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Mannich bases of scutellarein as thrombin-inhibitors: Design, synthesis, biological activity and solubility

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ABSTRACT

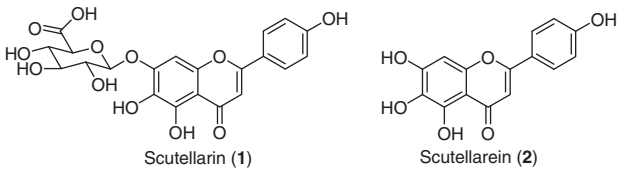
Two series of 8-aminomethylated derivatives were prepared by Mannich reaction of scutellarein (**2**) with appropriate aliphatic amines, alicyclic amines and formaldehyde. All the compounds were tested for their thrombin inhibition activity through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). The antioxidant activities of these target products were assessed by 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay using 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay method and the solubility were assessed by ultraviolet (UV). The results showed that morpholinyl aminomethylene substituent derivative (**3d**) demonstrated stronger anticoagulant activity, better water solubility and good antioxidant activity compared with scutellarein (**2**), which warrants further development as a agent for ischemic cerebrovascular disease treatment.

1. Introduction

Nowadays, ischemic cerebrovascular disease is a common and frequently-occurring disease that seriously endangers human health. It is one of the leading causes of death and disability worldwide.[1] Increasing evidence suggests a critical role of thrombin in ischemic cerebrovascular disease,[2] thrombin is generated in response to vascular injury, it acts as a multifunctional serine protease and catalyzes the proteolytic cleavage of the soluble plasma-protein fibrinogen to form insoluble fibrin leading to clot formation. In addition, thrombin also serves as a potent platelet agonist and amplifies its own generation by feedback activation of several steps in the coagulation cascade.[3] Secondly, oxidative stresses are major causes of ischemic cerebrovascular disease,[4,5] reactive oxygen species (ROS) including the superoxide anion radical ($O_2^{-}\cdot$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and nitric oxide ($NO^{\cdot}$) are constantly generated by various physiological functions in the human body.[6] The excessive production of ROS may result in increased levels of low-density lipoprotein (LDL), oxidative modification of LDL, and an impairment of endothelial derived relaxing factor (EDRF, nitric oxide, NO)-mediated bioactions.[7]

On this basis, some researchers have recently proposed and searched some natural product with anticoagulant capacity and antioxidant activity for the treatment of cerebrovascular disease.[8] Scutellarin(4′,5,6-trihydroxyflavone-7-glucuronide) (Fig. 1), the major anti-oxidant constituent in breviscapine extracted from Chinese herb of *Erigeron breviscapus* (vant.) Hand.–Mazz., showed the effectiveness on dilating blood vessels, improving microcirculation, increasing cerebral blood flow, and inhibiting platelet aggregation since the 1970s.[9] In addition, it has been clinically used to treat acute cerebral infarction and paralysis induced by cerebrovascular diseases such as hypertension, cerebral thrombosis, cerebral haemorrhage in China since 1984.[10]

However, scutellarin has low water-solubility,[11] and the bioavailability of scutellarin was very low with the absolute bioavailability in Beagle dog administered orally was rarely 0.4%.[12] Interestingly, some researchers found that scutellarin was mainly



Scutellarin (**1**) Scutellarein (**2**)

Figure 1. Chemical structures of scutellarin (**1**) and scutellarein (**2**).

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absorbed in the form of its hydrolyzed product scutellarein (**2**) (Fig. 1) by intestinal,[13] furthermore, in the clinical trials,[12] a large amount of scutellarein (**2**) was found in urine and plasma after oral administration of breviscapine, indicating that breviscapine was firstly hydrolyzed into aglycone when reaching colon and was then absorbed as scutellarein (**2**) as the real bioactive components in the body. Pharmacodynamics confirmed that scutellarein (**2**) had better protective effect than scutellarin (**1**) in rat cerebral ischemia,[14] scutellarein (**2**) can prevent thrombosis and platelet aggregation, and improve the characteristics of hemorheology in rats.[15] In our previous studies, we found that scutellarein (**2**) had stronger scavenging capacities toward DPPH, $ABTS^{+\cdot}$, ·OH free radicals than scutellarin (**1**), and had better protective effect on H_2O_2-induced cytotoxicity in PC12 cells.[16,17] These results suggested that scutellarein (**2**) can be a promising lead compound for the discovery of potent agent with thrombin-inhibitory and antioxidant activities for the treatment of ischemic cerebrovascular disease.

Firstly, our docking studies of scutellarein (**2**) with thrombin (2R2M)[18] showed that the B ring and C ring in ligand could interact well with S1 pocket and S2 pocket, respectively (Fig. 2), however, A ring partly interact with the S3 pocket in thrombin, so the inhibitory activity could be improved if a side chain is introduced at position 8 in the A ring by the Mannich reaction. Furthermore, Mannich bases have been associated with increased bioactivity.[19] The presence of a Mannich base group in many natural products may increase biological potency due to the greater number of molecular sites for electrophilic attack by cellular constituents, as well as due to the cascade effect of preferential chemosensitization. Aminoalkylation of aromatic substrates by the Mannich reaction has considerable importance for the synthesis and modification of biologically active compounds. This technique provides convenient access to many useful synthetic building blocks, because the resulting amino group can be easily converted to various functionalities, particularly, to quaternary ammonium salts to increase water solubility.

Herein, we describe the synthesis and in vitro biological evaluation of Mannich bases of scutellarein with electrophilic substitution at C-8, together with a discussion of structure–activity relationships. The thrombin inhibition activity of all the new analogs were evaluated through the analyzation of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB). The antioxidant activities of these target products were assessed by 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay using 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay method and the water solubility were assessed by ultraviolet (UV).

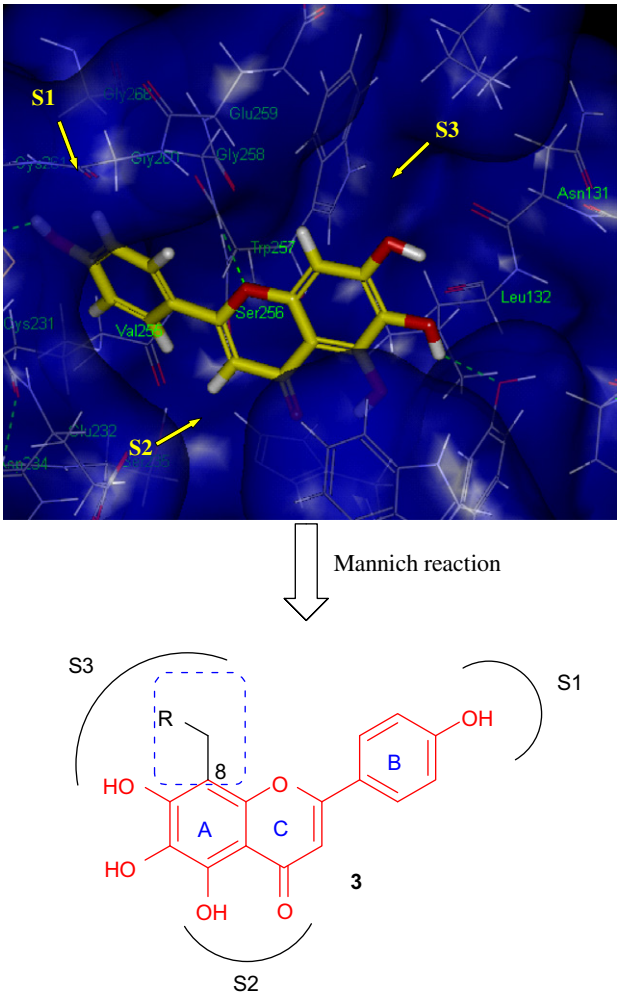


Figure 2. The strategy for the design of compound **3**.

2. Results and discussion

2.1. Chemistry

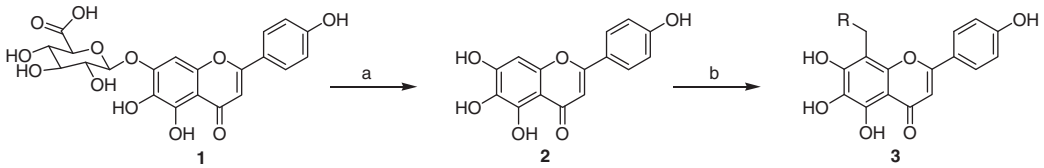
In general, Mannich bases of scutellarein derivatives **3a**–**3g** were synthesized via a two-step procedure as described in Scheme 1. According to our previous procedure,[16,17] the scutellarein (**2**) could be obtained from the hydrolysis of scutellarin (**1**) by refluxing it with 6 N HCl in 90% ethanol under the N_2 protection. Then our strategy for the synthesis of the targeted analogues was achieved by the Mannich reaction of the scutellarein (**2**) with formaldehyde in the presence of the secondary amines in methonal (Scheme 1). The classical conditions of the Mannich reaction for the hydroxyl compounds are based on the ratio of substrate, amine and formaldehyde in alcohol with prolonged heating.[20] In our case, scutellarein (**2**), formaldehyde and secondary amines were in 1:1:1.1 ratio, respectively, and stirred at 30 °C for 30 min–24 h to afford the C8-aminomethylated derivatives. The structures of the resulting Mannich bases were confirmed by ^{1}H NMR and mass analysis. The ^{1}H NMR spectra of compounds **3a**–**3g** clearly indicated the absence of the signal at d 6.57 for H-8 proton of the flavanoid ring system. It should be noted that in all cases the electrophilic substitution occurred solely in ring A even at the large excess of the reagent and under more severe conditions.[21]

2.2. Biological activity

2.2.1. Anti-thrombic activity

Because the thrombin inhibition activity can be assessed by assaying the prolongation of the plasma clotting time of TT, APTT, INR increasement of PT, and reduction of FIB content according to our previous studies,[18] so the thrombin time of different compounds was investigated for TT, PT, APTT and FIB. The results were shown in Table 1.

There were two series of Mannich bases of scutellarein, one series was aliphatic amine (methyl, ethyl and isopropyl group) substituents (**3a**–**3c**), and the other series was alicyclic amine (morpholinyl or piperzinyl ring) substituents (**3d**–**3g**). As shown in Table 1, both series showed stronger thrombin inhibition activity compared with scutellarein (**2**). In the series of aliphatic amine substituents Mannich bases, the most active compound was ethyl amine Mannich base (**3b**), this compound significantly prolonged TT and APTT, increased PT and decreased FIB content compared to scutellarein (**1**). When the ethyl group changed into methyl



3a. R=Dimethyl amino (C_2H_6N)
3b. R=Diethyl amino ($C_4H_{10}N$)
3c. R=Diisopropyl amino ($C_6H_{14}N$)
3d. R=Morpholinyl (C_4H_8NO)
3e. R=N-methyl piperzinyl ($C_5H_{11}N_2$)
3f. R=N-ethyl piperzinyl ($C_6H_{13}N_2$)
3g. R=N-n-butyl piperzinyl ($C_8H_{17}N_2$)

Scheme 1. Reagents and conditions: (a) Concentrated hydrochloric acid, EtOH, N_2, 120 °C, 17.0%; (b) MeOH, formaldehyde–water (37%), amine, 30 °C, 30.7–41.3%.

Table 1
Effect of Mannich bases of scutellarein on thrombin time

Compd. (25 μM)	Plasma coagulation parameters			
	TT (S)	APTT(s)	PT(s)	FIB(g/L)
2	10.25 ± 0.24	33.36 ± 2.47	7.34 ± 0.30	5.96 ± 0.64
3a	12.58 ± 0.42	34.23 ± 2.34	7.45 ± 0.42	5.56 ± 0.36
3b	12.63 ± 0.35	34.62 ± 4.45	7.55 ± 0.39	5.36 ± 0.26
3c	11.75 ± 0.80	34.52 ± 4.22	7.47 ± 0.27	5.54 ± 0.52
3d	14.48 ± 0.48	35.48 ± 4.26	7.84 ± 0.31	5.26 ± 0.31
3e	14.40 ± 0.14	35.42 ± 3.38	7.71 ± 0.19	5.46 ± 0.46
3f	14.13 ± 0.19	35.15 ± 2.51	7.41 ± 0.27	5.54 ± 0.20
3g	13.45 ± 0.37	35.01 ± 3.58	7.40 ± 0.37	5.56 ± 0.33

Data represent mean ± S.D. $n = 4$.

group or isopropyl group, the thrombin inhibition activity decreased, such as in the case of **3a**, it shortened TT and APTT, decreased PT and increased FIB compared to **3b**. This result revealed that the presence of ethyl aminomethylene group substitution at C8 position was very important in showing thrombin inhibition activity. In the series of alicyclic amine Mannich bases, our data indicated that morpholinyl aminomethylene substituent (**3d**) showed the most active thrombin inhibitory activity among all the synthesized Mannich bases of scutellarein derivatives, when the morpholinyl group changed into piperzinyl group, the thrombin inhibition activity decreased, for example, **3e** shortened TT and APTT, decreased PT and increased FIB compared to **3d**. One interesting phenomenon for the piperzinyl ring substituent was that the thrombin inhibition activity decreased as the atom numbers of the substituent in the piperzinyl ring became more, for instance, the *n*-butyl piperazinyl substituent **3g** shortened TT and APTT, decreased PT and increased FIB compared to the methyl substituent **3e**.

In the thrombin inhibition activity tests, **3d** showed the strongest inhibitory activity on thrombin, so **3d** was selected for the subsequent molecular docking experiment with thrombin (2R2M) (Fig. 3). The thrombin docking showed that the morpholinyl aminomethylene substituent at C7 in **3d** occupied the deep S3 pocket (composed of Asn131, Leu132, Iie209, Gly258, Glu259, Ser256, Try257) of thrombin. As displayed in Figure 4, ligand **3d** formed three hydrogen bonds with the active site residues of 2R2M in the binding mode, and the active site residues were Asp229, Ala230 and Tyr83. These results confirmed our strategy for designing Mannich bases of scutellarein as thrombin-inhibitors.

2.2.2. Antioxidants

Antioxidants protect the aging brain against oxidative damage associated with pathological changes of ischemic cerebrovascular disease. So the in vitro antioxidant activity[22] of scutellarein derivatives was evaluated by DPPH[23] radical-scavenging activity assays using MTT assay method. DPPH assay measured the hydrogen-donating ability of antioxidants to convert the stable DPPH free radical into 1,1-diphenyl-2-picrylhydrazine.[24] The reaction was accompanied by a change in color from deep-yellow to light-yellow and was monitored spectrophotometrically. The IC_{50} value is defined as the concentration of sample that causes 50% loss of the radical.

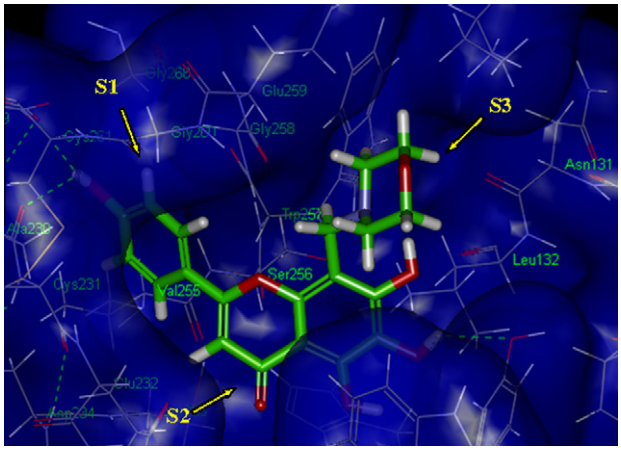


Figure 3. **3d** docked into the pockets of thrombin (2R2M).

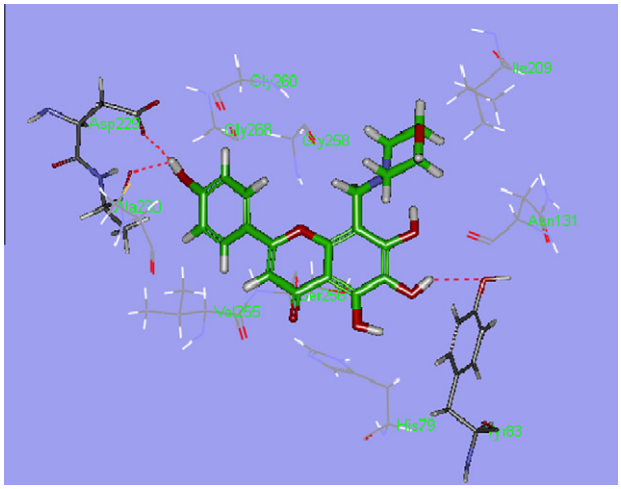


Figure 4. **3d** docked into residues of 2R2M, hydrogen bonding interactions are shown as red dashed lines.

The data obtained was depicted in Table 2. Although these compounds showed no more antioxidant activity than scutellarein (**2**), they still exhibited some interesting inhibition of oxidative activity. From the results obtained in the DPPH assay, we can infer that, compounds **3a**–**3c**, where the C8 position on the A-ring was occupied by aliphatic aminomethylene groups, showed a higher antioxidant activity than the compounds **3d**–**3e** where the C8 position on the A-ring was occupied by alicyclic aminomethylene groups, such as in the case of **3a** and **3g**, where the IC_{50}s were 26.46 μM and 32.70 μM, respectively. Among all the synthesized Mannich bases

Table 2
In vitro antioxidant activity in DPPH (IC_{50} in μM) radical-scavenging assay and the solubility in water of Mannich derivatives

Compd.	Antioxidant antivity IC_{50}[a](μM)	Water solubility	
		Solubility [a] (μM)	Fold increase[b]
2	24.04	6.95	1.0
3a	26.46	8.58	1.23
3b	24.96	7.71	1.11
3c	30.08	7.97	1.15
3d	30.89	11.62	1.67
3e	32.47	7.28	1.05
3f	32.44	7.07	1.02
3g	32.70	9.11	1.31

[a] Values are the means of at least two independent determinations; errors are within ±20%.
[b] Fold increase in water solubility relative to scutellarein (**2**).

of scutellarein, the most active compound was **3b**, with its IC_{50} for DPPH was 24.96 μM.

2.2.3. Solubility

The aqueous solubility of the synthesized Mannich bases of scutellarein has been determined using UV spectrophotometer.[25–27] As presented in Table 2, the water solubitily of the scutellarein derivatives has been greatly increased compared to scutellarein (**2**). The best soluble compound was morpholinyl aminomethylene substituent (**3d**) with its solubility in water was 11.62 μg/ml, which was increased 1.67-fold compared to that of scutellarein (**2**). Among the aliphatic amine substituents Mannich bases of scutellarein, methyl amine (**3a**), ethyl amine (**3b**) and isopropyl amine (**3c**) exhibited remarkable increases in solubility and showed 1.23-fold, 1.11-fold and 1.15-fold, respectively. Among the alicyclic amine Mannich bases of scutellarein **3d**–**3g**, introduction of methyl piperzinyl ring (**3e**) and ethyl piperzinyl ring (**3f**) showed similar solubility to that of scutellarein (**2**), with their values were 7.28 μg/ml and 7.07 μg/ml, respectively. Interestingly, replacement of methyl and ethyl group in the piperzinyl ring by the larger *n*-butyl group (**3g**) further increased experimental solubility about 1.31-fold compared to that of scutellarein (**2**).

3. Conclusion

Scutellarin has been clinically used to treat acute cerebral infarction and paralysis induced by cerebrovascular diseases such as hypertension, cerebral thrombosis, cerebral haemorrhage in China for many years,[10] and it showed the effectiveness on dilating blood vessels, improving microcirculation, increasing cerebral blood flow, and inhibiting platelet aggregation.[9] However, scutellarin has pharmacokinetic problems such as low solubility in water and fast metabolism which results in its low bioavailability. In this study, based on the metabolic mechanism of scutellarin in vivo, we took scutellarien (**2**) as a promising lead compound, designed and synthesized new Mannich base derivatives of scutellarein such as aliphatic amine substituents (**3a**–**3c**) and alicyclic amine substituents (**3d**–**3g**) to improve biological activities of scutellarien (**2**). In particular, morpholinyl aminomethylene substituent (**3d**), demonstrated stronger anticoagulant activity, better water solubility and good antioxidant activity compared with scutellarein (**2**), which warrants further development as a agent for ischemic cerebrovascular disease treatment.

4. Experimental

4.1. General methods

Reagents and solvents were purchased from commercial sources and used without further purification unless otherwise specified. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel cannula. Organic solutions were concentrated by rotary evaporation below 45 °C at approximately 20 mm Hg. All non-aqueous reactions were carried out under anhydrous conditions using flame-dried glassware within an argon atmosphere in dry and freshly distilled solvents, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.15∼0.20 mm Yantai silica gel plates (RSGF 254) using UV light as the visualizing agent. Chromatography was performed on Qingdao silica gel (160∼200 mesh) using petroleum ether (60∼90) and ethyl acetate as the eluanting solvent. The melting points (Mp) were measured on a WRS-1B apparatus and were not corrected. ^{1}H NMR spectra were obtained using a Bruker AV-300 (300 MHz) and AV-500 (500 MHz). Chemical shifts were recorded in ppm downfield from tetramethylsilane. *J* values were given in Hz. Abbreviations used were s (singlet), d (doublet), t (triplet), q (quartet), b (broad), and m (multiplet). ESI-MS spectra were recorded on a Waters Synapt HDMS spectrometer.

4.2. Synthesis of Mannich bases of scutellarein

4.2.1. Synthesis of scutellarien (2)

To a stirring mixture of **1** (10.0 g, 20.6 mmol) and concentrated hydrochloric acid (120 mL) in ethanol (120 mL) was added water (10 mL), the reaction mixture was refluxed under a N_2 atmosphere for 36 h. After cooled down to the room temperature, the mixture was poured into water. The solid obtained was filtered followed by silica gel column chromatographic purification of the residue using 50% ethyl acetate in petroleum ether afforded the compound **2** in 17.0% yield as yellow solid, mp 160–162 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.78 (s, 1H, C_5-OH), 10.44 (s, 1H, $C_{4'}$-OH), 10.29 (s, 1H, C_7-OH), 8.71 (s, 1H, C_6-OH), 7.90 (d, J = 8.8 Hz, 2H, $C_{2'}C_{6'}$-H), 6.91 (d, J = 8.8 Hz, 2H, $C_{3'}C_{5'}$-H), 6.74 (s, 1H, C_3-H), 6.57 (s, 1H, C_8-H). ESI-MS: m/z 287 $[M+H]^+$.

4.2.2. General procedure for the preparation of Mannich bases of scutellarein 3a–3g

To a solution of scutellarein (**2**) (0.35 mmol) in MeOH (7.5 mL) was added 37% formaldehyde–water (27.5 μL, 0.35 mmol, 1.0 equiv), after vigorous stirring at 30 °C for 30 min, different amines (0.39 mmol, 1.1 equiv) were added and the mixture was stirred for 30 min–24 h after the reaction was completed. The resulting mixture was evaporated under vacuo and the crude product was purified by silica gel column chromatography (20% MeOH in CH_2Cl_2) to yield the desired Mannich bases of scutellarein **3a–3g** (yields 30.7–41.3%).

4.2.2.1. 8-(*N*,*N*-Dimethyl)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3a). Yellow solid, 32.6% yield, mp 216–218 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 13.11 (s, 1H, C_5-OH), 10.25 (s, 1H, $C_{4'}$-OH), 8.05 (s, 1H, C_6-OH), 7.95 (d, J = 9.0 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.93 (d, J = 9.0 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H), 6.96 (s, 1H, C_3-H), 4.13 (s, 2H, CH_2), 2.56 (s, 6H, 2 × CH_3). ESI-MS: m/z 344 $[M+H]^+$.

4.2.2.2. 8-(*N*,*N*-Diethyl)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3b). Yellow solid, 41.3% yield, mp 206–207 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.75 (s, 1H, C_5-OH), 7.91 (d, J = 9.0 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.94 (d, J = 9.0 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H), 6.68 (s, 1H, C_3-H), 4.28 (s, 2H, CH_2), 2.90–2.97 (q, 4H, 2 × CH_2), 1.17–1.22 (t, 6H, 2 × CH_3). ESI-MS: m/z 372 $[M+H]^+$.

4.2.2.3. 8-(*N*,*N*-Diisopropylamine)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3c). Yellow solid, 39.6% yield, mp 223–225 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.78 (s, 1H, C_5-OH), 7.95 (d, J = 8.5 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.93 (d, J = 8.5 Hz, 2H, $C_{3'}$-H,

$C_{5'}$-H), 6.88 (s, 1H, C_3-H), 4.28 (s, 2H, CH_2), 2.30–2.34 (m, 2H, 2 × CH), 1.19–1.23 (d, 12H, 4 × CH_3). ESI-MS: m/z 400 $[M+H]^+$.

4.2.2.4. 8-(Morpholine base)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3d). Yellow solid, 32.7% yield, mp 208–210 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.89 (s, 1H, C_5-OH), 10.29 (s, 1H, $C_{4'}$-OH), 7.94 (d, J = 9.0 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.95 (d, J = 9.0 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H), 6.75 (s, 1H, C_3-H), 3.94 (s, 2H, CH_2), 3.58–3.63 (t, 4H, 2 × CH_2), 2.52–2.63 (t, 4H, 2 × CH_2). ESI-MS: m/z 386 $[M+H]^+$.

4.2.2.5. 8-(*N*-Methyl piperazine base)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3e). Yellow solid, 35.8% yield, mp 215–217 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.86 (s, 1H, C_5-OH), 10.28 (s, 1H, $C_{4'}$-OH), 7.92 (d, J = 8.5 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.93 (d, J = 8.5 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H), 6.73 (s, 1H, C_3-H), 3.95 (s, 2H, CH_2), 2.60–2.64 (t, 4H, 2 × CH_2), 2.46–2.50 (t, 4H, 2 × CH_2), 1.22 (s, 3H, CH_3). ESI-MS: m/z 399 $[M+H]^+$.

4.2.2.6. 8-(*N*-Ethyl piperazine base)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3f). Yellow solid, 38.4% yield, mp 221–223 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.83 (s, 1H, C_5-OH), 7.93 (d, J = 8.7 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.95 (d, J = 8.7 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H), 6.73 (s, 1H, C_3-H), 4.02 (s, 2H, CH_2), 2.66–2.70 (t, 4H, 2 × CH_2), 2.40–2.44 (t, 4H, 2 × CH_2), 2.33–2.38 (q, 2H, CH_2), 0.97–1.02 (t, 3H, CH_3). ESI-MS: m/z 413 $[M+H]^+$.

4.2.2.7. 8-(*N*-Butyl piperazine base)-methyl-amino-5,6,7,4′-tetrahydroxyflavone (3g). Yellow solid, 34.3% yield, mp 217–219 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.87 (s, 1H, C_5-OH), 10.26 (s, 1H, $C_{4'}$-OH), 7.93 (d, J = 8.5 Hz, 2H, $C_{2'}$-H, $C_{6'}$-H), 6.94 (d, J = 8.5 Hz, 2H, $C_{3'}$-H, $C_{5'}$-H,), 6.73 (s, 1H, C_3-H), 4.01 (s, 2H, CH_2), 2.63–2.67 (m, 4H, 2 × CH_2), 2.30–2.42 (m, 4H, 2 × CH_2), 1.38–1.41 (t, 2H, CH_2), 1.23–1.31 (m, 4H, 2 × CH_2), 0.86–0.89 (t, 3H, CH_3). ESI-MS: m/z 441 $[M+H]^+$.

4.3. DPPH radical scavenging activity

DPPH radical scavenging activity was determined using the method according to our procedure[16,17] with minor modifications. The solution of the sample (100 μL) in dimethylsulfoxide (DMSO) was added to 100 μL of DPPH radical in ethanol (0.2 mM) in 96-well plate. The sample solution refers to the tested compounds and the reference antioxidants at various concentrations, as well as DMSO as a control. The solutions of the tested compounds had concentrations ranging from 15.6 μM to 1000 μM. The reaction leading to the scavenging of DPPH radical was completed within 30 min at 25 °C. The absorbance of the mixture was then measured at 517 nm with a microplate reader. The inhibition of DPPH radical was expressed as percentage: scavenging rate (%) = $(1-A_{test}/A_{control}) \times 100$, where A_{test} was the absorbance of a sample at a given concentration after 10 min reaction time and $A_{control}$ was the absorbance recorded for 100 μL DMSO. The IC_{50} value was defined as the concentration of sample that causes 50% loss of the DPPH radical.

4.4. Water solubility

The solubility of scutellarein derivatives in water was determined using the known method[25–27] with minor modifications. The UV/UV device is composed of a UV source (for UV photodegradation) and a UV absorption detector (for on-line UV measurement). The UV source is a low pressure mercury lamp and the UV detector is a UV-vis spectrophotometer Anthelie (Secomam) controlled by software Dathelie, version 4.1f. The pathlength of the Suprasil quartz cell is 10 mm and the scan speed is 2000 nm min^{-1}. Under the influence of UV radiation, scutellarein was monitored by UV absorption spectrophotometry at the wavelength of maximum absorbance (334 nm) from the whole spectrum using a multicomponent exploitation method. Each tested compound (300 μg) was dissolved in 25 mL CH_3OH. The solutions of the tested compounds had concentrations ranging from 3 μg/mL to 12 μg/mL. Different concentration solutions of each compound were determined by UV scanning, and the absorbances were obtained. The results showed a good linear relationship, then all the standard curves were completed. Each tested compound (120 μg) was ultrasound dissolved in 10 mL pure water for 1 h at room temperature. The solutions were stranded for 30 min and centrifuged at the speed of 30000 r/min. The aqueous solution of each compound was determined by UV scanning, and the absorbances were obtained. Then through the analyzation of standard curve, all the compounds in the water solubility were obtained.

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