2.7
10	3′-Methoxy-4′-hydroxy	64.1 ± 9.2	0.7 ± 0.9
11	2′-Hydroxy	30.7 ± 10.9	>64
12	4′-Hydroxy	42.3 ± 6.8	3.5 ± 3.1

[a] Data ($n = 4$) are shown as means ± SD.
[b] IC_{50} values (μM) of **7a**, **7b**, and **11** for pro-MMP-9 production on PMA-treated SRA01/04 cells were newly determined. Other IC_{50} values were previously reported in Refs. 19 and 20. For experimental details, see also Ref. 20.

Acknowledgments

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24. Two striking similarities can be found between this cell line and the non-immortalized lens epithelial cells in artificially induced pathological conditions, that is, (1) their proliferative ability, and (2) their potential for matrix metalloproteinase (MMP)-9 production. We found that the inhibitors of mitogen-activated protein kinase (MAPK), such as SP600125 and SB203580, inhibit both cell proliferation and MMP-9 production in SRA01/04 cells. Since MAPKs (e.g., ERK, p38, and JNK) are also known to be involved both in the migration of other human lens epithelial cell lines[37–40] and in the proliferation of non-immortalized LECs stimulated by cytokines,[41,42] these phenomena may be because of closely related molecular mechanisms.
25. Experimental procedure in Figure 3A: The human lens epithelial cell line SRA01/04 was a kind gift from Dr. Nobuhiro Ibaraki (IBARAKI eye clinic, Tochigi, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 20% (v/v) heat-inactivated (56 °C for 30 min) fetal bovine serum (Biowest, Nuaille, France) and PSN antibiotic mixture (Invitrogen, penicillin/streptomycin/neomycin: 100 μg/mL each) at 37 °C in a humidified 5% CO_2 atmosphere. Upon reaching confluence, the SRA01/04 cells were subcultured (3×10^3 cells/well) and then treated with DMEM in the presence of various concentrations of nobiletin or the congeners for up to 4 days. The cell proliferation was analyzed with the Alamar Blue assay. Briefly, SRA01/04 cells were treated with flavonoids for up to 4 days in 96-well multiplates. For the last 2 h of the treatment, Alamar Blue® (Invitrogen) was added to the cells, after which the fluorescence intensity (FI) of the incorporated reagent was measured at 530 nm (excitation) and 590 nm (emission). The results were expressed as the inhibition of cell proliferation calculated as the ratio [($FI_{530/590}$ treated (Day 4) − $FI_{530/590}$ treated (Day 0)/ $FI_{530/590}$ control (Day 4) − $FI_{530/590}$ control (Day 0)) × 100].
26. Experimental procedure in Figure 3B: SRA01/04 cells (2×10^5 cells/dish) plated onto 60-mm dishes were harvested and lysed in RIPA buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM Tris–HCl (pH 7.5), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. The total cell protein (7 μg) was separated by 12.5% SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and then hybridized with mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Sigma, St. Louis, MO) or mouse anti-β-actin antibody (Sigma) overnight at 4 °C. After incubation with the alkaline-phosphatase-conjugated secondary antibody for 1 h at room temperature, immunoreactive PCNA or β-actin was visualized indirectly using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as chromogenic substrates. The protein concentration of the cellular fraction was measured by using a bicinchoninic acid (BCA) protein analysis kit (Pierce Biotechnology, Rockford, IL).
27. Statistical analysis: A one-way analysis of variance (ANOVA) was used for statistical analysis. The Fisher test was applied when multiple comparisons were performed.
28. ^{1}H NMR spectrum and melting point of **7a** are in full accord with those reported previously.[31] *Compound* **7a**: ^{13}C NMR ($CDCl_3$) δ 177.5, 158.6, 158.2, 151.0, 148.9, 148.3, 143.7, 138.0, 132.0, 129.7, 128.8, 115.6, 115.1, 111.2, 103.9, 62.2, 61.9, 61.8, 61.7, 55.9 (2C); HRMS calcd. for $[M+H]^+$ of $C_{21}H_{22}O_8$: 403.1387; found: 403.1389.
29. Compound **7b** (new compound): mp 246–248 °C (decomp.); ^{1}H NMR (DMSO-d_6) δ 9.85 (2H, br s), 7.11 (1H, t, *J* = 8.0 Hz), 6.43 (2H, d, *J* = 8.0 Hz), 6.10 (1H, s), 4.00 (3H, s), 3.85 (3H, s), 3.84 (3H, s), 3.78 (3H, s); ^{13}C NMR (DMSO-d_6) δ 176.3, 159.6, 157.3, 151.3, 148.7, 148.0, 143.9, 138.2, 132.2, 115.0, 114.9, 108.8, 107.1, 62.4, 62.2, 62.1, 61.9; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_8$: 375.1074; found: 375.1070.
30. Compound **11**: mp 229–231 °C [Lit.[32] 228-230 °C]; ^{1}H NMR (DMSO-d_6) δ 12.17 (1H, s), 10.25 (1H, s), 7.96 (1H, d, *J* = 8.0 Hz), 7.71 (1H, s), 7.42 (1H, ddd, *J* = 1.6 Hz, 8.0 Hz, 8.0 Hz), 7.12 (1H, br d, *J* = 8.0 Hz), 7.02 (1H, br t, *J* = 8.0 Hz), 4.15 (3H, s), 4.00 (3H, s), 3.98 (3H, s); ^{13}C NMR (DMSO-d_6) δ 182.8, 161.9, 157.0, 152.6, 148.4, 145.4, 135.7, 133.1, 132.6, 128.3, 119.6, 117.1, 61.8, 61.46 61.45, 60.6; HRMS calcd. for $[M-H]^-$ of $C_{18}H_{16}O_7$: 343.0823; found: 343.0816.
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