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The comparison of neuroprotective effects of isoliquiritigenin and its Phase I metabolites against glutamate-induced HT22 cell death

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ABSTRACT

It is becoming increasingly important to investigate drug metabolites to evaluate their toxic or preventive effects after administration of the parent compound. In our previous study, isoliquiritigenin isolated from Glycyrrhizae Radix effectively protected mouse-derived hippocampal neuronal cells (HT22) against 5 mM glutamate-induced oxidative stress. However, there is little information on the protective effects of the metabolites of isoliquiritigenin on HT22 cells. In this study, isoliquiritigenin and its Phase I metabolites were prepared and their neuroprotective activities on glutamate-treated HT22 cells were compared. The prepared metabolites were liquiritigenin (**1**), 2′,4,4′,5′-tetrahydroxychalcone (**2**), sulfuretin (**3**), butein (**4**), davidigenin (**5**), and *cis*-6,4′-dihydroxyaurone (**6**). Among the six metabolites, **4** showed better neuroprotective effects than the parent compound, isoliquiritigenin. Our study suggests that the neuroprotective effect of isoliquiritigenin could be elevated by its active metabolite **4,** which is a chalcone containing a catechol group in the B ring.

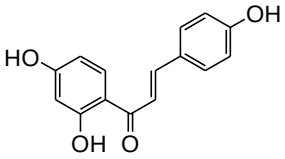
Keywords:

Isoliquiritigenin; Phase I metabolites; Butein; Neuroprotection; HT22; Glutamate

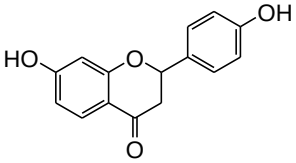
Isoliquiritigenin (ISOLIQ), a kind of chalcone, is known as a key component of Glycyrrhizae Radix (licorice roots),[1-3] and has various biological effects such as anti-cancer,[4-5] anti-inflammatory,[6] and neuroprotective[1-3] effects. By inducing mitochondrial dysfunction, ISOLIQ promotes apoptosis of the prostate cancer cells, MAT-LyLu and DU145, which are derived from rat and human, respectively.[5] It also induces heme-oxygenase (HO)-1 expression by activating extracellular signal-regulated kinases (ERK) 1/2, which are associated with lipopolysaccharide (LPS)-induced nitric oxide (NO) and tumor necrosis factor (TNF)-α production in murine macrophage cells (RAW 264.7).[6] In addition, ISOLIQ attenuates 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in mouse embryonic substantia nigra-derived cell line (SN4741) by regulating mitochondrial function and *c*-Jun *N*-terminal kinase (JNK) and p38 phosphorylation.[2] In our previous study, ISOLIQ was isolated from the ethanolic extract of *Glycyrrhiza uralensis* and used as a neuroprotectant against 5 mM glutamate-induced oxidative stress in a mouse-derived hippocampal neuronal cell line (HT22).[3]

The investigation of drug metabolic fate and metabolites is important in the drug discovery field, especially for toxicological studies, tracking of the active materials after biotransformation, and the information for further drug design.[7-8] The metabolism of ISOLIQ *in vitro* has been evaluated by HPLC-MS analysis in several studies, where the Phase I metabolites of ISOLIQ were confirmed in human and rat liver microsomes; they are liquiritigenin (**1**), 2′,4,4′,5′-tetrahydroxychalcone (**2**), sulfuretin (**3**), butein (**4**), davidigenin (**5**), *cis*-6,4′-dihydroxyaurone (**6**), and *trans*-6,4′-dihydroxyaurone (**7**) in human and rat liver microsomes.[9-10] The major metabolites are **1**, **3**, **4**, and **7**, and the minor ones are **2**, **5**, and **6**. The biological effects of each metabolite have been investigated separately in many studies; they include osteoblastic cell protection,[11] anti-inflammation,[12-13] neuroprotection,[14] anti-leukemia,[15] hepatocytes protection,[16] and anti-cancer[17] effects. However, there is little information on the comparison of metabolites' biological activities in the same batch, especially on hippocampal

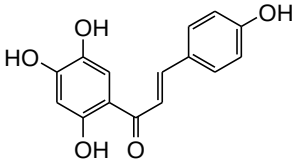
neuronal cells. Therefore, we synthesized or isolated ISOLIQ Phase I metabolites (Figure 1) and examined their effect on glutamate-induced HT22 cell death.



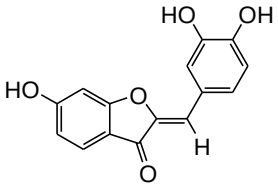
Isoliquiritigenin



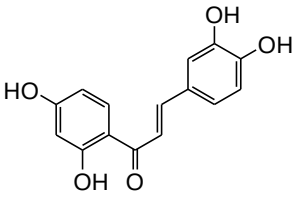
Liquiritigenin (**1**)



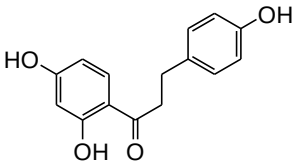
2′,4,4′,5′-Tetrahydroxychalcone (**2**)



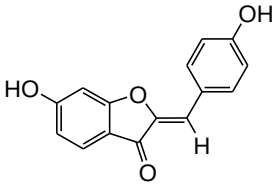
Sulfuretin (**3**)



Butein (**4**)



Davidigenin (**5**)



cis-6,4′-Dihydroxyaurone (**6**)

Figure 1. The chemical structures of ISOLIQ and the prepared Phase I metabolites **1** - **6**.

ISOLIQ was synthesized by aldol condensation as previously described.[18] The purified yellow powder was used for ^{1}H and ^{13}C NMR analysis and the spectral data were compared with those of in the literature.[1]

1 was purified from the ethanolic extract of *Glycyrrhiza uralensis* by silica gel column chromatography in the previous study and identified its structure without determination of stereochemistry.[19]

2 was synthesized[20] and its NMR spectral data is shown in Table 1. ^{1}H NMR showed typical 1,4-disubstituted benzene ring at δ 7.60 and 6.85 (d, *J* = 8.4 Hz each). Two resonances with large coupling constants (*J* = 15.3 Hz) at δ 7.77 and 7.50 suggested that the presence of a *trans*-olefinic moiety. In ^{13}C NMR, fifteen carbons including one carbonyl carbon at δ 191.8 were detected. Four oxygenated aromatic carbons were detected at δ 160.1, 159.9, 154.5, and 150.9. The peak at δ 144.1 could be assigned to a β-position to the carbonyl group. Combined these NMR data, **2** was identified as 2′,4,4′,5′-tetarhydroxychalcone.

Table 1

^{1}H and ^{13}C NMR spectral data of synthesized isoliquiritigenin (ISOLIQ) metabolite **2** in CD_3OD (δ in ppm; *J* Hz)

	2	
Position	^{1}H	^{13}C
1′	-	112.0
2′	-	154.5
3′	6.34 s	102.8
4′	-	159.9

5′	-	138.1
6′	7.39 s	114.2
1	-	191.8
2	7.77 d (15.3)	144.1
3	7.50 d (15.3)	117.1
1″	-	126.5
2″	7.60 d (8.4)	130.3
3″	6.85 d (8.4)	115.6
4″	-	160.1
5″	6.85 d (8.4)	115.6
6″	7.60 d (8.4)	130.3

Chemical shifts (δ; ppm) of ^{1}H NMR (500 MHz) were referenced using tetramethylsilane (TMS) as an internal standard, and those of ^{13}C NMR (125 MHz) were referenced to the solvent.

3 was synthesized according to the previous report[21] with some modifications.[22]

4 was synthesized by referring to the procedure of ISOLIQ preparation. However, 18.36 mM (2.54 g) 3,4-dihydroxybenzaldehyde was used as a reactant instead of 4-hydroxybenzaldehyde. Butein (200.00 mg, 0.73 mM) was yielded in the form of an orange powder. The ^{1}H and ^{13}C NMR spectral data of the product were compared with those in previous literature.[23]

5 and **6** were synthesized according to the literature.[9]

To compare the protective effect of seven compounds against glutamate-induced HT22 cell death, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazlium bromide (MTT) cell viability

assay, detection of reactive oxygen species (ROS), and Western blotting experiments were carried out by using reported methods.[19, 24]

It is known that a high concentration of glutamate over millimolar can cause oxidative stress via inhibition of cystine uptake, leading to a lack of glutathione in the cells.[25] In this study, HT22 cells were treated with 5 mM glutamate for 12 h to induce cell death. With this treatment, the cell viability was reduced to 43.35 ± 1.48% compared to the vehicle control (100.00 ± 2.73%). The cell viability was recovered by 78.93 ± 1.24% in the 10 μM ISOLIQ-pretreated group. Metabolites **2**, **3**, and **4** showed higher protective efficacy than ISOLIQ, with treated cells showing viabilities of 83.51 ± 2.13, 93.73 ± 2.34, and 92.46 ± 1.56%, respectively, at the same concentration (Figure 2).

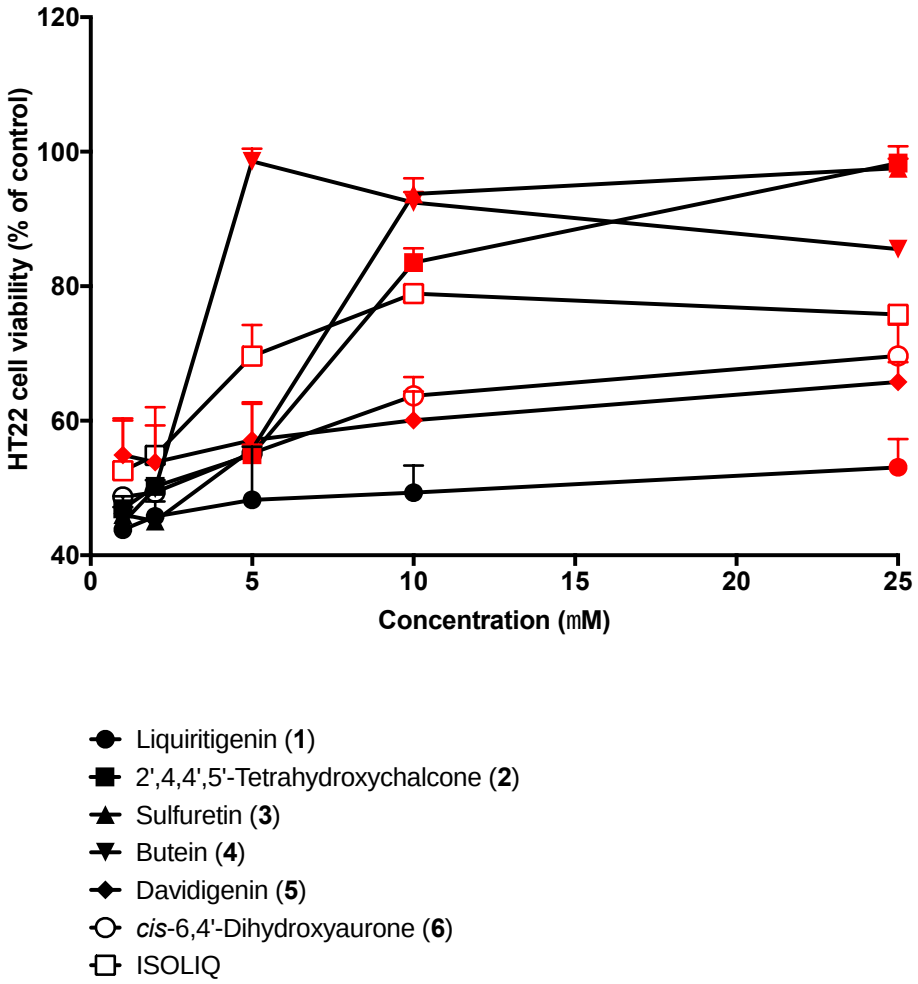
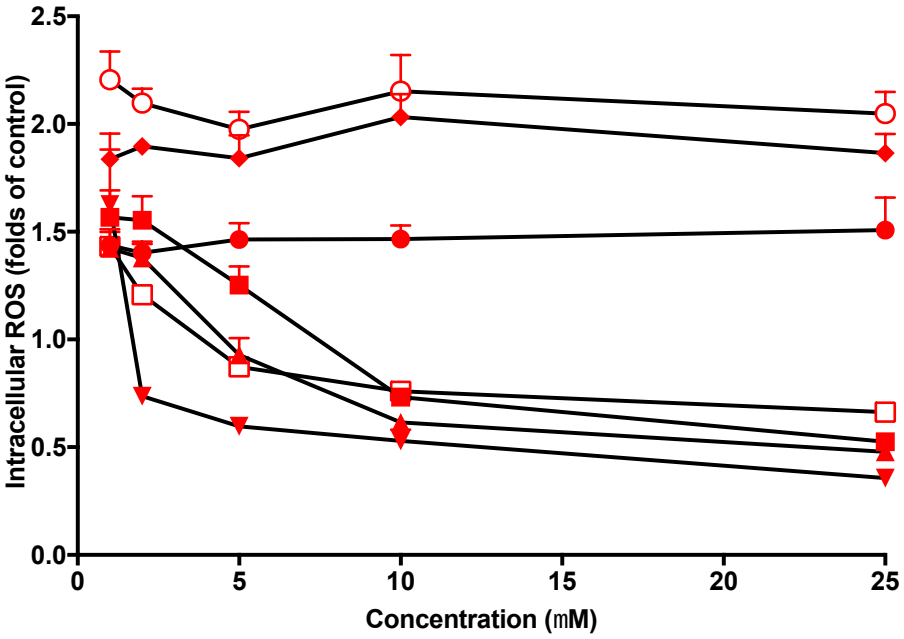


Figure 2. The comparison of HT22 cell protective effects between ISOLIQ and its metabolites on 5 mM glutamate-induced cell death. The cell viability was reduced to 43.35 ± 1.48% compared to the vehicle control (100.00 ± 2.73%). Quercetin (10 μM) was used as a positive control (93.67 ± 2.50%). The symbols marked as red indicates $p < 0.05$ compared to the 5 mM glutamate-only. The value of butein (**4**) showed statistical difference compared to other metabolites and ISOLIQ at 5 μM.

The intracellular ROS production was significantly increased to 241.63 ± 7.98% after administration of 5 mM glutamate, whereas the group treated with 5 μM ISOLIQ showed a decrease to 96.19 ± 2.28% compared to the vehicle control (100.00 ± 8.06%) measured by a microplate reader. In particular, 5 μM of **2**, **3**, and **4** significantly decreased the intracellular ROS production to 137.94 ± 9.50, 102.25 ± 8.57, and 59.79 ± 4.07%, respectively (Figure 3A). In the flow cytometry analysis, 5 mM of glutamate increased intracellular ROS production, 3.95 ± 0.01-fold compared to vehicle control (1.00 ± 0.01-fold), while 5 μM ISOLIQ and metabolites **2**, **3**, and **4** attenuated the glutamate-induced ROS generation to 1.44 ± 0.01, 2.39 ± 0.03, 1.82 ± 0.01, and 0.69 ± 0.03-fold, respectively (Figure 3B). From these results, it was confirmed that **4** is the most effective compound among the tested metabolites and the parent compound ISOLIQ at 5 μM.

A)



- Liquiritigenin (**1**)
- 2',4,4',5'-Tetrahydroxychalcone (**2**)
- Sulfuretin (**3**)
- Butein (**4**)
- Davidigenin (**5**)
- *cis*-6,4'-Dihydroxyaurone (**6**)
- ISOLIQ

B)

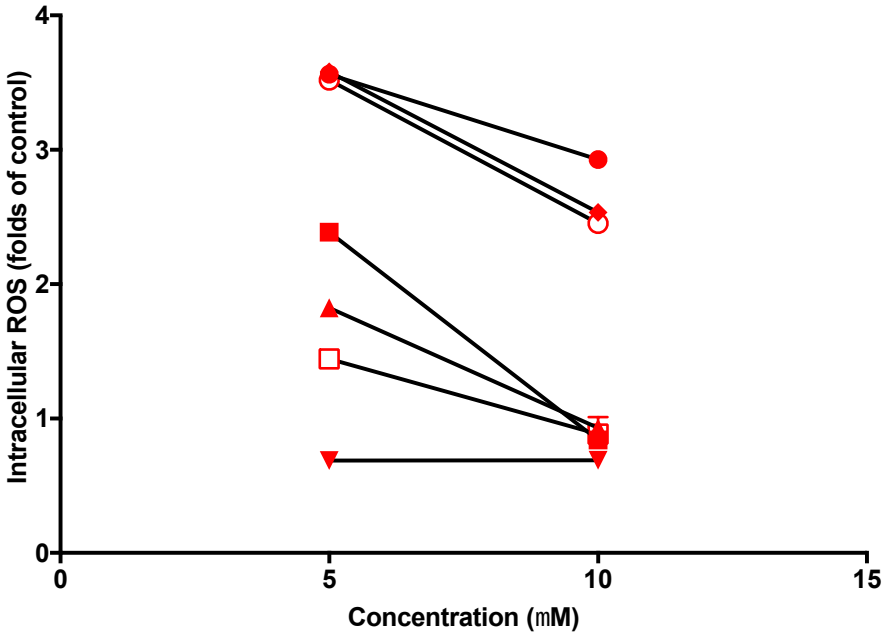
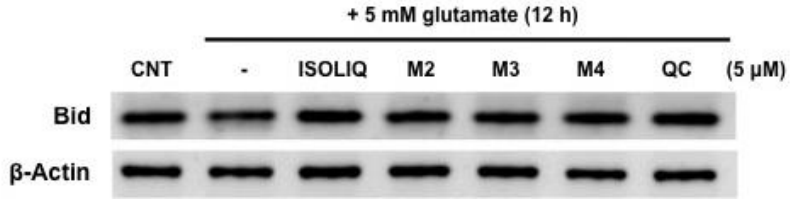


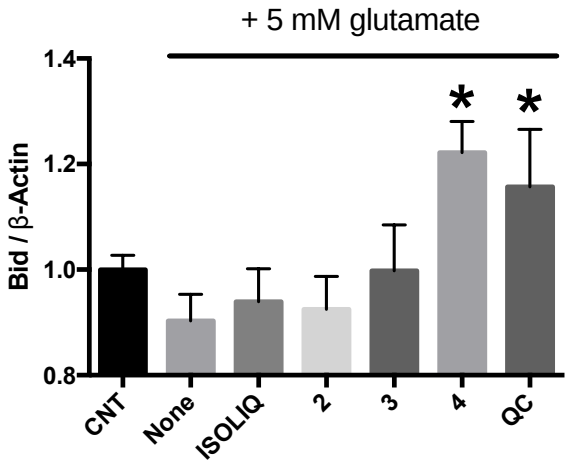
Figure 3. The inhibitory effects of ISOLIQ and its metabolites on 5 mM glutamate-induced intracellular ROS production measured by a microplate reader (A) and flow cytometry (B). The intracellular ROS production was significantly increased to 241.63 ± 7.98% by glutamate compared to the vehicle control (100.00 ± 8.06%) measured by a microplate reader. In addition, glutamate increased intracellular ROS production to 3.95 ± 0.01-fold compared to vehicle control (1.00 ± 0.01-fold) in the flow cytometry analysis. Quercetin (10 μM) was used as a positive control (53.84 ± 4.98% in Figure 3A; 0.79 ± 0.03-fold in Figure 3B). The symbols marked as red indicates $p < 0.05$ compared to the 5 mM glutamate-only. The value of butein (**4**) showed statistical difference compared to other metabolites and ISOLIQ at 5 μM.

To investigate the HT22 protective mechanism of the most active compounds, ISOLIQ, **2**, **3**, and **4**, Western blotting was performed. The reduced full-length Bid level by 5 mM glutamate

(0.90 ± 0.05-fold; control, 1.00 ± 0.03-fold) was effectively recovered by **2**, **3**, and **4** at 5 μM. Especially, the Bid level of **4**-treated cells was higher (1.22 ± 0.06-fold) than those of ISOLIQ- and QC-treated cells (0.94 ± 0.06 and 1.16 ± 0.11-fold, respectively), at 5 μM. The phosphorylated p38 (pp38) was also elevated by 5 mM glutamate (2.46 ± 0.96-fold) as compared to the control (1.00 ± 0.38-fold). However, **2**, **3**, and **4** significantly decreased pp38 level to 1.21 ± 0.50, 1.09 ± 0.52, and 0.75 ± 0.45-fold, respectively. Moreover, the pp38 level of **4**-treated cells was lower than that of parent compound ISOLIQ-treated cells (1.03 ± 0.43-fold). Glutamate also increased the phosphorylated ERK (pERK) level to 3.01 ± 0.30-fold (control, 1.00 ± 0.08-fold), while that was attenuated by **2** (1.64 ± 0.11-fold), **3** (2.02 ± 0.08-fold), and **4** (1.49 ± 0.31-fold), respectively. However, the pERK level was not reduced by three metabolites as much as ISOLIQ (0.80 ± 0.06-fold) or QC (0.77 ± 0.04-fold). In addition, **2**, **3**, and **4** significantly suppressed the phosphorylation of JNK induced by 5 mM glutamate (1.84 ± 0.11-fold; control, 1.00 ± 0.13-fold) to 1.08 ± 0.03, 0.72 ± 0.15, and 0.73 ± 0.36-fold, respectively. The pJNK levels of **3**- or **4**-treated cells were lowered than that of ISOLIQ-treated cells (1.00 ± 0.03-fold; Figure 4). These data demonstrated that **4** had better HT22 protective effects than its parent compound ISOLIQ, on glutamate-induced cell death resulted by the ROS production, Bid reduction, and the phosphorylation of p38 and JNK, except for phosphorylation of ERK.

A)





B)

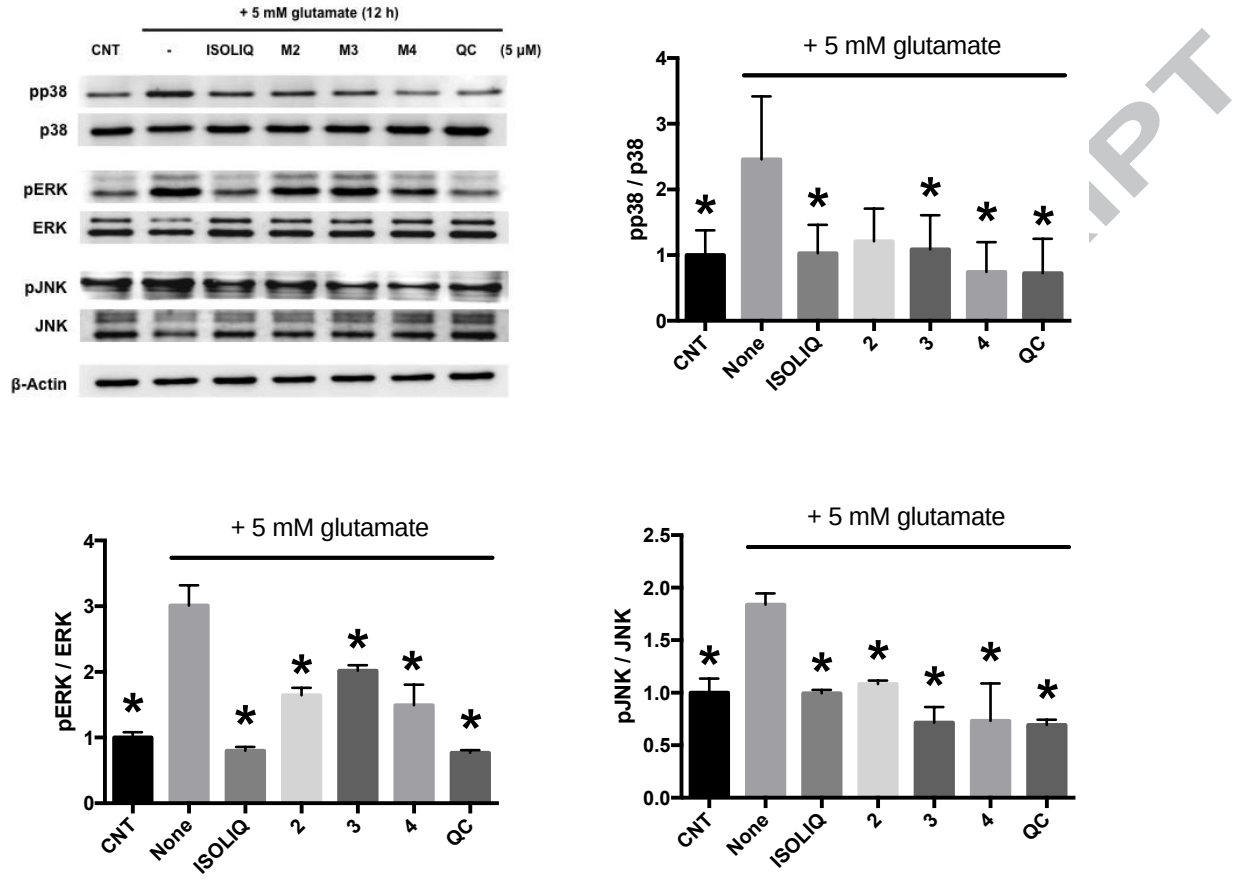


Figure 4. The inhibitory effects of ISOLIQ and its three metabolites (**2** - **4**) on 5 mM glutamate-induced reduction of full-length Bid (A) and the phosphorylation of mitogen-activated protein kinases (MAPKs; B). *, $p< 0.05$ compared to the 5 mM glutamate only.

We demonstrated that butein (**4**), one of the major Phase I metabolite of ISOLIQ,[9-10] has strong protective activities against 5 mM glutamate-induced cytotoxicity in HT22 cells. Compared with other compounds, the presence of catechol moiety in B ring was believed to be the key pharmacophore, as previous study indicated that this moiety is responsible for radical

scavenging,[26] as well as an inhibitory effect on lipid peroxidation.[27] In addition, it has been discussed that the HT22 cell protection efficacy against glutamate toxicity would be increased by the presence of two hydroxyl groups attached on C3′ and C4′ positions (a catechol-bearing B ring), compared to only one hydroxyl on C4′.[28] It was also reported that catechol type compounds exhibited neuroprotective effects via conversion of catechol to a quinone, a kind of electrophile.[29] The electrophiles adduct with the cysteine residues on the Keap1, leading to the stabilization of nuclear factor erythroid 2-related factor 2 (Nrf2) following its translocation from cytosol into the nucleus to bind to the antioxidant-responsive element (ARE). The ARE plays a vital role in the induction of detoxificative Phase II enzymes, such as heme oxygenase-1 (HO-1), γ-glutamyl cysteine ligase (γ-GCL), and NADPH quinone oxidoreductase 1 (NQO1).[30-32] The cytoprotective mechanisms are subsequently enhanced via regulation of the intracellular redox status.[30-32] There is literature describing that a catechol moiety-bearing diterpene isolated from *Rosmarinus officinalis*, carnosic acid, showed the neuroprotective effects against glutamate-induced toxicity through ARE activation and Phase II enzyme induction mediated by the transformation of catechol to quinone in the carnosic acid structure.[33-34] Metabolite **2**, which has a catechol group on the A ring but not the B ring, also recovered the HT22 cells but the protective activity was the lowest among three metabolites **2**, **3**, and **4**, indicating that the position of catechol moiety is more important rather than its presence.

In this study, HT22 cell protective effects of the parent compound ISOLIQ and its metabolites were compared for the first time. In addition, the metabolite **2** was firstly synthesized and identified by spectroscopic analysis. Further study on the structure-activity relationship (SAR) would be helpful for the discovery of ISOLIQ-derived anti-neurodegenerative drug candidates.

Acknowledgements

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20. Chloromethyl methyl ether (0.14 mL, 1.79 mM) was added to a stirred solution of 2,4,5-trihydroxyacetophenone (50.00 mg, 0.30 mM) and *N*,*N*-diisopropylethylamine (0.31 mL, 1.79 mM) in acetonitrile. The mixture was stirred overnight. In a separate flask, a mixture of 4-hydroxybenzaldehyde (37.00 mg, 0.30 mM), *N*,*N*-diisopropylethylamine (0.11 mg, 0.60 mM), and chloromethyl methyl ether (0.05 mL, 0.60 mM) in acetonitrile was stirred for 3 h. After the given reaction time, volatiles in the each of the flasks were removed under reduced pressure, and the residues were each dissolved in ethanol (1.00 mL each). The ethanolic solutions were mixed together, and a solution of KOH (168.00 mg, 3.00 mM) in water (0.10 mL) was added. The combined mixture was stirred overnight, and then diluted with ethyl acetate. The mixture was then treated with 1 N-HCl and extracted with ethyl acetate. Volatiles were removed under the reduced pressure, and the residue was dissolved in methanol (2.00 mL). 2 N-HCl (0.30 mL) was added and the reaction mixture was refluxed for 1 h. The resulting mixture was diluted with water and extracted with ethyl acetate. Volatiles were removed under the reduced pressure, and the residue was purified with preparative HPLC (YMC-Actus Triart C18, gradient, acetonitrile/water with 0.1% acetic acid) to yield 2',4,4',5'-tetrahydroxychalcone (**2**) as a yellow solid (38.00 mg, 47%); R_f 0.3 (acetone : *n*-hexane = 1 : 1); IR (ATR-IR) ν_{max} 3333, 3224, 1512, 1160 cm^{-1}; HR-MS calculated for $C_{15}H_{12}O_5$ 272.0685, found 272.0683.

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22. KOH (2.00 g) in water (2.00 mL) was added to a stirred solution of 6-hydroxy-2,3-dihydrobenzo[*B*]furan-3-one (200 mg, 1.33 mM) and 3,4-dihydroxybenzaldehyde (219.00 mg, 1.59 mM) in ethanol (4.00 mL). The mixture was refluxed for 3 h. The resulting mixture was diluted with water and washed with dichloromethane. 1 N-HCl was added to the aqueous phase, and the mixture was extracted with ethyl acetate. The organic phase was dried on anhydrous $MgSO_4$, and volatiles were removed under reduced pressure. The remaining residue was purified with SiO_2 chromatography to yield sulfuretin (**3**) as yellow solid (305.00 mg, 85%); R_f 0.2 (ethyl acetate : *n*-hexane = 1 : 1).

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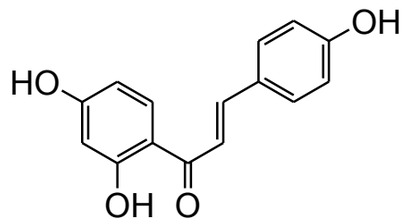
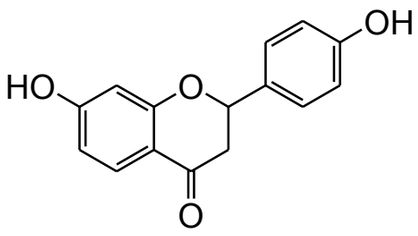
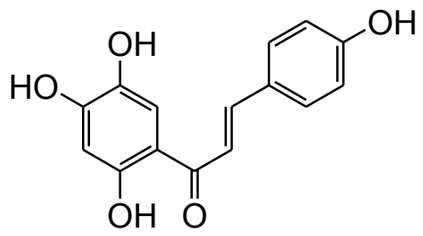


Isoliquiritigenin

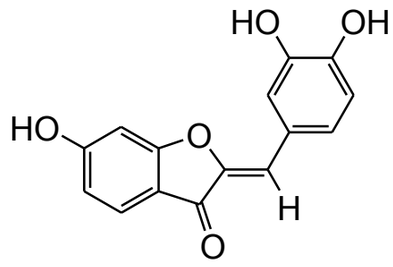
Phase I metabolites



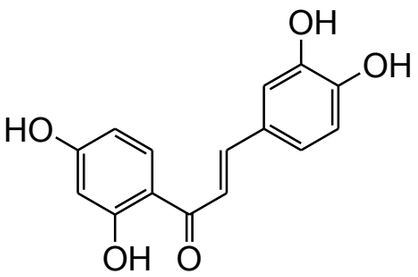
Liquiritigenin (1)



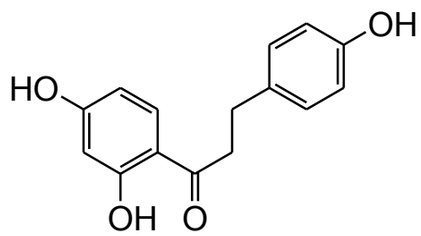
2′,4,4′,5′-Tetrahydroxychalcone (2)



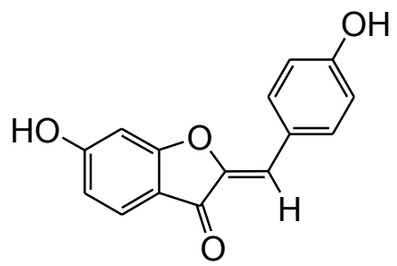
Sulfuretin (3)



Butein (4)



Davidigenin (5)



***cis*-6,4′-Dihydroxyaurone** (6)

The comparison of HT22 cell protective effects against glutamate toxicity

Butein (4) is the most active compound

Highlights

- Isoliquiritigenin (ISOLIQ) and its six Phase I metabolites were prepared.
- The neuroprotective effects of seven compounds were compared on glutamate toxicity.
- Butein (**4**) showed better effects than the parent compound, ISOLIQ.
- **4** is a chalcone containing a catechol group in the B ring.
- The neuroprotective effect of ISOLIQ could be elevated by its metabolite **4**.