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Short communication

Active components of Dengzhan Shengmai ameliorate cognitive impairment by facilitating hippocampal long-term potentiation via the NMDA receptor-mediated Ca^{2+}/CaMKII pathway

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Abnormal synaptic plasticity causes cognitive deficits. Hippocampal long-term potentiation (LTP) is a critical synaptic plasticity process [1]. Rescuing impaired LTP is challenging; hence, novel agents are required for LTP facilitation. Chinese medicine Dengzhan Shengmai (DZSM) has shown notable clinical efficacy against cognitive deficits [2]. However, it remains unclear how DZSM modulates cognition. Our previous study [3] revealed the influence of DZSM on glutamatergic and GABAergic synapses following chronic cerebral hypoperfusion (CCH), which motivated us to assess how DZSM affects synaptic functions.

We performed brain transcriptomic analysis (Table S1 and Sections S1 and S2 in the Supplementary data) in CCH rats to confirm the effect of DZSM on synaptic functions (Figs. 1A and S1A–C). This was then validated by recording field excitatory postsynaptic potentials (fEPSPs) in rat hippocampal slices. LTP was significantly attenuated ($P < 0.01$) in CCH rats as evidenced by the decreased fEPSP slope, indicating cognitive impairment. DZSM restored impaired LTP ($P < 0.01$), and LTP did not significantly differ between the DZSM-treated and Sham groups (Figs. 1B, 1C, and S1D). These findings demonstrate that DZSM rescued CCH-induced LTP deficits.

A practical approach was developed to identify active components of DZSM that facilitate LTP under CCH conditions (Fig. 1D). CCH injury is associated with several factors such as inflammation, excitotoxicity, and energy deprivation. To focus on LTP, we conducted *in vitro* investigations with SH-SY5Y cells as an excitotoxicity model, as this model shares the molecular pathway with LTP, specifically the *N*-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway (Section S3 in the Supplementary data) [4]. NMDA receptors not only play pivotal roles in synaptic plasticity but also contribute to excitotoxicity. Normal NMDA receptor activation promotes LTP. However, excessive activation causes Ca^{2+} overload, inhibition of CaMKII activation, and ultimately LTP deficits. Among over 200 components in DZSM, 14 components detected in the brain and plasma of CCH rats [5] (Fig. S2) were screened for anti-excitotoxicity activity in SH-SY5Y cells. This screening identified an active component group (ACG). Further *in vitro* and *in vivo* studies were performed for elucidating mechanisms of active components in facilitating LTP through the NMDA receptor-mediated Ca^{2+}/CaMKII pathway.

NMDA or glutamate (Glu)-induced excitotoxic models were established using SH-SY5Y cells. Ten components (marked in red in Fig. S2), including 5-*O*-caffeoylquinic acid (5-CQA), caffeic acid (CA), 4-*O*-caffeoylquinic acid (4-CQA), scutellarin (Scu), 3,4-*O*-dicaffeoylquinic acid (3,4-CQA), apigenin-7-*O*-glucuronide (Api), ginsenoside Rc (Rc), schizandrol (SolA), schizandrin A (SchA), and schizandrin B (SchB), exhibited protective effects against excitotoxic damage (Fig. S3A). Ifenprodil, an NMDA receptor subunit 2B (NR2B)-specific inhibitor, was included as a positive control (Fig. S3B). The ACG comprised 10 components in fixed ratios (Table S2). The ACG showed similar anti-excitotoxicity activity in SH-SY5Y cells as DZSM (Figs. S3C and D), suggesting that ACG largely retained the efficacy of DZSM.

We then aimed to elucidate the mechanism of active components against LTP impairment and excitotoxicity. Effects of the 10 active components on CaMKII phosphorylation at Thr286 residue

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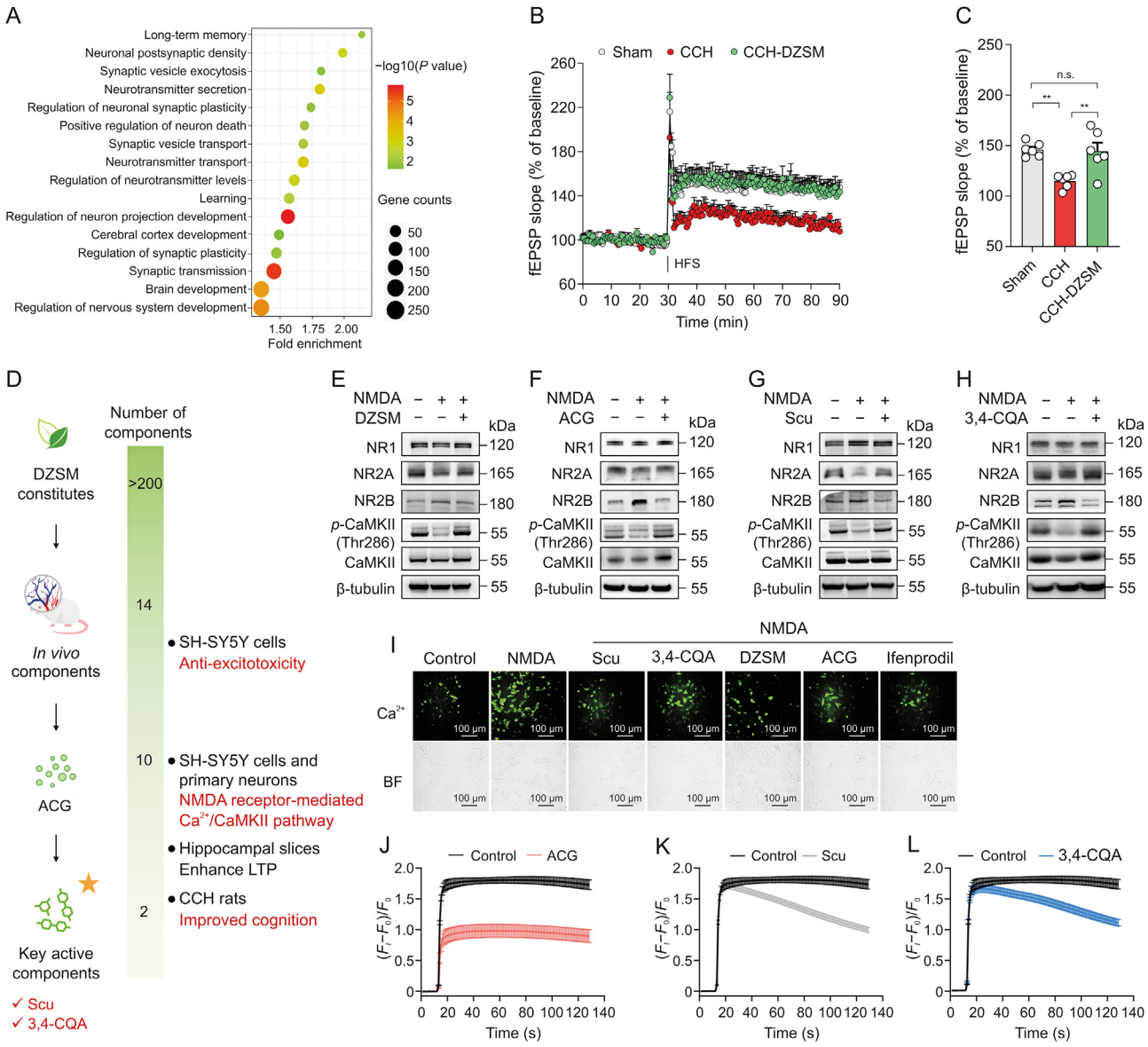

Fig. 1. Dengzhan Shengmai (DZSM) and its active components rescue chronic cerebral hypoperfusion (CCH)-associated hippocampal long-term potentiation (LTP) deficits and regulate the *N*-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway *in vitro*. (A) Bubble plot of the selected Gene Ontology (GO) terms for differentially expressed genes (DEGs) in the brain tissues of CCH rats and DZSM-treated CCH rats. The bubble size represents the gene counts of each term. The color intensity of the bubble indicates the *P* value of each term. (B) Time course for the normalized slope of field excitatory postsynaptic potentials (fEPSPs) before and after inducing an LTP. We induced LTP by high-frequency stimulation delivered at time 30 (line) at CA3-CA1 synapses in hippocampal slices ($n = 6$). Results are shown as the percentage of baseline fEPSP slope (=100%). (C) Averaged fEPSP slope recorded in the last 10 min. Data are indicated as mean ± standard error of the mean (SEM). n.s.: no significant difference. One-way analysis of variance (ANOVA) and Dunnett's test, $^{**}P < 0.01$. (D) A practical approach for identifying DZSM active components that facilitate LTP under CCH conditions. (E–H) Effects of 10 μg/mL DZSM (E), 10 μg/mL active component group (ACG) (F), 10 μmol/L scutellarin (Scu) (G), and 10 μmol/L 3,4-*O*-dicaffeoylquinic acid (3,4-CQA) (H) on the expression of NMDA receptor subunit 1 (NR1), NR2A, NR2B, phospho-CaMKII (Thr286) (*p*-CaMKII (Thr286)), and CaMKII proteins in SH-SY5Y cells after NMDA exposure. (I) Representative images of intracellular Ca^{2+} fluorescence labeled with Ca^{2+} binding dye Fluo-4 acetoxymethyl (Fluo-4 AM). (J–L) Representative traces of NMDA-triggered Ca^{2+} response in cortical neurons activated by 10 μmol/L glycine and 30 μmol/L NMDA with or without 10 μg/mL ACG (J), 10 μmol/L Scu (K), and 10 μmol/L 3,4-CQA (L). The *y*-axis $[(F_t–F_0)/F_0]$ indicates the ratio of fluorescence intensity at different time points (F_t) to the baseline (F_0). HFS: high-frequency stimulation; BF: bright field.

(*p*-CaMKII (Thr286)), a critical molecular switch in LTP induction, were evaluated (Section S3 in the Supplementary data). Five components, including three caffeoyl compounds (4-CQA, 5-CQA, and 3,4-CQA) and two flavonoids (Scu and Api), effectively reversed the NMDA-induced inhibition of CaMKII phosphorylation (Figs. S4A and B). Scu and 3,4-CQA were chosen as representative flavonoids and caffeoyl compounds, respectively, to further investigate their effects on the NMDA receptor-mediated Ca^{2+}/CaMKII pathway. Treatment with DZSM (Figs. 1E and S4D), ACG (Figs. 1F and S4E), Scu (Figs. 1G and S4F), and 3,4-CQA (Figs. 1H and S4G) in NMDA-damaged SH-SY5Y cells decreased NR2B expression and increased the NMDA receptor subunit 2A (NR2A)/NR2B ratio, without affecting NMDA receptor subunit 1 (NR1) expression, thus indicating reduced susceptibility to excitotoxicity (Section S3 in the Supplementary data). Additionally, these treatments reversed Ca^{2+} overload (Figs. 1I and S4I) and increased *p*-CaMKII (Thr286) levels

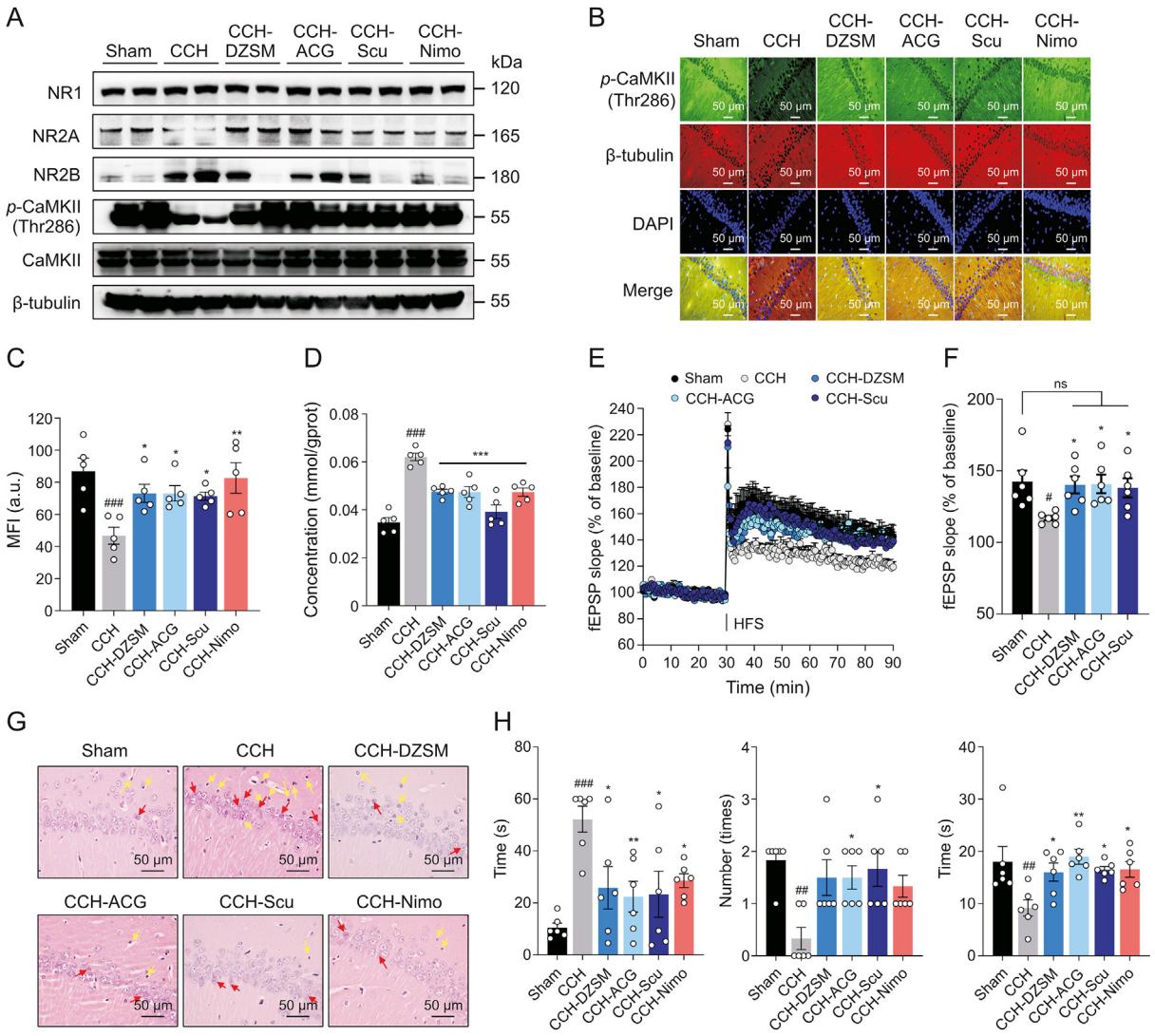

Fig. 2. Dengzhan Shengmai (DZSM) and its active components ameliorate cognitive impairment by regulating the *N*-methyl-D-aspartate (NMDA) receptor-mediated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) pathway in the hippocampus of chronic cerebral hypoperfusion (CCH) rats. (A) Representative results of Western blotting assay of the protein bands of NMDA receptor subunit 1 (NR1), NR2A, NR2B, phospho-CaMKII (Thr286) (*p*-CaMKII (Thr286)), CaMKII, and β-tubulin from the hippocampal lysates of rats in each group. (B) Representative immunofluorescence images of *p*-CaMKII (Thr286) (green), β-tubulin (red), and 4,6-diamino-2-phenyl indole (DAPI) (blue) in the rat hippocampal CA1 regions ($n = 5$). (C) Estimation of the mean fluorescent intensity (MFI) of *p*-CaMKII (Thr286) in rat hippocampal slices ($n = 5$). (D) Ca^{2+} levels in rat hippocampus tissues ($n = 5$). (E) Time course for the normalized slope of field excitatory postsynaptic potentials (fEPSPs) before and after inducing long-term potentiation (LTP). We induced LTP using high-frequency stimulation delivered at time 30 (line) at CA3-CA1 synapses in the hippocampal slices of the indicated groups ($n = 6$). Results are shown as the baseline fEPSP amplitude percentage (=100%). (F) Averaged fEPSP slope recorded in the last 10 min ($n = 6$). (G) Representative images of hematoxylin-eosin (HE)-stained hippocampal sections. Red and yellow arrows indicate neuronal damage and microglial infiltration, respectively. (H) The escape latency (left), target crossings (middle), and time spent in the target quadrant (right) of rats in each group in a probe trial (Day 6) of the Morris water maze test ($n = 6$). Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Dunnett's test for data in the left and right panels and nonparametric test (Kruskal-Wallis test) for data in the middle panel. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, vs. the Sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, vs. the CCH group. ACG: active component group; Scu: scutellarin; Nimo: nimodipine; HFS: high-frequency stimulation.

(Figs. 1E–H and S4D–G). Similar effects were observed with ifenprodil treatment (Figs. S4C and H). These findings strongly support the regulatory effects of DZSM and its active components on the NMDA receptor-mediated Ca^{2+}/CaMKII pathway.

Based on the inhibition of NMDA receptor-mediated Ca^{2+} influx, Scu and 3,4-CQA were initially hypothesized to directly block NMDA receptors. However, unlike ifenprodil, the acute application of Scu and 3,4-CQA did not affect NMDA receptor currents in rat hippocampal neurons, thus suggesting that they may indirectly inhibit NMDA channels (Figs. S5A and B). Nonetheless, chronic exposure (24 h) to ACG, Scu, and 3,4-CQA significantly suppressed the NMDA-triggered Ca^{2+} response (Figs. 1J–L, S5C, and S5D). Whole-cell patch recordings confirmed that these active components effectively decreased NMDA receptor-mediated current densities (Figs. S5E and F), thus indicating a reduction in available NMDA channels. These observations suggested that active components may decrease NMDA receptor surface density and indirectly inhibit its functions by normalizing NR2B expression and the NR2A/NR2B ratio, thereby preventing NMDA receptor-mediated excitotoxicity and rescuing LTP deficits.

We further evaluated the effects of DZSM, ACG, and Scu on the NMDA receptor-mediated Ca^{2+}/CaMKII pathway *in vivo*. CCH rats

were treated with DZSM, ACG, Scu, or nimodipine (Nimo) as a positive control (Fig. S6A). Consistent with *in vitro* findings, CCH increased NR2B expression, decreased the NR2A/NR2B ratio, induced Ca^{2+} overload, and reduced *p*-CaMKII (Thr286) levels in the hippocampus. DZSM attenuated these effects by decreasing NR2B expression, increasing the NR2A/NR2B ratio, mitigating Ca^{2+} overload, and increasing *p*-CaMKII (Thr286) levels (Figs. 2A, 2D, and S6C). Fluorescence intensities of *p*-CaMKII (Thr286) in hippocampal slices were higher in DZSM-treated rats (Figs. 2B, 2C, and S7–S10). ACG and Scu treatments exhibited similar *in vivo* efficacies to DZSM (Figs. 2A–D, S6C, and S7–S10).

To validate the cognitive efficacy of ACG and Scu, fEPSP recordings were performed in rat hippocampal slices. Both ACG and Scu treatments significantly restored impaired LTP to levels comparable to those achieved with DZSM treatment ($P < 0.05$; Figs. 2E, 2F, and S6B), supporting the effectiveness of our approach (Fig. 1D) in identifying active components facilitating LTP. Hematoxylin-eosin (HE) staining revealed CCH-induced neuronal damage and microglial infiltration in the hippocampus, which were alleviated by DZSM, ACG, or Scu treatments (Figs. 2G and S11). Moreover, rats treated with DZSM, ACG, or Scu performed better in the water maze test (Figs. 2H and S6D). Notably, the cognitive efficacies of ACG and Scu were comparable to that of Nimo, which is known to enhance cognition in dementia patients. These findings demonstrate that ACG and Scu effectively enhance cognition in CCH rats, with Scu exhibiting similar *in vivo* efficacy as ACG and DZSM.

The mechanism of the regulation of DZSM and its active components in LTP was presented in Fig. S12. However, this is only one of the factors affecting LTP. Scu and 3,4-CQA enhanced SH-SY5Y cell survival after oxygen and glucose deprivation (OGD) (Fig. S13). Thus, DZSM may contribute to LTP by acting against OGD-induced injury. This emphasizes the advantage of Chinese medicines with multiple active compounds in enhancing LTP by targeting multiple pathways.

In conclusion, this study shows that flavonoids and caffeoyl compounds are the key active components of DZSM that improve cognition. These components facilitate hippocampal LTP via the NMDA receptor-mediated Ca^{2+}/CaMKII pathway, thus suggesting potential clinical applications in CCH therapy. Active components such as Scu and 3,4-CQA provide valuable insights for developing therapeutic agents that target cognitive impairment.

CRediT author statement

Congyu Ma: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing - Original draft preparation, Reviewing and Editing; **Ning Sheng:** Conceptualization, Investigation, Methodology, Resources, Validation; **Jun Wu:** Data curation, Formal analysis, Methodology, Resources, Visualization, Writing - Original draft preparation; **Lulu Wang:** Investigation, Methodology, Validation; **Jiandong Jiang:** Conceptualization, Writing - Reviewing and Editing, Project administration, Funding acquisition; **Haibo Yu:** Conceptualization, Validation, Writing - Reviewing and Editing, Supervision; **Jinlan Zhang:** Conceptualization, Investigation, Validation, Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2023.09.016.

References

[1] A. Citri, R.C. Malenka, Synaptic plasticity: Multiple forms, functions, and mechanisms, Neuropsychopharmacology 33 (2008) 18–41.
[2] H. Lu, J. Zhang, Y. Liang, et al., Network topology and machine learning analyses reveal microstructural white matter changes underlying Chinese medicine Dengzhan Shengmai treatment on patients with vascular cognitive impairment, Pharmacol. Res. 156 (2020), 104773.
[3] N. Sheng, H. Zheng, M. Li, et al., 4,5 caffeoylquinic acid and scutellarin, identified by integrated metabolomics and proteomics approach as the active ingredients of Dengzhan Shengmai, act against chronic cerebral hypoperfusion by regulating glutamatergic and GABAergic synapses, Pharmacol. Res. 152 (2020), 104636.
[4] X. Fan, W.Y. Jin, Y.T. Wang, The NMDA receptor complex: A multifunctional machine at the glutamatergic synapse, Front. Cell. Neurosci. 8 (2014), 160.
[5] C. Ma, N. Sheng, Y. Li, et al., A comprehensive perspective on the disposition, metabolism, and pharmacokinetics of representative multi-components of Dengzhan Shengmai in rats with chronic cerebral hypoperfusion after oral administration, J. Ethnopharmacol. 307 (2023), 116212.