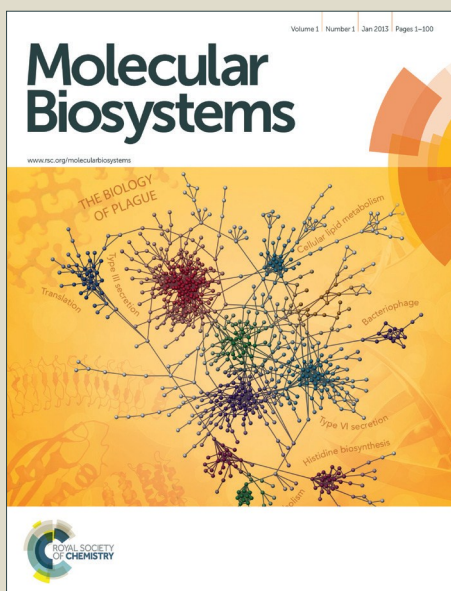


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1 **Identification of Key Active Constituents of Buchang Naoxintong Capsules with Therapeutic**  
2 **Effects Against Ischemic Stroke by Using an Integrative Pharmacology-based Approach**

3 **Short title: Active Constituents of Buchang Naoxintong Capsules**

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17

1 **Abstract**

2 Integrative pharmacology has been used to identify the key active constituents (KACs) of  
3 Buchang Naoxintong capsules (BNCs), a traditional Chinese medical preparation; this approach  
4 involves the evaluation of the content profiles and drug-like properties of the BNC constituents  
5 and development of an ingredient-target network. In this study, we used a sensitive analytical  
6 method to simultaneously identify and quantify 16 constituents of BNCs. Metabolism of these  
7 constituents by gut microbiota and human oral bioavailability were predicted using an *in silico*  
8 approach, followed by construction of networks to analyze the interactions between BNC  
9 constituents, their molecular targets, and proteins known to be the molecular targets for Food and  
10 Drug Administration-approved colitis medications. Finally, an animal model of ischemic stroke  
11 was used to verify the therapeutic effects of the KACs of BNCs. Amygdalin and paeoniflorin were  
12 identified as the KACs because they were the 2 most abundant BNC constituents, had appropriate  
13 drug-like properties, and produced therapeutic effects against cerebral ischemia. Amygdalin  
14 produced an anti-cerebral ischemia effect, likely by interacting with the glucocorticoid receptor  
15 (NR3C1) and serpin peptidase inhibitor, clade C (antithrombin), member 1 (SERPINC1). These  
16 results form the basis for conducting studies to identify KACs in traditional medicinal  
17 preparations; such studies might improve quality control and allow the *in vivo* evaluation of  
18 synergistic interactions between complex mixtures of compounds.

19 **Keywords:** integrative pharmacology, quantitative analysis, pharmacokinetics, network  
20 pharmacology, Buchang Naoxintong capsule

21

1 **Author Summary**

2 Lack of correlations among chemical profiles, drug metabolism, and pharmacological actions has  
3 become a significant obstacle for researchers and clinicians aiming to modernize traditional  
4 Chinese medicine (TCM). However, this issue can be effectively addressed by utilizing integrative  
5 pharmacology, an approach involving systematic identification of active constituents involved in  
6 the effectiveness of TCM formulations, as well as their molecular mechanisms, via the integration  
7 of knowledge of chemical profiles, drug-like properties, and the ingredient-target network. In this  
8 study, we applied the integrative pharmacology approach to identify the key active constituents  
9 (KACs) of the Buchang Naoxintong capsules that are effective against ischemic stroke by  
10 integrating quantitative determinations of multiple constituents, *in silico* pharmacokinetics  
11 modeling, and network pharmacology. The predictions obtained using this approach were  
12 validated by animal experiments by using the middle cerebral artery occlusion (MCAO) model,  
13 which indicated that amygdalin and paeoniflorin were 2 KACs of the Buchang Naoxintong  
14 capsules. To our knowledge, this is the first report of an anti-cerebral ischemia effect by  
15 amygdalin. These results showed that integrative pharmacology is a powerful tool for identifying  
16 the active constituents of TCM formulations and their molecular mechanisms.

17

18

## 1 **Introduction**

2 Traditional Chinese medicine (TCM) is one of the oldest medical systems in the world [1].  
3 Recently, the focus of health care paradigm has shifted from disease to TCM therapy with a  
4 holistic approach. Some researchers utilize omics and systems biology techniques to elucidate the  
5 detailed molecular mechanisms underlying the effectiveness and promote the modernization of  
6 TCMs [2,3]. At present, chemical analysis, *in vivo* drug metabolism evaluation, and  
7 pharmacological action elucidation are performed separately because of the complexity of TCM  
8 formulations. The lack of correlations among these studies has led to the formation of three  
9 disciplines (chemistry, pharmacokinetics and pharmacodynamics) in the “three parallel universes”;  
10 this has restricted the acceptance of TCMs within Western biomedical practices [4]. This  
11 limitation can be addressed using integrative pharmacology and systems pharmacology, which  
12 allow the determination of the active constituents of TCM formulations and their molecular  
13 mechanisms by integrating the knowledge of chemical profiles, drug-like properties, and  
14 ingredient-targeted networks [5-7]. High-throughput qualitative and quantitative analytical  
15 techniques, including ultra-high-pressure liquid chromatography (UHPLC) coupled with linear ion  
16 trap/Orbitrap (LTQ-Orbitrap) or a triple quadrupole electrospray tandem mass spectrometry  
17 (QQQ), are powerful tools for elucidating the chemical profiles of TCM formulations [8, 9].  
18 Information on the chemical constituents of TCM ingredients is critical for the accurate  
19 assessments of the therapeutic effects of each constituent. However, studies simultaneously  
20 evaluating several TCM ingredients are limited by the lack of standard substances and appropriate  
21 analytical methods for evaluating multiple constituents. Further, most TCM formulations are  
22 consumed orally. Therefore, TCMs need to undergo intestinal metabolism and absorption to entry  
23 the body and produce therapeutic action [10,11]. However, experimental determination of these  
24 processes can be costly and time consuming. For the rapid estimation of TCM absorption and  
25 metabolism in humans, high-throughput *in silico* screening methods involving the use of Caco-2  
26 cell monolayers and P450 enzymes are considered the most advanced *in vitro* approaches for  
27 assessing new chemical entities [12,13]. Systems biology and network pharmacology are valuable  
28 tools for better understanding the molecular mechanisms underlying the therapeutic effects of  
29 TCM formulations [14-16]; however, their results need to be experimentally validated.

1 Cardiovascular diseases (CVDs) are the most common cause of death worldwide, especially in  
2 developing countries [17]. Buchang Naoxintong capsule (BNC) is one of the TCM prescriptions  
3 (BuYangHuanWuTang) and is used to treat CVDs in China. Every BNC weighs 0.4 g and consists  
4 of 16 TCMs: *Astragalus membranaceus* (16.2%), *Salvia miltiorrhiza* (6.6%), *chuanxiong hort*  
5 (6.6%), *Radix paeoniae rubra* (6.6%), *Szechwan lovage rhizome* (6.6%), *Semen persicae* (6.6%),  
6 *Carthamus tinctorius* L. (3.2%), frankincense (3.2%), myrrh (3.2%), *Spatholobus suberectus*  
7 Dunn (4.9%), *Achyranthes bidentate* (6.6%), cassia twig (6.6%), *Morus alba* L. (6.6%), *Pheretima*  
8 *hupeiensis* (6.6%), *Buthus martensii* (3.2%), and *Whitmania pigra Whitman* (6.6%) [18]. The  
9 standardized procedure for the preparation of this TCM is as follows: the 16 TCMs are weighted  
10 precisely, and then the two herbs (*Pheretima hupeiensis* and *Scorpionidae*) and the remaining 14  
11 ingredients are separately mixed and ground into fine powders. The powders are then mixed and  
12 filled in the capsules. In our previous chemical analysis, the UHPLC-LTQ-Orbitrap method was  
13 used as a rapid and high-throughput identification or tentative characterization method that  
14 allowed the simultaneous analysis of a total of 178 components, including 21 flavones, 6 flavone  
15 glycosides, 18 phenanthraquinones, and 22 terpenoids, from the BNCs [19]. Further, the  
16 UPLC-DAD method was used to quantify 5 constituents of BNCs and obtain the intestinal  
17 absorption profiles of 4 of these constituents [20,21].

18 BNCs produce various pharmacological actions, including lipid concentration reduction,  
19 anti-platelet effect enhancement, dendritic cell maturation inhibition, and drug-metabolizing  
20 enzyme CYP<sub>2C19</sub> activity enhancement [22-24]. However, the key active constituents (KACs) of  
21 BNCs that are beneficial in the cases of CVDs and their molecular mechanisms of action are still  
22 unclear. In particular, since the ingredient-target network is not known, the molecular mechanisms  
23 of BNC action in CVDs are not yet known. Therefore, in the present study, the integrative  
24 pharmacology approach was applied to rapidly identify KACs of BNCs and their potential  
25 molecular mechanisms in ischemic stroke; quantitative analysis of multi-constituents; absorption,  
26 distribution, metabolism, and excretion (ADME) prediction; network analysis; and experimental  
27 validation were also performed. The workflow is shown in **Figure 1**.

## 28 **Results and Discussion**

29 **Selection of candidate constituents for quantitative analysis and optimization of sample**  
30 **preparation**

1 Because of the lack of standards and appropriate quantitative methods, contents of only 16 of the  
2 178 BNC components qualitatively identified in our previous study [19] (gallic acid, danshensu,  
3 hydroxysafflor yellow A, chlorogenic acid, amygdalin, protocatechuic aldehyde, (-)-epicatechin,  
4 caffeic acid, albiflorin, ononin, paeoniflorin, rutin, salvianolic acid A, cinnamic acid,  
5 formononetin, and dihydrotanshinone I) were determined using the RRLC-QQQ method in this  
6 study; the structures of these components are shown in **Figure 2**. The best quantitative results  
7 were obtained by using variables such as solvent, procedure, and extraction time.

8 Ultrasonic extraction was found to be more convenient and effective than refluxing and Soxhlet  
9 extraction because of its simpler extraction process, lower extraction temperature, and higher  
10 extraction recoveries of the 16 constituents. In a preliminary experiment, different extraction  
11 solvents such as methanol, ethyl acetate, and methylene dichloride were investigated, and  
12 methanol showed the best extraction efficiency. Thus, the main parameters that independently  
13 influenced the ultrasonic-assisted extraction of constituent-enriched BNC extract were  
14 investigated for the optimum methanol concentration (100%, 75%, and 50%), solvent volumes (25,  
15 50, and 100 mL), and extraction times (30, 40, 50, and 60 min). The results suggested that the  
16 compounds of interest were completely extracted from the BNC samples within 40 min when 50  
17 mL of 100% methanol was used.

#### 18 **Optimization of RRLC-QQQ conditions**

19 Good chromatographic behavior and satisfactory MS response were obtained by  
20 systematically optimizing the RRLC-QQQ conditions. Liquid chromatographic conditions,  
21 including the stationary phase, mobile phase, column temperature, and flow rate, were  
22 investigated. The results indicated that the Eclipse XDB-C18 column (Agilent Technologies, Palo  
23 Alto, CA) with a small (1.8  $\mu\text{m}$ ) particle size showed better separation efficiency in a shorter time,  
24 and the aqueous phase with 0.1% formic acid (A)-acetonitrile (B) was better than the other mobile  
25 phases. Further, 0.1% formic acid could eliminate the peak tailing of the target compounds. The  
26 optimal gradient elution procedure (described in the methods section) was determined by  
27 comparing different procedures. In particular, the organic phase proportion decreased to the initial  
28 ratios (15% B) after 8.1 min and could continue to elute the residual target constituents and  
29 balance the chromatographic column such that better separation within shorter analytical time  
30 could be obtained.

1 The MS conditions were also optimized systemically. The richest relative abundance of  
 2 precursor and product ions were obtained by optimizing the parameters for fragmentor energy and  
 3 collision energy to establish multiple reaction monitoring (MRM) transitions for all target  
 4 compounds. The representative MRM chromatograms and total ion chromatograms of the 16 tested  
 5 BNC constituents are shown in **Tab. 1** and **Figure S1**, respectively.

6

7 **Tab. 1.**

8 Retention time (RT) and related mass spectrometry (MS) data of the 16 target compounds detected  
 9 using rapid resolution liquid chromatography (RRLC)-MS

Maker	RT (min)	Ionization mode	Quasi-molecular ions	Quantitative ion (m/z)	Fragmentor (V)	CE (V)
1	3.33	ESI-	169.0	125.1	100	10
2	3.72	ESI-	197.1	135.0	100	10
3	4.33	ESI-	611.1	325.0	250	30
4	5.32	ESI-	353.0	191.0	100	15
5	6.10	ESI-	502.2	323.2	100	10
6	6.99	ESI-	137.3	108.2	100	25
7	7.52	ESI-	289.1	245.0	100	5
8	7.87	ESI-	179.0	135.0	100	10
9	8.02	ESI-	525.1	121.2	150	20
10	8.58	ESI-	475.1	267.2	100	5
11	8.59	ESI-	525.0	449.0	100	5
12	8.71	ESI-	609.0	300.0	200	40
13	8.76	ESI-	493.1	295.0	150	15
14	9.34	ESI-	147.0	103.0	100	5
15	9.56	ESI-	267.1	252.0	100	15
16	11.52	ESI+	279.0	261.0	150	15

Electrospray ionization (ESI)+ and ESI- represent the positive and negative modes of ESI, respectively.

10

11



1

## 2 **Method validation and quantification**

3 In this study, the calibration curves of the 16 compounds showed good linearity ( $R^2 \geq 0.9933$ ). The  
4 LODs were in the range of 0.14–5.62 ng/mL, whereas the LOQs ranged from 0.48 to 18.72  $\mu\text{g/mL}$   
5 (**Tab. S1**). The relative standard deviation values (%RSD) of the 16 BNC constituents were  
6 0.44%–4.14% for intra-day precision and 2.19%–4.86% for inter-day precision. Further, the  
7 method showed good repeatability, with RSDs for the 16 compounds ranging from 0.61% to  
8 4.31%. The RSD values of the 16 compounds were less than 5%, indicating good stability of the  
9 methanol solution within the experimental period (**Tab. S2**). The mean recoveries of the 16  
10 compounds ranged from 92.8% to 104.3% (RSD,  $\leq 4.94\%$ ; **Tab. S3**).

11 The established RRLC-QQQ method was successfully applied to quantitatively and  
12 simultaneously analyze the 16 compounds of BNCs in 15 batches. The levels of the 16 compounds  
13 were determined in triplicate by using calibration curves generated using external standards (**Tab.**  
14 **2**). The results indicated that the contents of the 16 compounds varied considerably, ranging from  
15 4.20% to 1,938.42  $\mu\text{g/g}$ . Amygdalin and paeoniflorin were the most abundant compounds, and  
16 their concentrations ranged 1,955.11–2,323.14  $\mu\text{g/g}$  and 1,430.18–1,938.42  $\mu\text{g/g}$ , respectively.  
17 Among the other analytes, gallic acid, danshensu, hydroxysafflor yellow A, chlorogenic acid, and  
18 (-)-epicatechin were identified as the primary active compounds in the BNCs; they were present at  
19 a concentration of more than 100  $\mu\text{g/g}$ . Certain trace constituents, including protocathechuic  
20 aldehyde, caffeic acid, rutin, and formononetin, were also quantified. The RSDs of the  
21 determinations of the 16 compounds ranged from 6.56% to 18.02% in the 15 batches of BNC  
22 analysis, suggesting that the chemical profiles of the BNCs were stable across the different  
23 batches of the product, probably because of the similar plant origins, growth locations, and  
24 manufacturing techniques.

25

T sample ( $\mu\text{g}\cdot\text{g}^{-1}$ ) and oral bioavailability of 16 constituents

	5	6	7	8	9	10	11	12	13	14	15	16
	2218.51	13.56	101.64	10.89	65.92	7.57	1647.68	12.60	56.57	27.42	16.80	14.27
	2105.23	12.20	109.01	9.42	62.67	7.46	1628.94	10.01	45.65	20.61	14.79	11.69
	2155.17	14.67	103.52	11.19	69.94	7.79	1683.80	10.28	44.32	25.64	13.48	13.53
	2128.13	12.16	102.33	11.36	79.66	6.20	1703.77	10.46	52.42	22.62	12.99	13.94
	2311.84	15.98	112.80	10.23	74.18	7.39	1820.96	10.55	52.09	21.92	14.22	13.56
	1972.93	13.91	88.01	10.11	73.79	5.81	1597.93	9.55	59.36	30.67	14.83	14.36
	2440.25	13.88	96.62	9.80	74.41	4.20	1651.85	10.20	61.87	36.88	14.51	13.06
	2312.88	15.05	101.81	10.93	80.06	6.03	1744.54	10.13	55.31	29.45	15.50	12.50
	1955.11	11.21	103.18	10.48	80.81	7.20	1732.12	11.65	49.21	19.23	13.17	14.39
	2245.75	12.35	113.59	11.77	90.91	7.11	1927.47	12.57	51.17	21.59	14.68	15.62
	2323.14	15.25	116.32	13.06	90.54	6.25	1938.42	11.25	56.15	24.86	13.88	15.73
	2108.98	10.85	104.90	10.94	79.31	5.84	1751.82	9.94	49.48	24.64	13.16	14.50
	2031.28	10.50	87.05	7.30	54.66	7.02	1430.18	8.31	43.22	24.57	13.47	10.67
	2315.52	14.20	130.66	10.43	43.82	4.63	1431.05	7.59	51.42	25.75	14.62	15.94
	2078.71	11.69	94.37	10.21	77.95	6.78	1696.92	11.49	53.24	21.93	12.94	12.06
$\pm 25.4$	$2180.23 \pm 143.2$	$13.16 \pm 1.7$	$104.39 \pm 11.2$	$10.54 \pm 1.2$	$73.24 \pm 12.6$	$6.48 \pm 1.1$	$1692.50 \pm 144.9$	$10.44 \pm 1.4$	$52.10 \pm 5.3$	$25.18 \pm 4.5$	$14.20 \pm 1.1$	$13.72 \pm 1.5$
	6.56	13.07	10.74	11.92	17.13	16.38	8.56	13.18	10.02	18.02	7.54	11.11
	1.6	99.9	71.6	41.1	13.9	79.6	44.7	0.1	6.7	99.1	87.2	5.5

Numbers of the BNC capsules obtained from Buchang Pharmaceutical Co. Ltd.; compounds 1–16 represent the following 16 markers: 1, gallic acid; 2, danshensu; 3, amygdalin; 6, protocatechuic aldehyde; 7, (-)-epicatechin; 8, caffeic acid; 9, albiflorin; 10, ononin; 11, paeoniflorin; 12, rutin; 13, salvianolic acid A; 14, cinnamic acid; 15,

1 **Reports of the biotransformation of the primary BNC constituents by gut microbiota from**  
 2 **the literature and *in silico* prediction of oral bioavailability**

3 Most TCMs are orally administered as decoctions and might be transformed by intestinal bacteria  
 4 before absorption in the gastrointestinal tract [25]. Therefore, intestinal flora plays an important  
 5 role in the metabolism of TCM compounds [26,27]. Recently, metabolic studies of human  
 6 intestinal flora have attracted wide attention and provided new insights into the therapeutic  
 7 mechanisms of TCMs [28]. A literature search was conducted using electronic databases,  
 8 including Medline (Ovid), PubMed, CNKI, Wanfang, and Weipu, to obtain the data regarding the  
 9 biotransformation of the six quantified BNC constituents (chlorogenic acid, rutin, amygdalin,  
 10 ononin, albiflorin and paeoniflorin) by gut microbiota. The metabolic pathways of several BNC  
 11 constituents are summarized in **Tab. 3**, and the structures of these constituents are shown in  
 12 **Figure 2**. Following deglycosylation, amygdalin, rutin, ononin, and chlorogenic acid were  
 13 metabolized to mandelonitrile, quercetin, formononetin, and caffeic acid, respectively, and  
 14 albiflorin and paeoniflorin were converted to metabolites 5 (M5) and 6 (M6), respectively, by the  
 15 gut microbiota; deglycosylation is considered a critical step in the absorption and metabolism of  
 16 dietary flavonoid glycosides in humans [29-37]

17  
 18 **Tab. 3.**

19 Biotransformation of the six constituents in the gastrointestinal tract identified by comparison with  
 20 literature searches

Prototype	Metabolite (s)	OB (F%)	Reference (s)
chlorogenic acid	caffeic acid,	40.5	Ref 28-30
rutin	quercetin	94.3	Ref 31,32
amygdalin	mandelonitrile	99.4	Ref 33.
ononin	formononetin	87.2	Ref 34
albiflorin	Metabolites 5 (M5)	99.9	Ref 35, 36
paeoniflorin	Metabolites 6 (M6)	99.8	

21 OB: oral bioavailability

22

23 After the TCM components are metabolized by the gastrointestinal tract, they are absorbed  
 24 into the body. Therefore, their oral bioavailability, one of the most important pharmacokinetic

1 parameters, was measured to determine the oral dose of each compound that was sufficient to  
2 produce a pharmacological effect. High oral bioavailability suggests that the bioactive molecules  
3 have the potential to be used as therapeutic agents [38]. Recently, numerous studies have been  
4 conducted to develop *in silico* quantitative models for predicting oral bioavailability [39]. The  
5 module for determining oral bioavailability involves a combination of probabilistic and  
6 mechanistic models to produce quantitative predictions of bioavailability in humans after oral  
7 administration (%F); these modules are widely used in research and drug development [40,41]. In  
8 this study, the pharmacokinetics (PK) explorer module included in the commercially available  
9 ACD/Percepta software was used to calculate the absorbed fraction and to predict the oral  
10 bioavailability of the 16 quantified BNC constituents and the 6 metabolites. In all, 7 BNC  
11 constituents, including protocatechuic aldehyde, (-)-epicatechin, caffeic acid, ononin, paeoniflorin,  
12 cinnamic acid, and formononetin, showed good oral bioavailability (F%,  $\geq 40$ ; **Tab. 2 and 3**).  
13 Some components, including chlorogenic acid, rutin, amygdalin, and albiflorin, were converted to  
14 metabolites by the gut microbiota, which improved their oral bioavailability. Thus, paeoniflorin  
15 and its metabolite (M6) had good oral bioavailability.

#### 16 **Identification of KACs of BNCs for the treatment of ischemic stroke**

17 Numerous KACs generally exist in the TCM product; they have the appropriate pharmacokinetic  
18 properties and possess therapeutic activity [42]. Therefore, the selection of the 16 BNC  
19 constituents and determination of their contents in different batches of BNCs was a precondition  
20 for the evaluation of BNC KACs. *In silico* prediction of the molecular targets for the 16 BNC  
21 constituents and construction and analysis of the constituent-target-disease network is a  
22 cost-effective method of predicting the KACs of TCM formulations such as the BNC.

23 **Putative targets for the 16 BNC constituents.** Based on our previously developed target  
24 prediction system [43], we identified 80 known drugs with a similar chemical structure as those of  
25 the BNC constituents/metabolites and their known targets (n = 227); they were considered as the  
26 putative targets of BNC constituents/metabolites. Their details are shown in **Table S4**. Of the 227  
27 putative targets of BNC constituents, 18 (7.93%; **Tab. S5**) were known therapeutic targets for the  
28 treatment of ischemic stroke. Notably, 14 putative targets of mandelonitrile were known  
29 therapeutic targets for the treatment of ischemic stroke, suggesting that M1 played an important  
30 role in the therapeutic effect of BNCs.

1 **Network construction and analysis.** The bioactive BNC constituents responsible for the  
2 therapeutic effect in ischemic stroke patients were identified by first constructing a BNC  
3 component/metabolite-putative target-known ischemic stroke target network (**Fig. 3** and **Tab. S6**).  
4 The putative targets of gallic acid (constituent ID: 1), danshensu (2), chlorogenic acid (4),  
5 protocatechuic aldehyde (6), (-)-epicatechin (7), caffeic acid (8), paeoniflorin (11), mandelonitrile  
6 (M1), and quercetin (M3) directly interacted with known therapeutic targets for the treatment of  
7 ischemic stroke.

8       Next, a PPI network consisting of putative targets of BNC constituents, known therapeutic  
9 targets for ischemic stroke, and other human proteins was constructed to identify the bioactive  
10 BNC constituents responsible for the therapeutic effect on ischemic stroke (**Fig. 4** and **Tab. S7**).  
11 The PPI network consisted of 184 nodes and 423 edges. Four topological features (degree,  
12 betweenness, closeness, and k-value) were calculated to screen the major putative targets. In all,  
13 47 major nodes with higher degree, betweenness, closeness, and k-values than the corresponding  
14 median values were identified. Among them, 37 were recognized as the major putative targets of  
15 BNC constituents. Notably, the glucocorticoid receptor (NR3C1)—a major putative target of  
16 paeoniflorin and mandelonitrile—and serpin peptidase inhibitor, clade C (antithrombin), member  
17 1 (SERPINC1)—a major putative target of amygdalin—had the highest betweenness values,  
18 suggesting their large influence on the transfer of information through the network, under the  
19 assumption that the information transfer follows the shortest paths. Detailed information on the  
20 topological features of the 47 major nodes in the PPI network is shown in **Table S8**. Detailed  
21 information on the 37 major putative targets of BNC constituents is shown in **Table S9**.

22       Further, the edge-betweenness values of each interaction in the BNC putative target/known  
23 ischemic stroke therapeutic target/other human protein PPI network were calculated. The NR3C1-  
24 TAT (Tyrosine aminotransferase), NR3C1-HSD17B6, and SERPINC1-FTCD  
25 (Formimidoyltransferase-cyclodeaminase) interactions had the three highest edge-betweenness  
26 values, suggesting their crucial roles in connecting the other edges in the network (**Tab. 4**).

27  
28  
29  
30

1 **Tab. 4.**

2 Top 10 edges with the highest edge-betweenness value

NodeA	NodeB	Edge-betweenness
NR3C1	TAT	1332.089
NR3C1	HSD17B6	1036
SERPINC1	FTCD	966.4062
SLC6A3	COMT	951.6876
ANXA1	MAPK1	900.9778
DRD5	SLC6A3	867.4568
RXRA	ALAS1	835.7336
TAT	PTGS2	744.5632
DDC	COMT	736.5258
RXRA	MAPK1	715.9232

3

4 Our results suggested that amygdalin and paeoniflorin might be important bioactive  
5 constituents of BNC according to the following observations. First, amygdalin and paeoniflorin  
6 were the most abundant BNC constituents, with concentrations greater than 1,400  $\mu\text{g/g}$  in all the  
7 tested samples. Second, *in silico* pharmacokinetics prediction indicated that paeoniflorin had good  
8 drug-like properties, including oral bioavailability of 44.7%. Although amygdalin had poor  
9 absorption and low oral bioavailability, it was easily deglycosylated and converted to  
10 mandelonitrile, which had a very good oral bioavailability ( $F = 99.4\%$ ). Third, amygdalin and  
11 paeoniflorin, or their metabolites had many putative targets that directly interacted with other  
12 known therapeutic targets for ischemic stroke. Finally, NR3C1 and SERPINC1, respectively  
13 identified as the putative targets of amygdalin and paeoniflorin, or their metabolites, had the most  
14 important topological features in the network.

15 **Experimental validation of the active BNC constituents in a model of ischemic stroke**

16 In rats and mice, the middle cerebral artery occlusion (MCAO) model has been used to represent  
17 cerebrovascular stroke; BNC was found to increase the number of masculine nerve cells of  
18 calcitonin gene-related peptide (CGRP) and improve learning and memory capacity in this animal

1 model [44,45]. In this study, the predicted outcomes on ischemic stroke after treatment with the  
2 primary active BNC constituents were investigated by using the MCAO model (**Fig. 5 (a, b)**). The  
3 sham group did not show any infarction volumes, and their neurobehavior was completely normal.  
4 In contrast, the control group showed excessive infarction volumes, and their neuronal functions  
5 were obviously abnormal, but the positive group (GBE) showed significant curative effect after  
6 treatment with BNC components. The optimal dose was determined by administering the 16 BNC  
7 constituents at three concentrations: high (High\_16-MS), medium (Medium\_16-MS), and low  
8 concentrations (Low\_16-MS). The Medium\_16-MS group showed an infarction rate of  $10.50 \pm$   
9  $12.87$  and a neurological behavior score of  $1.58 \pm 0.79$ , indicating that this concentration produced  
10 the best curative effect.

11 Next, the bioactivities of the primary active BNC constituents (amygdalin and paeoniflorin)  
12 and the mixture of the 14 BNC constituent standards (14-MS, excluding amygdalin and  
13 paeoniflorin) were determined at doses equivalent to those of the medium-dose 16-MS. The  
14 infarction rates and neurological deficit scores of the groups treated with amygdalin were  $14.32 \pm$   
15  $12.80\%$  and  $1.83 \pm 0.41$ , respectively, and those of the groups treated with paeoniflorin were  $18.36$   
16  $\pm 7.50\%$  and  $1.67 \pm 0.52$ , respectively. These results suggested that amygdalin and paeoniflorin  
17 significantly decreased the infarction volume ( $p < 0.05$ ) and improved neurological scores  
18 compared with those of the control group. However, 14-MS had very low bioactivity in MCAO  
19 rats and showed small effects on infarction volume and neurological function. These findings  
20 indicated that amygdalin and paeoniflorin are the KACs of BNCs that play critical roles in the  
21 therapeutic effects in ischemic stroke patients. Previous studies showed that paeoniflorin produces  
22 a delayed protective effect in ischemia-injured rats by inhibiting MAPK/NF- $\kappa$ B-mediated  
23 peripheral and cerebral inflammatory responses, corroborating our network pharmacology results  
24 [46,47]. However, to our knowledge, this is the first report of the anti-cerebral ischemia effect of  
25 amygdalin, which needs to be investigated further for elucidating its underlying molecular  
26 mechanism.

27

## 28 **Conclusions**

29

30 Identification of the KACs in TCM formulations is critical for ensuring their efficacy, safety, and

1 quality. In this study, an integrative pharmacology approach involving a combination of  
2 quantitative analysis, pharmacokinetics prediction, network pharmacology, and experimental  
3 validation was used to identify KACs and the molecular mechanisms involved in the therapeutic  
4 effects of BNCs in ischemic stroke patients. First, a RRLC-QQQ technique was used to  
5 simultaneously quantify 16 constituents from 15 commercial samples of BNCs. Next, an *in silico*  
6 pharmacokinetics model was applied to predict the oral bioavailability of the tested BNC  
7 constituents and 6 plausible metabolites produced by the intestinal flora, which suggested that the  
8 BNC constituents had good oral bioavailability. In addition, pharmacological networks were  
9 constructed to analyze and visualize the synergistic interactions among the BNC  
10 constituents/metabolites, putative BNC targets, and known therapeutic targets of drugs used to  
11 treat ischemic stroke patients. Amygdalin and paeoniflorin were identified as KACs of BNCs  
12 based on their high abundance in the product, appropriate drug-like properties, and importance to  
13 the bioactivity of the mixture of BNC constituents. Finally, the MCAO model was used to show  
14 that amygdalin and paeoniflorin significantly decreased the infarction volume and improved  
15 neurological scores in ischemic rats. To our knowledge, this is the first report of the anti-cerebral  
16 ischemia effect of amygdalin and the involvement of NR3C1 and SERPINC1 in its therapeutic  
17 effects. Future studies are warranted to determine the molecular mechanisms underlying the  
18 therapeutic effects of BNC constituents, with a focus on compound-target interactions and the  
19 synergistic interactions of active constituents at the systemic level.

20

## 21 **Materials and Methods**

### 22 **Ethics statement**

23 All animal studies were approved by the committee of ethical regulations of the Laboratory  
24 Animal Ethics Committee of the Institute of Basic Theory of TCM, China Academy of Chinese  
25 Medical Sciences. Animal care and welfare and experimental procedures complied with the Guide  
26 for the Care and Use of Laboratory Animals (National Research Council of the USA, 1996) and  
27 related ethical regulations of the China Academy of Chinese Medical Sciences. The approval code  
28 of the Ethics Committee was SYXK (Jing) 2005–2008, and the period of validity was from  
29 August 2011 to August 2015. All efforts were made to minimize the number of animals used and  
30 their suffering.



1 **Reagents and materials**

2 HPLC-grade acetonitrile and formic acid were obtained from Fisher Scientific Company (Thermo  
 3 Fisher, USA). Analytical-grade formic acid was purchased from Sinopharm Chemical Regent  
 4 Beijing. Co., Ltd. (Beijing, China). Purified water was purchased from the Wahaha Company  
 5 (Hangzhou, China). Reference standards of hydroxysafflor yellow A, chlorogenic acid, amygdalin,  
 6 protocatechuic aldehyde, L-epicatechin, caffeic acid, albiflorin, paeoniflorin, ononin, rutin,  
 7 cinnamic acid, and formononetin were purchased from the National Institute for the Control of  
 8 Pharmaceutical and Biological Products (Beijing, China). Gallic acid, tanshinol, salvianolic acid A,  
 9 and dihydrotanshinone I were obtained from Chengdu Must Bio-technology Co., Ltd. (Chengdu,  
 10 China). The purities of all the standards were at least 98% and suitable for quantitative analysis. The  
 11 *Ginkgo biloba* extract (GBE), trade name Jinnaduo capsule, was purchased from Dr. Willmar  
 12 Schwabe, GmbH & Co. KG (Batch No., H20140768; Germany). Fifteen commercial samples of  
 13 Buchang Naoxintong capsules (BNCs) were obtained from Buchang Pharmaceutical Co. Ltd.  
 14 (Xian, China). Detailed information regarding the BNCs is provided in **Tab. 5**.

15 **Tab. 5.**

16 A summary of the tested samples of BNC capsules from Buchang Pharmaceutical Co. Ltd.

Sample NO.	Batch NO.	Production date
S1	131117	October 06,2013
S2	131118	October 06,2013
S3	131120	October 07,2013
S4	131121	October 07,2013
S5	131122	October 07,2013
S6	131125	October 08,2013
S7	131126	October 08,2013
S8	131127	October 08,2013
S9	131129	October 09,2013
S10	131130	October 09,2013
S11	131131	October 09,2013
S12	131134	October 10,2013
S13	131135	October 10,2013
S14	131137	October 10,2013
S15	131138	October 10,2013

## 1 Samples and standard solution preparations

2 **Preparation of standard solutions.** A stock solution containing the 16 standard components was  
3 weighed, dissolved in methanol, and diluted serially to provide standard solutions at a range of  
4 concentrations, which were used to establish calibration curves. A working solution of each  
5 standard was also prepared. All solutions were stored at 4°C.

6 **Preparation of sample solutions.** The capsule shell was removed, and the BNC powder (0.5 g)  
7 was weighed precisely. The samples were ultrasonically extracted in 50 mL methanol at room  
8 temperature for 40 min, settled to a volume of 50 mL, and filtered through 0.22- $\mu$ m nylon  
9 membrane filters. The filtrates were analyzed immediately.

## 10 Chromatographic and mass spectrometric conditions

11 Rapid resolution liquid chromatography (RRLC) was performed using an Agilent 1200 Rapid  
12 Resolution Liquid Chromatograph (Agilent, MA, USA) equipped with an online vacuum degasser,  
13 a binary pump, an autosampler, and a thermostated column compartment. The analytes were  
14 separated on a ZORBAX Eclipse XDB-C18 column (4.6 mm  $\times$  50 mm, 1.8  $\mu$ m) at 25°C with a  
15 mobile phase flow rate of 0.20 mL/min. The linear gradient conditions for solvent A (0.1% aqueous  
16 formic acid) and solvent B (acetonitrile) were as follows: 0–4 min, 15–20% B; 4–5 min, 20–90% B;  
17 5–8 min, 90% B; 8–8.1 min, 90–15% B; 8.1–15 min, 15% B. The injection volume for each analysis  
18 was 5.0  $\mu$ L.

19 An Agilent G6410 Triple Quadrupole mass spectrometer equipped with an electrospray ion  
20 source (ESI; Agilent, MA, USA) was used to quantitatively analyze the 16 BNC components.  
21 ESI-MS spectra were acquired in both positive and negative ion modes. The drying gas temperature  
22 was maintained at 300°C at a flow rate of 12 L/min. The nebulizing gas ( $N_2$ ) pressure was set at 15  
23 psi. The capillary voltage was 4,000 V for negative and positive modes. The dwell time was 200 ms.  
24 Mass analyzers Q1 and Q3 operating at unit mass resolution were used for all MRM transitions.

25

## 26 Method validation

27 For each standard compound, linearity, the limit of detection (LOD), the limit of quantification  
28 (LOQ), repeatability, stability, precision, and accuracy were experimentally verified.

29 The linearity of the optimized analytical method was determined by plotting a series of  
30 concentrations of standard solutions containing at least 7 non-zero concentrations. Each

1 calibration curve for each analyte was analyzed individually by fitting the area ratio response by  
2 using least-square weighted ( $1/x_2$ ) linear regression and excluding the point of origin. The LOD  
3 and LOQ, and the signal-to-noise ratio (S/N) of 3 and 10, respectively, were determined by serial  
4 dilution of each standard solution under the described conditions.

5 The precision of the optimized analytical method was validated by determining the intra- and  
6 inter-day variance. Intra-day precision was assessed by measuring a standard mixture solution  
7 consisting of the 16 markers 6 times per day under the optimized conditions. Inter-day precision was  
8 evaluated twice per day on 3 consecutive days. Intra-day and inter-day precisions were calculated  
9 as relative standard deviation (RSD) and analyzed using one-way analysis of variance (ANOVA)  
10 with day as the grouping variable.

11 The repeatability of the optimized analytical method was tested by preparing and analyzing 6  
12 independent working solutions of the same sample (S15, batch no. 131138). The stability of the  
13 sample solutions was tested in triplicate at room temperature every 6 h for 24 h.

14 In the recovery test, known amounts of the mixed standard solutions with 3 different  
15 concentration levels (high, medium, and low) were added to known amounts of BNC sample (S15,  
16 batch no. 131138); subsequently, the samples were extracted and analyzed using the optimized  
17 analytical method. The experiment was performed in triplicate for each concentration of standard  
18 solution. The average recoveries were estimated using the following equation:

19 
$$\text{Recovery (\%)} = [(\text{detected amount} - \text{original amount}) / \text{spiked amount}] \times 100.$$

## 20 **Literature search for metabolites of intestinal flora and oral bioavailability prediction by** 21 **using *in silico* pharmacokinetic models**

22 The metabolites of the 16 BNC constituents produced by the intestinal flora were obtained by  
23 searching electronic databases, including PubMed (1950-2014), Medline (1950-2014), and CNKI  
24 (China Journals of Full-text database; 1989-2014). Structural information for each component was  
25 downloaded from ChemSpider (<http://www.chemspider.com/>) or drawn using Chemdraw 12.0 and  
26 saved in “.mol” format. The oral bioavailability of the constituents and their metabolites was

1 predicted *in silico* by using the PK explorer module of ACD/Percepta software 5.07 (ACD/Labs,  
2 Toronto, Canada).

### 3 **Known therapeutic targets of drugs used to treat ischemic stroke**

4 The known therapeutic targets of drugs used to treat ischemic stroke were obtained from 2  
5 resources: the DrugBank database [48] (<http://www.drugbank.ca/>, version: 3.0) and the Online  
6 Mendelian Inheritance in Man (OMIM) database [49] (<http://www.omim.org/>, last updated:  
7 October 31, 2013). Only drug-target interactions for drugs approved by the Food and Drug  
8 Administration (FDA) for treating ischemic stroke and human gene/protein targets were selected.  
9 In all, 70 therapeutic targets of drugs used to treat ischemic stroke were identified. The OMIM  
10 database was searched with the keyword “ischemic stroke”; the search yielded 72 therapeutic  
11 targets of drugs used to treat ischemic stroke (detailed information **in Tab. S10**). After redundant  
12 entries were removed, 62 therapeutic targets of drugs used to treat ischemic stroke were  
13 considered in this study.

### 14 **Protein-protein interaction data**

15 Protein-protein interaction (PPI) data were imported from 8 existing PPI databases: Human  
16 Annotated and Predicted Protein Interaction Database (HAPPI) [50], Reactome [51], Online  
17 Predicted Human Interaction Database (OPHID) [52], InAct [53], Human Protein Reference  
18 Database (HPRD) [54], Molecular interaction Database (MINT) [55], Database of Interacting  
19 Proteins (DIP) [56], and PDZBase [57]. Detailed information on the PPI databases is shown in  
20 **Table S11**.

### 21 **Pharmacological mechanism analysis**

22 **Prediction of putative BNC targets.** As described in our previous study [58], the Drug Similarity  
23 Search tool in the Therapeutic Targets Database (TTD,

1 <http://xin.cz3.nus.edu.sg/group/cjttd/ttd.asp>, version 4.3.02, released on Feb 25, 2014) was used to  
2 identify drugs similar to the BNC via structural similarity comparisons. Only drugs with a high  
3 structural similarity score ( $>0.70$ , moderately similar to very similar) with the 16 BNC  
4 constituents and their metabolites in the gastrointestinal tract were selected. The therapeutic  
5 targets of the identified drugs were also included as putative BNC targets.

6 **Network construction and analysis.** The 16 BNC components and their metabolites and putative  
7 targets, as well as known therapeutic targets for drugs used to treat ischemic stroke, were used to  
8 construct a BNC component/metabolite/putative target/known ischemic stroke target network and  
9 a BNC putative target/known ischemic stroke therapeutic target/other human protein PPI network.  
10 Navigator software (version 2.2.1) and Cytoscape (version 2.8.1) were utilized to visualize the  
11 networks.

12 **BNC component/metabolite/putative target/known ischemic stroke target network.** This  
13 network was constructed by linking the 16 BNC components, their metabolites, and their putative  
14 targets that interacted with known therapeutic targets of drugs used to treat ischemic stroke.

15 **BNC putative target/known ischemic stroke therapeutic target/other human protein PPI**  
16 **network.** This network was constructed by linking putative BNC targets, known ischemic stroke  
17 therapeutic targets, and other human proteins that directly interact with putative BNC targets and  
18 targets of ischemic stroke treatments. According to our previous study [59, 60], 5 topological  
19 features (degree, betweenness, closeness, k-value, and edge betweenness) were chosen to identify  
20 the major putative targets by selecting those having values of the 4 features higher than the  
21 corresponding median values.

22 **Defining features set.** The definitions of the 5 measures of topology for each node “i” are as  
23 follows. Degree was defined as the number of links to node i. Betweenness was defined as the  
24 number of edges running through node i. Closeness was defined as the inverse of the sum of the  
25 distances from node i to all other nodes; it can be regarded as a measure of the time required to  
26 spread sequentially information from node i to all other nodes. Degree, betweenness, and  
27 closeness centralities are correlated with a protein’s topological importance in a PPI network [61].

28 K-core analysis is an iterative process in which nodes are sequentially removed from a network,  
29 from the least-connected node to the most-connected node [62]. The core of maximum order is  
30 defined as the main (highest) k-core in a network. A k-core sub-network of an original network

1 can be generated by recursively deleting vertices with a degree less than  $k$  from the network,  
 2 resulting in a series of sub-networks that gradually reveal the globally central region of the  
 3 original network. The  $k$ -value is a measure of the centrality of node  $i$ . Edge betweenness is defined  
 4 as the frequency at which an edge belongs to the shortest paths between all pairs of vertices in a  
 5 network [63]. The edges with the highest betweenness values are most likely to lie between  
 6 sub-graphs.

7 The edge betweenness for edge  $e$  was defined as

$$8 \quad EB(e) = \sum_{vi \in V} \sum_{vj \in V \setminus \{vi\}} \frac{\sigma_{vij}(e)}{\sigma_{vij}} \dots \dots \dots fl$$

9 where  $\sigma_{vij}$  is the number of the shortest paths between nodes  $V_i$  and  $V_j$  in the network, and  
 10  $\sigma_{vij}(e)$  is the number of the shortest paths between  $V_i$  and  $V_j$ .

11

## 12 **Experimental validation**

13 **Animals and stroke models.** Wild-type healthy male C57BL/6 mice weighing 19–21 g (6-  
 14 to 8-week old) were obtained from the Experimental Animal Center of the Peking University  
 15 Health Science Center (Laboratory animal certificate: scxk 2012-0001; Beijing, China).  
 16 Anesthesia was induced by 5% isoflurane and maintained using 1% to 2% isoflurane during  
 17 surgery. In the middle cerebral artery occlusion (MCAO) model [40], focal cerebral ischemia was  
 18 generated by occluding the left MCA by inserting an intraluminal 5-0 nylon monofilament suture  
 19 through the common carotid artery to the branch point of the MCA for 6 h. The core body  
 20 temperature was maintained at 36.5–37.2°C throughout the experiments, and the arterial pO<sub>2</sub>,  
 21 pCO<sub>2</sub>, and pH were maintained within normal ranges. For measuring acute infarct size, mice were  
 22 euthanized with isoflurane 6 h after MCAO. Brains were collected and sectioned coronally at  
 23 1-mm intervals to produce 5 sections. Each section was stained with a solution of 0.5%  
 24 2,3,4-triphenyltetrazolium-chloride (TTC). A computerized image analysis system (ImageJ,  
 25 version 6.0) was used to measure the area of infarction in the inner section. Infarct volumes were  
 26 calculated from the ischemic area and the thickness of each slice. The neurological function of  
 27 each mouse was measured 6 h after left MCA occlusion according to the following 0–4-point

1 graded scoring system: 0 = no deficit; 1 = forelimb weakness and torso turning to the ipsilateral  
2 side when held by the tail; 2 = circling to the affected side; 3 = falling in the direction contralateral  
3 to the infarct; and 4 = depressed level of consciousness without spontaneous movement, as  
4 described previously [64,65].

5 **Bioassay of the mixtures of 16 BNC constituents and 2 primary active BNC constituents.** The  
6 16 BNC constituents were weighed and dissolved in a solution of 0.5% Tween-80 in water. The  
7 ratio of the 16 standards in the standard solution was based on the average relative content of each  
8 constituent in the 15 tested batches of BNCs. Before the experiment, the animals were weighed  
9 and randomly assigned to 9 groups (10 mice in each group). Group 1 (High\_16-MS) was orally  
10 administered the mixture of the 16 standards, which contained gallic acid (4.7 mg/kg), danshensu  
11 (1.5 mg/kg), hydroxysafflor yellow A (4.2 mg/kg), chlorogenic acid (1.9 mg/kg), amygdalin (25.8  
12 mg/kg), protocatechuic aldehyde (0.2 mg/kg), (-)-epicatechin (1.2 mg/kg), caffeic acid (0.1  
13 mg/kg), albiflorin (0.9 mg/kg), ononin (0.1 mg/kg), paeoniflorin (20.0 mg/kg), rutin (0.1 mg/kg),  
14 salvianolic acid A (0.6 mg/kg), cinnamic acid (0.3 mg/kg), formononetin (0.2 mg/kg), and  
15 dihydrotanshinone I (0.2 mg/kg). Group 2 (Medium\_16-MS) was orally administered the mixture  
16 of the 16 standards at 50% of the dose administered to group 1. Group 3 (low dose) was orally  
17 administered the mixture of the 16 standards at 25% of the dose administered to the group 1.  
18 Group 4, as a positive control group; mice in this group were orally administered normal saline.  
19 Group 5, as a positive control group was orally administered GBE at a dose of 71.5 mg/kg. Group  
20 6 was orally administered a mixture of 14 standards (14-MS, excluding amygdalin and  
21 paeoniflorin) at the medium dose administered to group 2. Group 7 was orally administered  
22 amygdalin at a dose of 12.9 mg/kg. Group 8 was orally administered paeoniflorin at a dose of 10.0  
23 mg/kg. Group 9 was the sham group.

#### 24 **Statistical analysis**

25 All data of bioassay experiments are represented as mean  $\pm$  standard deviation. Student's *t*-test  
26 was used when the analysis involved the comparison of two means, and a *P* value of less than 0.05  
27 was considered significant. The data were analyzed using SPSS 16.0 software.

#### 28 **Conflict of interests**

1 The authors declare that they have no conflict of interests.

2 **Author contributions**

3 Conceived and designed the experiments: HX, FL, HY. Performed the experiments: YZ, YS, QJ,

4 DL, YZ. Written the manuscript: HX, YZ.

5

6



1

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### Figure captions

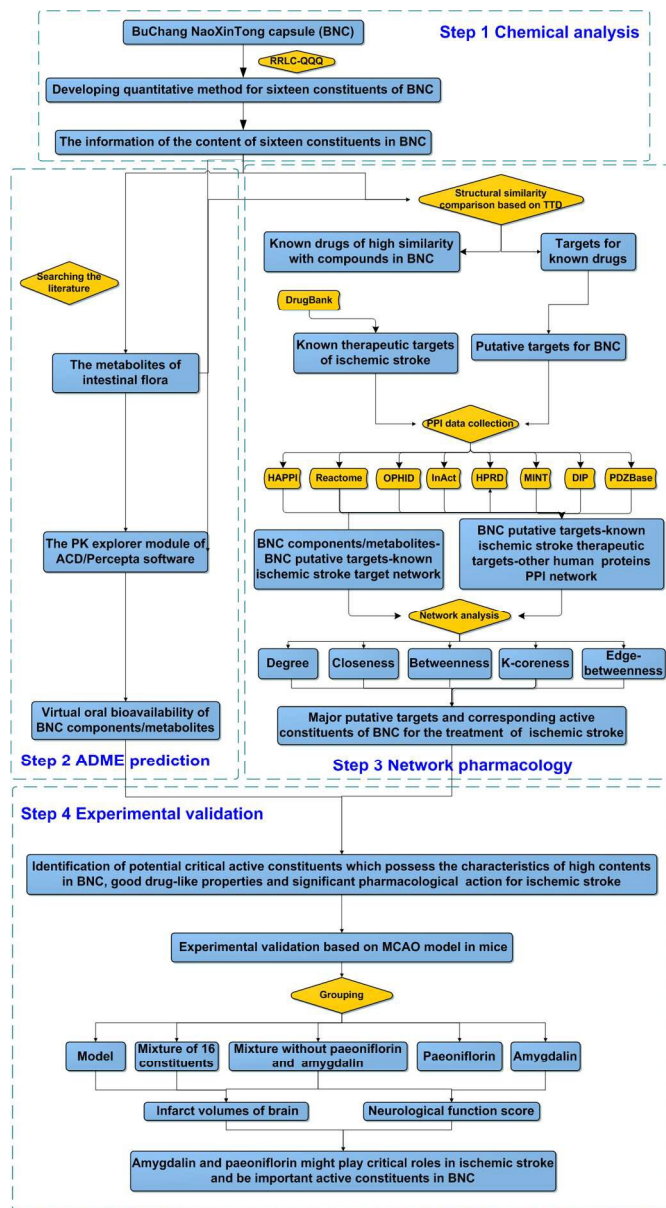
**Figure 1. Schematic diagram of the analytical approach.** Schematic diagram of the analytical approach used to identify the critical active constituents of Buchang Naoxintong capsules with therapeutic effects against ischemic stroke by integrating quantitative analysis, pharmacokinetic prediction, network analysis, and experimental validation.

**Figure 2. Chemical structures of the 16 constituents and 4 metabolites of Buchang Naoxintong capsules.**

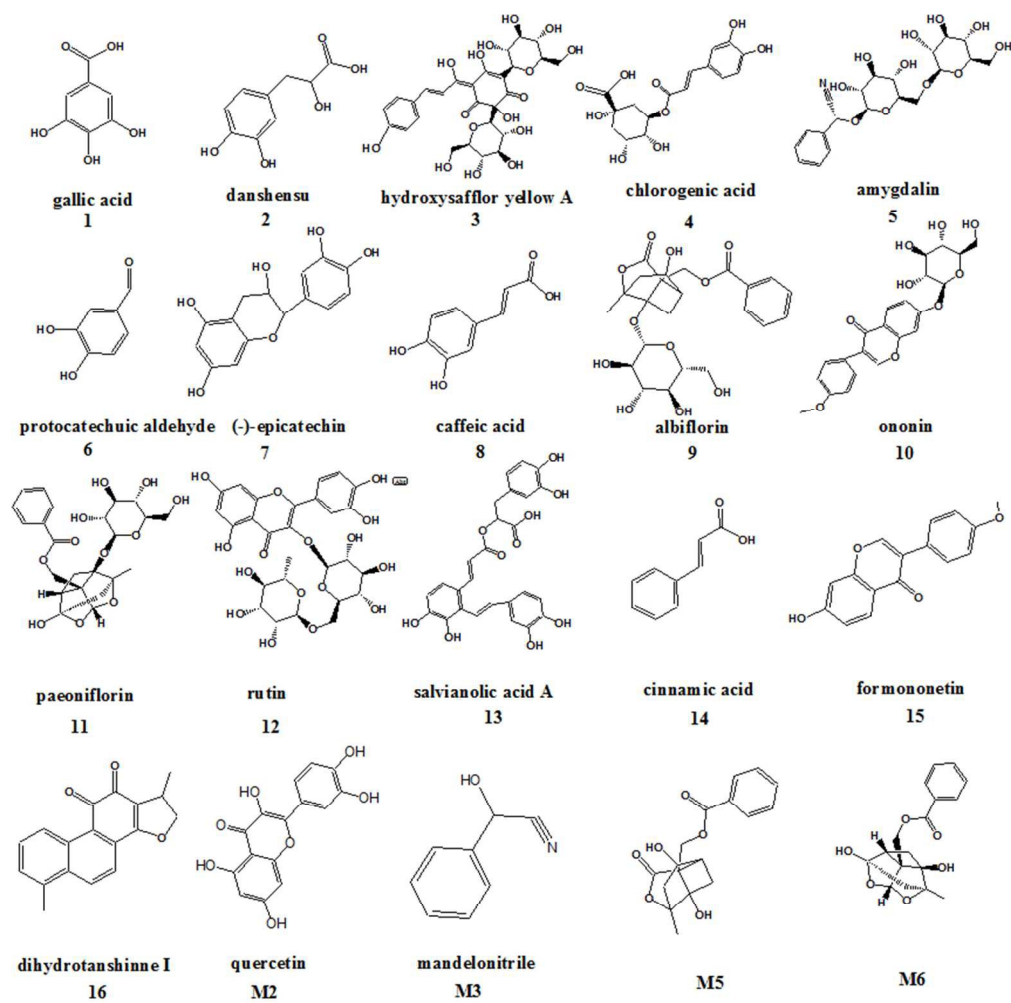
**Figure 3. Network of interactions between Buchang Naoxintong capsule (BNC) constituents and their predicted targets.** Interactions between predicted targets and known targets of drugs used to treat ischemic stroke patients were obtained from DrugBank. The network consists of 38 nodes and 51 edges. Blue nodes represent BNC constituents; red nodes, predicted targets of BNC constituents; yellow nodes, known targets of drugs used to treat ischemic stroke patients obtained from DrugBank.

**Figure 4. Network of interactions between predicted targets of Buchang Naoxintong capsule (BNC) constituents, known targets of drugs used to treat ischemic stroke patients obtained from DrugBank, and other human genes.** The network consists of 184 nodes and 423 edges. Red nodes represent predicted targets of BNC constituents; yellow nodes, targets of drugs used to ischemic stroke patients obtained from DrugBank; green nodes, other human genes; yellow nodes with red rings, known targets of drugs used to treat ischemic stroke patients that are also predicted targets of BNC constituents. Nodes with gene names refer to key nodes with topological importance according to their degree, closeness, betweenness, and k-value.

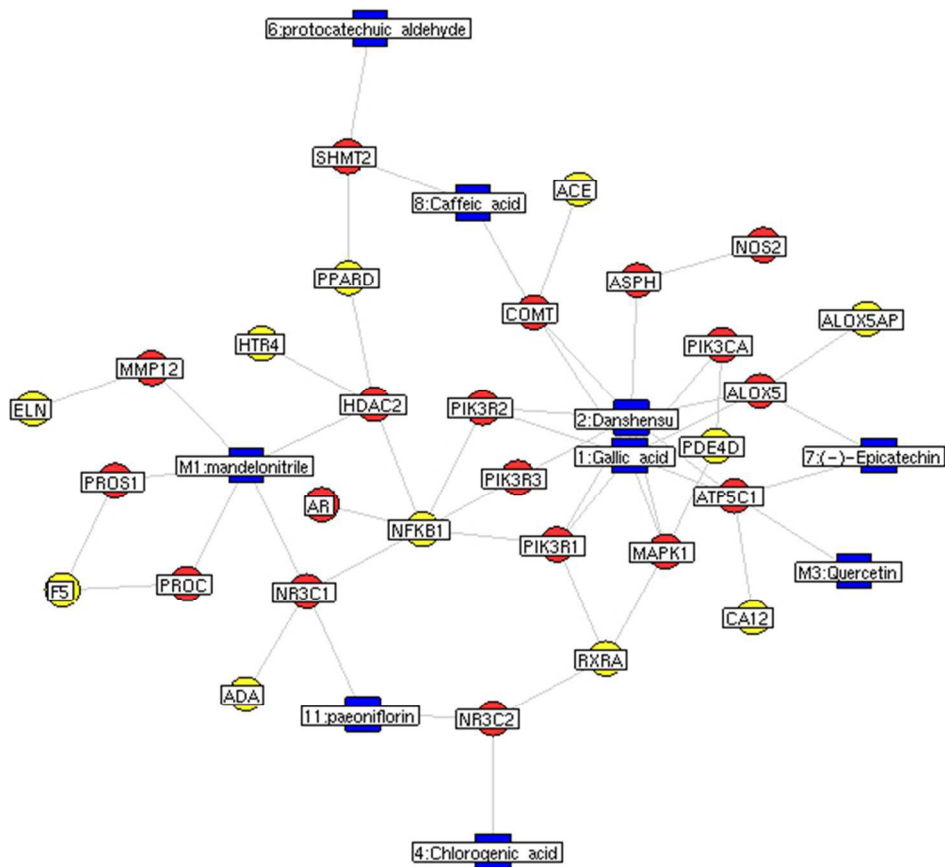
**Figure 5. Evaluation of the effects of different mixtures/active Buchang Naoxintong capsule (BNC) constituents in the middle cerebral artery occlusion (MCAO) model.** (a) Infarction rates; (b) neurological deficit scores. High\_16-MS, high dose of the mixture of the 16 standards; medium\_16-MS, medium dose of the mixture of the 16 standards; low\_16-MS, low dose of the mixture of the 16 standards; 14-MS, the mixture of 14 standards excluding amygdalin and paeoniflorin; and GBE, *Ginkgo biloba* extract.



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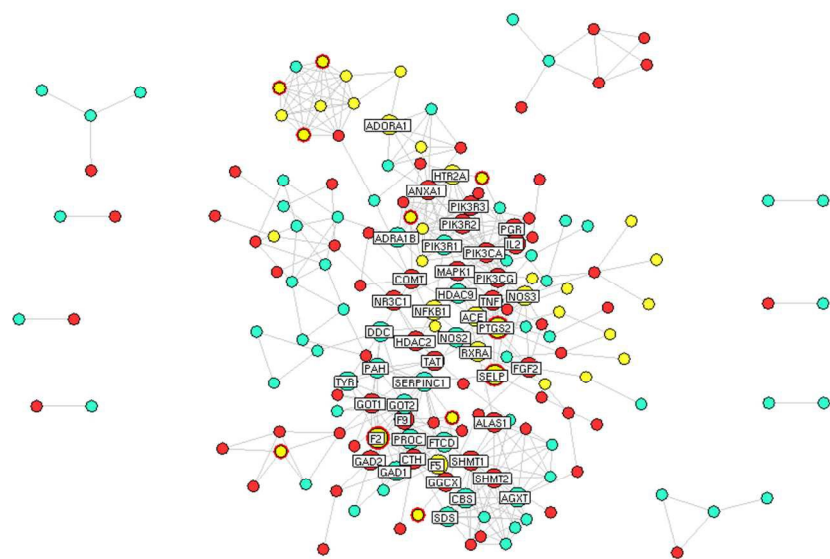


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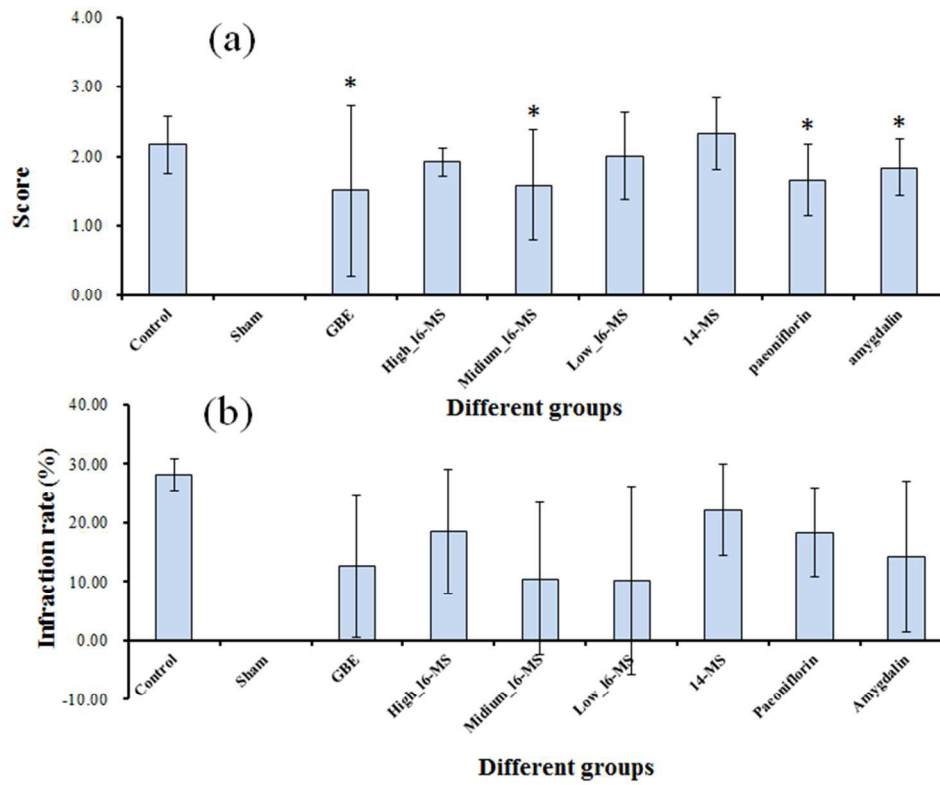


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159x129mm (120 x 120 DPI)