

Evaluation of the anti-inflammatory and antioxidant pharmacodynamic components of naoxintong capsules as a basis of broad spectrum effects

Bin Lv^a, Lina Deng^a, Tian Xie^a, Xing Wei^a, Xiao Liu^a, Wangxiao Tan^a, Xiaoying Wang^{a,b} and Xiumei Gao^a

^aState Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China; ^bCollege of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China

ABSTRACT

Context: Naoxintong capsule (NXT) is one of the most prevalent Traditional Chinese Medicine formulations in the treatment of coronary heart disease (CHD), yet the action of pharmacodynamic components remains unclear.

Objective: To determine the basis by which pharmacodynamic components of NXT may be effective in the treatment of CHD.

Materials and methods: The protective effect of NXT (0.01–100 µg/mL) on 293T and hy926 cells was determined by MTT assay for 24 h. Afterwards, to investigate the pharmacodynamic material basis of NXT in anti-inflammatory and antioxidant effects, based on previous UPLC/Q-TOF analysis, 293T and hy926 cells were divided into control (treated with solvent), model (incubated with TNF- α , LPS or H₂O₂), intervention (treated with UPLC components) and positive groups. After 24 h of treatment, all cells were tested to verify the screening results. MOE software was applied to dock bioactive compounds with phosphoinositide 3-kinase (PI3K), then the protein expression and phosphate levels were determined by western blotting.

Results: NXT could significantly inhibit the expression of NF- κ B, MMP-9 and NO in cells with IC₅₀ values of 0.1178, 0.1182 and 0.1094 µg/mL. Based on the screening results, six components of NXT were identified (calycosin, ferulic acid, salvianolic acid B, ononin, salvianolic acid E, and salvianolic acid F) which can inhibit NF- κ B, MMP-9, and NO simultaneously, while exerting cytoprotective effects by inhibiting the activation of the PI3K/AKT pathway under different conditions by virtue of their advantageous interaction with PI3K.

Conclusions: These ingredients have outstanding therapeutic potential and may provide a scientific basis for the future application and research of NXT.

ARTICLE HISTORY

Received 1 February 2020
Revised 6 November 2020
Accepted 23 December 2020



KEYWORDS

High-throughput screening;
PI3K/AKT pathway;
biological activity
ingredients

Introduction

Coronary heart disease (CHD) has high morbidity and mortality, and is one of the most serious diseases that has aroused widespread concern globally (Anderson et al. 2016). In China, CHD is one of the leading causes of death (Ma et al. 2016a). CHD is a chronic inflammatory disorder; plaque formation can cause the narrowing of the coronary artery, the blood supply to the heart is then reduced, thereby triggering CHD (Chhibber-Goel et al. 2016). It is well established that atheromatous, dyslipidemia, and endothelial dysfunction are the most important risk factors for CHD. In the early stages of CHD, certain risk factors such as hypercholesterolaemia, hypertension, chemical stimuli, and obesity can promote endothelial cell damage. After that, macrophages are aggregated to the injury sites, subsequently taken up by oxidized low density lipoprotein, transformed to foam cells, ultimately produce the early atherosclerotic plaques (Wang et al. 2015), and causing the impaired coronary microvascular function. In addition, ventricular hypertrophy, thrombus, myocardial oedema and smooth muscle dysfunction may also cause impairment of coronary microvascular function (Gutiérrez et al. 2013).

Inflammation plays an important role in CHD. Pro-inflammatory cytokines and activated neutrophils, can cause instability of atherosclerotic plaque, thrombosis, platelet aggregation (Lyu et al. 2015), and induce the damaged endothelial cells (Zhang and Li 2018). The transcription factor nuclear factor- κ B (NF- κ B) can increase the level of pro-inflammatory cytokines and exacerbate the inflammatory reaction. During the process of inflammation, macrophages secrete a large amount of matrix metalloprotein-9 (MMP-9), which plays a key role in the process of basal membrane neutrophil migration, vascular remodelling, platelet production, and plaque rupture (Busti et al. 2010). MMP-9 can degrade collagen and allow smooth muscle cells to migrate inside blood vessels, which can accumulate other cellular material, leading to tissue ischaemic events and atherosclerosis (Han et al. 2018). In addition, reactive oxygen species (ROS) are also an important factor in CHD. Excessive ROS can be accompanied by the production of reactive nitrogen species (RNS), a strong oxidant produced by the reaction of nitric monoxide (NO) and O²⁻ (Lubos et al. 2008). Under inflammatory conditions, the production of NO greatly increased, combined with other ROS, contributing to the formation of oxidative stress,

CONTACT Xiaoying Wang  wxy@tjutcm.edu.cn  College of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, 301617, China

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

promoting endothelial cell and vascular dysfunction, resulting in the formation of unstable plaque.

In the development of CHD, PI3K/AKT pathway shows an extremely important role. But it works two ways. On the one hand, it can serve to repress cardiomyocytes apoptosis by inhibiting the activities of pro-apoptotic proteins and protecting the heart against ischemia-reperfusion injury; on the other hand, it can actively promote platelet aggregation (Feng et al. 2019), involved in physiological cardiac hypertrophy (Song et al. 2015) and lead to the formation of unstable atherosclerotic plaques prone to rupture (Zhai et al. 2014). Phosphoinositide 3-kinase (PI3K) activates protein kinase B (AKT), promotes its phosphorylation, regulates the transcriptional activity of NF- κ B, or affects the upstream pathways (Hoesel and Schmid 2013). After activation, PI3K/AKT induces the expression of MMP-9, and enhanced chemokines released by endothelial cells (Dilly et al. 2013). Beyond that, activated AKT can directly phosphorylate endothelial nitric oxide synthase (eNOS), thereby promoting NO production (Abeyrathna and Su 2015), and give rise to oxidative stress injury. Therefore, selective inhibition of PI3K is of great significance in prevention and cure CHD.

In recent years, more and more traditional Chinese herbal preparations have been used to treat cardiovascular diseases. As a Traditional Chinese Medicine formula, Naointong capsule (NXT) was approved by the Sino Food and Drug Administration (Z20025001), and is currently used as a hospital prescription. NXT was developed from Buyang Huanwu decoction and is composed of 16 ingredients (Table 1). It has been widely used to treat CHD for more than 20 years in China (Yang et al. 2017). NXT can exert a cardioprotective effect by inhibiting NLRP3 inflammasome activation in the ischemia/reperfusion injury model (Wang et al. 2017). NXT can activate PPAR α pathway to protect cardiomyocytes, inhibit cardiac hypertrophy, and retard atherosclerosis (Xu et al. 2016; Yuan et al. 2017; Wang et al. 2018). NXT also can reduce the NO level and the expression of inducible nitric oxide synthase (iNOS) in the vessel wall to treat atheromatous (Zhong et al. 2013). In clinics, NXT can be used for carotid atherosclerosis and cerebral infarction (Liang et al. 2018), partly abrogate the impaired protective effect of high-density lipoprotein (HDL) on endothelial function (Lv et al. 2016), and improve adverse cardiovascular events, atherosclerosis, and the expression of inflammatory factors in patients with acute coronary syndrome undergoing PCI, when combined with Danhong injection (Lv et al. 2015; Zhao et al. 2018).

Table 1. The ingredients of NXT.

Ingredient name	Latin binomial	Abbreviations
Radix Astragali	<i>Astragalus membranaceus</i> (Fisch.) Bge.	RA
Radix Paeoniae Rubra	<i>Paeonia veitchii</i> Lynch	RPR
Radix Salviae Miltiorrhizae	<i>Salvia miltiorrhiza</i> Bunge	RSM
Radix Angelicae Sinensis	<i>Angelica sinensis</i> (Oliv.) Diels	RAS
Rhizoma Chuanxiong	<i>Ligusticum chuanxiong</i> Hort.	RCX
Radix Achyranthis Bidentatae	<i>Achyranthes bidentata</i> Blume	RAB
Flos Carthami	<i>Carthamus tinctorius</i> Linn.	FC
Frankincense	<i>Boswellia carterii</i> Birdw.	FK
Myrrha	<i>Commiphora myrrha</i> (Nees) Engl.	MRH
Caulis Spatholobi	<i>Spatholobus suberectus</i> Dunn	CS
Semen Persicae	<i>Prunus persica</i> (L.) Batsch	SP
Ramulus Mori	<i>Morus alba</i> L.	RM
Ramulus Cinnamomi	<i>Cinnamomum cassia</i> Presl	RC
Pheretima	<i>Pheretima aspergillum</i> (E.Perrier)	PT
Scorpio	<i>Buthus martensii</i> Karsch	SCP
Hirudo	<i>Hirudo nipponica</i> Whitman	HRD

Although NXT has been proven to be widely useful in the clinical treatment of cardiovascular diseases, due to the complex chemical composition, the mechanism of action and pharmacodynamic substance foundation of NXT are still unknown. In this study, based on previous work, we developed a multiple-high throughput screening method, explored the pharmacodynamic basis of NXT for inhibiting NF- κ B, MMP-9, and NO, established effective active ingredients by *in vitro* screening, explored the effect of the active components on PI3K/AKT signalling pathway, identified the possible mechanism of NXT in prevention and cure CHD, and provide a theoretical basis for the clinical application of NXT.

Materials and methods

Sample preparation

NXT (1 g) (Heze Buchang Pharmaceutical Co., Ltd., Heze, China) was ultrasonically dissolved in 10 mL of 75% methanol (Merck, Darmstadt, Germany) for 10 min, centrifuged to obtain the supernatant and stored at -20°C .

In a previous study, we identified 81 major compositions in NXT by Ultra-performance liquid chromatography/quadrupole time-of-flight (UPLC/Q-TOF) (Ma et al. 2016b). Based on the pre-existing condition, UPLC fractions were collected every 30 s and vacuum dried. The residues were stored at -20°C for the follow-up experiment.

Cell culture

Human embryonic kidney cells (HEK 293 cells, American Type Culture Collection, Rockville, MD, USA) were cultured in high-glucose Dulbecco's modified Eagle's Medium (DMEM) (Biological Industries Israel Beit Haemek Ltd., Israel), including 10% foetal bovine serum (FBS) (Biological Industries Israel Beit Haemek Ltd., Israel), 0.1 mg/mL streptomycin and 100 U/mL penicillin (Biological Industries Israel Beit Haemek Ltd., Israel). Cells passaged when they reached 70–80% confluence.

Human umbilical vein endothelial cell line EA.hy926, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), cultured similarly to HEK 293 cells. All the cells were confluent in 96-well plates for 12 h before use.

Cell viability assay

Cell viability was measured by the MTT (Sigma Aldrich, Steinheim, Germany) assay. After incubation with drugs, cells were treated with MTT (20 μL , 0.5 mg/mL) for 4 h. Formazan crystals were dissolved in 150 μL DMSO by shaking for 15 min, and measured the viability at 490 nm.

Screening of anti-NR- κ B components

Transfection

HEK 293 cells were seeded into a 96-well plate and transfected when the cell confluence was 50–70%. Cells were transfected with the NF- κ B luciferase reporter plasmid PGL 4.32 and the Renilla luciferase reporter vector plasmid pRL-TK at 100 and 9.6 ng per well, respectively. Transfection was performed for 24 h by using the transfection reagent PEI (PEI: pGL 4.32 = 8:1, W/W) before drug treatment.

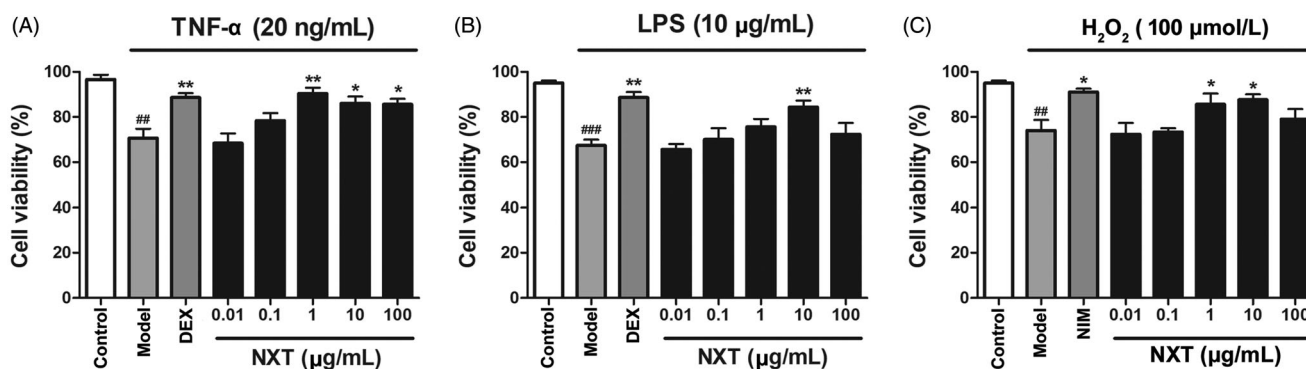


Figure 1. Cell viability assay. (A) TNF- α treated 293 T cell viability detected by MTT assay. (B) LPS treated 293 T cell viability detected by MTT assay. (C) H₂O₂ treated EA hy926 cell viability detected by MTT assay. The results are the mean \pm SEM ($n = 6$ per group); $##p < 0.01$, $###p < 0.001$, versus the control group; $*p < 0.05$, $**p < 0.01$, versus the model group.

Dual-luciferase assay

Hek 293 cells were incubated with DMEM containing TNF- α (20 ng/mL, Sigma, St. Louis, MO, USA) for 6 h. The cells pre-treated with dexamethasone (DEX, 10 μ mol/L, Sigma, St. Louis, MO, USA) and drugs (diluted by DMEM, the concentration was identical with the effective concentration of NXT we had detected before) for 24 h after transfection. All the chemical standards were purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Dual-Luciferase Reporter Assay Kit was used to detect the transcriptional activity of NF- κ B.

Screening of anti-MMP-9 components

EA.hy926 cells were treated for 24 h to DEX (10 μ mol/L) plus lipopolysaccharides (LPS, 10 μ g/mL, Peprotech, Rocky Hill, CT, USA), drugs (diluted by DMEM, the concentration was identical with the effective concentration of NXT) plus LPS (10 μ g/mL), LPS (10 μ g/mL), or DMEM before detection. Human MMP-9 ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the expression of MMP-9. The absorbance was measured at 450 nm.

Screening of anti-NO components

After incubation in 96-well plates, EA.hy926 cells were exposed for 24 h to Nimodipine (NIM, 10 μ mol/L, Sigma, St. Louis, MO, USA) plus H₂O₂ (100 μ mol/L, Sigma, St. Louis, MO, USA), drugs (diluted by DMEM) plus H₂O₂ (100 μ mol/L), H₂O₂ (100 μ mol/L), or cell culture medium before detection. Treatments were performed at room temperature in the absence of light. Human NO ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the release of NO from the supernatants of EA.hy926 cells after stimulation. The absorbance was measured at 450 nm.

Western-blot

Cells collected after incubation with drugs were prepared with RIPA buffer (Solarbio, Beijing, China). Proteins were separated and transferred to PEGF membranes (GE Healthcare Life Science, Boston, MA, USA), and incubated with different primary antibodies overnight at 4 $^{\circ}$ C. The HRP-conjugated secondary antibody was incubated for 50 min, and followed by detection with chemiluminescence (ECL, ProteinSimple, Santa

Clara, CA, USA). All the antibodies were bought from Cell Signalling Technology (Danvers, MA, USA).

Target prediction of active ingredients from NXT

We obtained the 3D structures of potential protein targets from Protein Data Bank (<http://www.rcsb.org/pdb>). The SYBYL software was used to construct and minimize the molecules and potential targets, and then docked by AutoDock version 4.2 using a hybrid Lamarckian genetic algorithm (LGA). Using the Molecular Operating Environment (MOE 2015.10) software was used to dock the molecules into the active site of the potential protein.

Statistical analysis

All the results were expressed as the mean values \pm standard error of the mean (SEM). Data were evaluated by one-way analysis of variance (ANOVA) with the Tukey's test for comparisons between groups using SPSS 16.0. Probability values of less than 0.05 were considered to be significant.

Result

Cell viability

According to Figure 1(A), the viability of the model group (treated with TNF- α) was decreased ($##p < 0.01$ vs. control). When it was treated by NXT and DEX, the cell viability increased significantly ($*p < 0.05$ for 10 μ g/mL, 100 μ g/mL NXT vs. model, $**p < 0.01$ for 1 μ g/mL NXT and DEX vs. model). In Figure 1(B), the viability of the LPS (10 μ g/mL) treated group was decreased ($###p < 0.001$ vs. control) but the intervention group could improve the viability of cells ($**p < 0.01$ for DEX and 10 μ g/mL NXT vs. model). H₂O₂ (100 μ mol/L) could reduce the viability ($**p < 0.01$ vs. control); the cell viability increased when treated by NIM and NXT ($*p < 0.05$ for NIM and 1 μ g/mL, 10 μ g/mL NXT vs. model) (Figure 1(C)).

Screening of NF- κ B inhibitory active ingredients in NXT

In Figure 2(A), we screening the optimal concentration of NXT to inhibit NF- κ B by dual-luciferase assay. NXT (1, 10 μ g/mL) could decrease the over-expression of NF- κ B induced by TNF- α ($***p < 0.001$, $**p < 0.01$, with IC₅₀ values of 0.1178 μ g/mL),

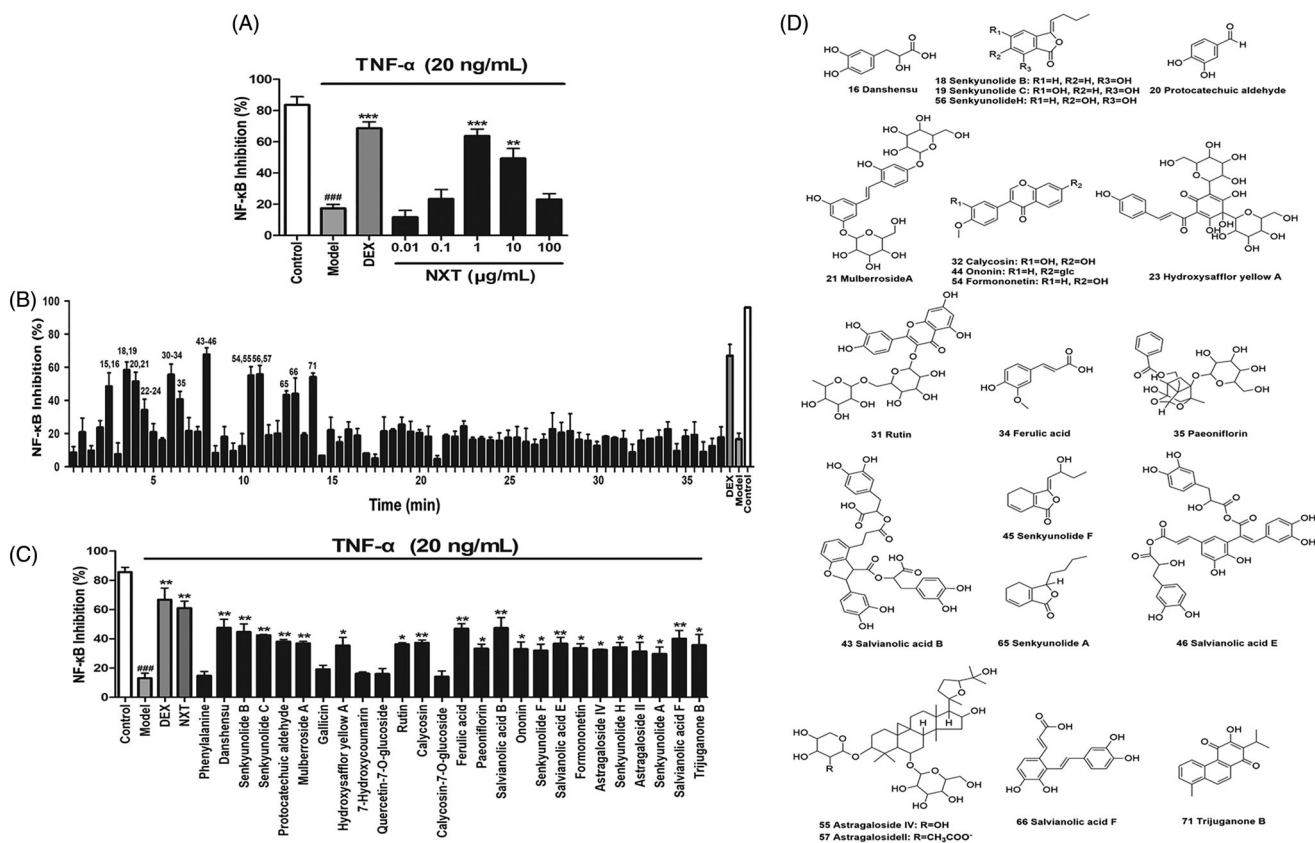


Figure 2. Bioactivity analysis of NXT on anti-NF- κB . (A) The inhibitory effect of NXT on NF- κB in 293 T cells. (B) The screening of potential NF- κB inhibitory active ingredients in NXT. (C) Verification the NF- κB inhibition active ingredients in NXT. (D) Chemical structures of the NF- κB inhibitory active ingredients in NXT. The results are the mean \pm SEM ($n = 6$ per group); $###p < 0.001$ versus the control group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, versus the model group.

and 1 $\mu\text{g/mL}$ was more effective. Through the dual-luciferase assay (Figure 2(B)), the UPLC fraction in 12 time periods we collected could inhibit NF- κB : 2–2.5 min, 3–3.5 min, 3.5–4 min, 4–4.5 min, 5.5–6 min, 6–6.5 min, 7.5–8 min, 10–10.5 min, 10.5–11 min, 12–12.5 min, 12.5–13 min and 13.5–14 min. The effective fractions were analysed by mass spectrometry, and we discovered 26 ingredients as potential NF- κB inhibitors: phenylalanine, danshensu, senkyunolide B, senkyunolide C, protocatechuic aldehyde, mulberroside A, gallicin, hydroxysafflor yellow A, 7-hydroxycoumarin, quercetin-7-*O*-glucoside, rutin, calycosin, calycosin-7-*O*-glucoside, ferulic acid, paeoniflorin, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, formononetin, astragaloside IV, senkyunolide H, astragaloside II, senkyunolide A, salvianolic acid F, and trijuganone B. The anti-NF- κB ingredients in NXT were verified (Figure 2(C)), and 21 ingredients were identified that can inhibit NF- κB (Table 2, Figure 2(D)).

The screening of MMP-9 inhibitory active ingredients in NXT

In Figure 3(A), we screening the effective concentration of NXT to inhibit MMP-9. NXT (1, 10 $\mu\text{g/mL}$) can inhibit MMP-9 induced by LPS ($**p < 0.01$, $*p < 0.05$, with an IC_{50} value of 0.1182 $\mu\text{g/mL}$), and 10 $\mu\text{g/mL}$ was the optimum concentration. Through the MMP-9 inhibition screening (Figure 3(B)), the UPLC fraction in 8 time periods were collected could inhibit MMP-9: 2–2.5 min, 3–3.5 min, 3.5–4 min, 4.5–5 min, 5.5–6 min, 7.5–8 min, 9.5–10 min and 12.5–13 min, and 22 compounds were discovered as potential MMP-9 inhibitors, phenylalanine, danshensu, senkyunolide B, senkyunolide C, protocatechuic aldehyde, mulberroside A, vanillic acid, benzoic acid, epicatechin,

quercetin-7-*O*-glucoside, rutin, calycosin, calycosin-7-*O*-glucoside, ferulic acid, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, pratensein, hydroxyl calendic acid, *trans*-oxyresveratrol, and salvianolic acid F. The anti-MMP-9 ingredients in NXT were verified with 293 T cells (Figure 3(C)), and 16 ingredients were identified that can inhibit MMP-9 (Table 3, Figure 3(D)).

The screening of NO inhibitory active ingredients in NXT

In Figure 4(A), NXT (1, 10 $\mu\text{g/mL}$) inhibits NO ($**p < 0.01$, $*p < 0.05$, with an IC_{50} value of 0.1094 $\mu\text{g/mL}$), and 10 $\mu\text{g/mL}$ was the most effective concentration as an antioxidant. Through the NO inhibition screening (Figure 4(B)), the UPLC fraction in 7 time periods found activation at: 5–5.5, 5.5–6, 7.5–8, 10–10.5, 12.5–13, 13.5–14, and 18–18.5 min. Seventeen compounds were discovered as potential NO inhibitors: catechin, albiflorin, quercetin-7-*O*-glucoside, rutin, calycosin, calycosin-7-*O*-glucoside, ferulic acid, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, formononetin, astragaloside IV, salvianolic acid F, trijuganone B, tanshinone IIA, and angelic acid; the biological active ingredients in NXT were verified (Figure 4(C)). Thirteen ingredients were identified that cause the NO inhibition (Table 4, Figure 4(D)).

Molecular docking of the biological activity ingredients in NXT with PI3K

As a result of screening, six components in NXT were determined to inhibit NF- κB , MMP-9, and NO: salvianolic acid B,

Table 2. NF- κ B-inhibiting compounds identified in Naoxintong Capsules.

Peak No.	RT (min)	Identification	Mode	MS (<i>m/z</i>)	Composition	Herbal source
16	2.459	Danshensu	Neg	198.0501	C ₉ H ₁₀ O ₅	RSM
18	3.438	Senkyunolide B	Neg	204.2374	C ₁₂ H ₁₂ O ₃	RCX
19	3.456	Senkyunolide C	Neg	204.2374	C ₁₂ H ₁₂ O ₃	RCX
20	3.600	Protocatechuic aldehyde	Neg	138.1185	C ₇ H ₆ O ₃	RSM, RC
21	3.974	Mulberroside A	Neg	568.5277	C ₂₆ H ₃₂ O ₁₄	RM
22	4.122	Gallicin	Neg	184.1453	C ₈ H ₆ O ₅	RPR
23	4.230	Hydroxysafflor yellow A	Pos/Neg	612.5364	C ₂₇ H ₃₂ O ₁₆	FC
31	5.952	Rutin	Neg	610.5203	C ₂₇ H ₃₀ O ₁₆	RA
32	5.970	Calycosin	Neg	284.2679	C ₁₆ H ₁₂ O ₅	RA
34	5.989	Ferulic acid	Neg	194.1815	C ₁₀ H ₁₀ O ₄	RA, RCX, RAS, RAB
35	6.321	Paeoniflorin	Pos	480.466	C ₂₃ H ₂₈ O ₁₁	RPR
43	7.540	Salvianolic acid B	Neg/Pos	718.6220	C ₃₆ H ₃₀ O ₁₆	RSM
44	7.688	Ononin	Pos	430.4107	C ₂₂ H ₂₂ O ₉	RA, CS
45	7.763	Senkyunolide F	Pos	206.1017	C ₁₂ H ₁₄ O ₃	RCX, RAS
46	7.855	Salvianolic acid E	Neg	718.1512	C ₃₆ H ₃₀ O ₁₆	RSM
54	10.240	Formononetin	Pos/Neg	268.2580	C ₁₆ H ₁₂ O ₄	RA
55	10.405	Astragaloside IV	Neg	784.4633	C ₄₁ H ₆₈ O ₁₄	RA
56	10.590	Senkyunolide H	Neg	220.2305	C ₁₂ H ₁₂ O ₄	RCX
57	10.978	Astragaloside II	Neg	826.4701	C ₄₃ H ₇₀ O ₁₅	RA
65	12.198	Senkyunolide A	Pos	192.2516	C ₁₂ H ₁₆ O ₂	RCX
66	12.975	Salvianolic acid F	Neg	314.0735	C ₁₇ H ₁₄ O ₆	RSM
71	13.917	Trijuganone B	Pos	280.1107	C ₁₈ H ₁₆ O ₃	RSM

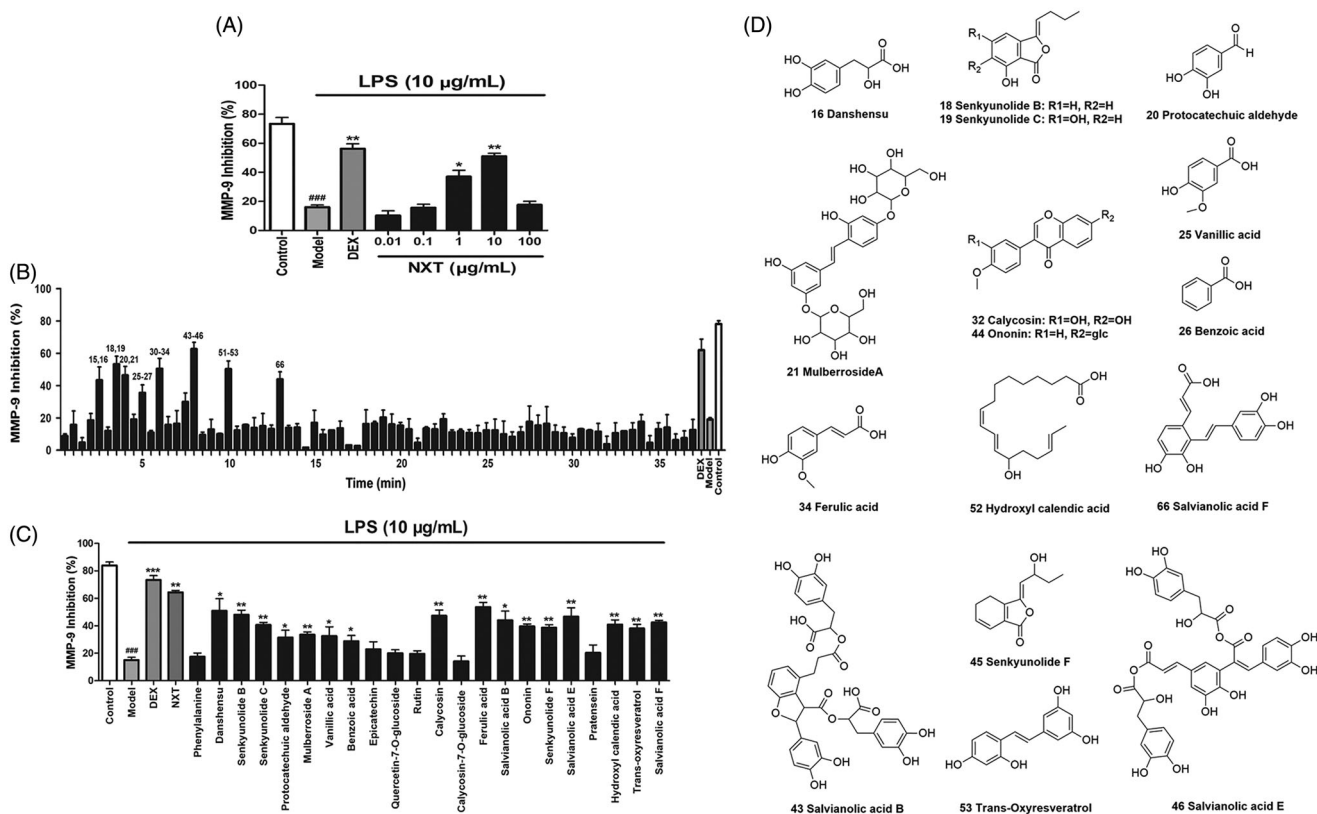


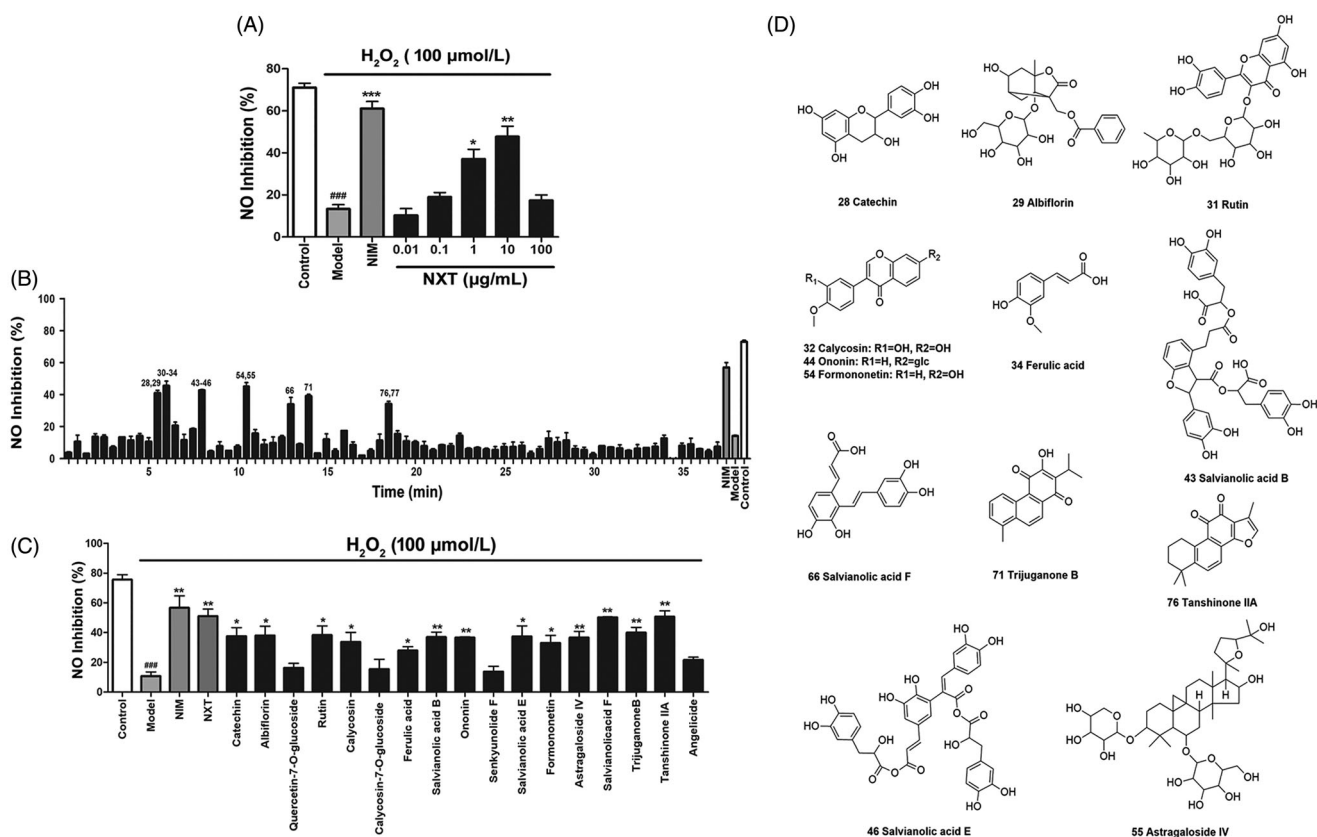
Figure 3. Bioactivity analysis of NXT on anti-MMP-9. (A) Effect of NXT on MMP-9 activation in LPS-stimulated 293T cells. (B) The screening of potential MMP-9 inhibitory active ingredients in NXT. (C) Verification the MMP-9 inhibition active ingredients in NXT. (D) Chemical structures of the MMP-9 inhibitory active ingredients in NXT. The results are the mean \pm SEM ($n = 6$ per group), $###p < 0.001$ versus the control group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, versus the model group.

ferulic acid, calycosin, salvianolic acid E, ononin, and salvianolic acid F (Figure 5(A)). The inhibition rate of the biological active ingredients is shown in Table 5. Based on reports, PI3K/AKT pathway plays a crucial role in CHD, so we determined whether the biological activity of NXT was related to the PI3K/AKT signalling pathway. We docked six biological active ingredients to the binding site of PI3K (PDB: 5ITD), and found that all the

ingredients had better associativity with PI3K. The binding energy is shown in Table 6. After that, we analysed and displayed a 3D map (Figure 5(B)) indicating the interaction of the biological active ingredients in NXT with PI3K. All the reactions take place in PIK helical domain and PI3K/PI4K domain, which are the activity region of PI3K. The ingredients established hydrogen bonds with the active site amino acids, such as ASP-

Table 3. MMP-9-inhibiting compounds identified in Naoxintong Capsules.

Peak No.	RT (min)	Identification	Mode	MS (<i>m/z</i>)	Composition	Herbal source
16	2.459	Danshensu	Neg	198.0501	C ₉ H ₁₀ O ₅	RSM
18	3.438	Senkyunolide B	Neg	204.2374	C ₁₂ H ₁₂ O ₃	RCX
19	3.456	Senkyunolide C	Neg	204.2374	C ₁₂ H ₁₂ O ₃	RCX
20	3.600	Protocatechuic aldehyde	Neg	138.1185	C ₇ H ₆ O ₃	RSM, RC
21	3.974	Mulberroside A	Neg	568.5277	C ₂₆ H ₃₂ O ₁₄	RM
25	4.565	Vanillic acid	Neg	168.1459	C ₈ H ₈ O ₄	RCX,RPR
26	4.694	Benzoic acid	Neg	122.1209	C ₇ H ₆ O ₂	RPR
32	5.970	Calycosin	Neg	284.2679	C ₁₆ H ₁₂ O ₅	RA
34	5.989	Ferulic acid	Neg	194.1815	C ₁₀ H ₁₀ O ₄	RA, RCX, RAS, RAB
43	7.540	Salvianolic acid B	Neg/Pos	718.6220	C ₃₆ H ₃₀ O ₁₆	RSM
44	7.688	Ononin	Pos	430.4107	C ₂₂ H ₂₂ O ₉	RA,CS
45	7.763	SenkyunolideF	Pos	206.1017	C ₁₂ H ₁₄ O ₃	RCX,RAS
46	7.855	Salvianolic acid E	Neg	718.1512	C ₃₆ H ₃₀ O ₁₆	RSM
52	9.611	Hydroxyl calendic acid	Neg	294.4342	C ₁₈ H ₃₀ O ₃	SP
53	9.648	<i>Trans</i> -Oxyresveratrol	Pos	244.2435	C ₁₄ H ₁₂ O ₄	RM
66	12.975	Salvianolic acid F	Neg	314.0735	C ₁₇ H ₁₄ O ₆	RSM

**Figure 4.** Bioactivity analysis of NXT on anti-NO. (A) Effect of NXT on NO activation in H₂O₂-stimulated EA hy926 cells. (B) The screening of potential NO inhibitory active ingredients in NXT. (C) Verification the NO inhibition active ingredients in NXT. (D) Chemical structures of the NO inhibitory active ingredients in NXT. The results are the mean ± SEM (n = 6 per group); ###*p* < 0.001 versus the control group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, versus the model group.**Table 4.** NO-inhibiting compounds identified in Naoxintong capsules.

Peak No.	RT (min)	Identification	Mode	MS (<i>m/z</i>)	Composition	Herbal source
28	5.157	Catechin	Neg	290.2674	C ₁₅ H ₁₄ O ₆	RPR
29	5.212	Albiflorin	Pos	480.4653	C ₂₃ H ₂₈ O ₁₁	RPR
31	5.952	Rutin	Neg	610.5203	C ₂₇ H ₃₀ O ₁₆	RA
32	5.970	Calycosin	Neg	284.2679	C ₁₆ H ₁₂ O ₅	RA
34	5.989	Ferulic acid	Neg	194.1815	C ₁₀ H ₁₀ O ₄	RA, RCX, RAS, RAB
43	7.540	Salvianolic acid B	Neg/Pos	718.6220	C ₃₆ H ₃₀ O ₁₆	RSM
44	7.688	Ononin	Pos	430.4107	C ₂₂ H ₂₂ O ₉	RA,CS
46	7.855	Salvianolic acid E	Neg	718.1512	C ₃₆ H ₃₀ O ₁₆	RSM
54	10.240	Formononetin	Pos/Neg	268.2580	C ₁₆ H ₁₂ O ₄	RA
55	10.405	Astragaloside IV	Neg	784.4633	C ₄₁ H ₆₈ O ₁₄	RA
66	12.975	Salvianolic acid F	Neg	314.0735	C ₁₇ H ₁₄ O ₆	RSM
71	13.917	Trijuganone B	Pos	280.1107	C ₁₈ H ₁₆ O ₃	RSM
76	18.076	Tanshinone IIA	Pos	294.3430	C ₁₉ H ₁₈ O ₃	RSM

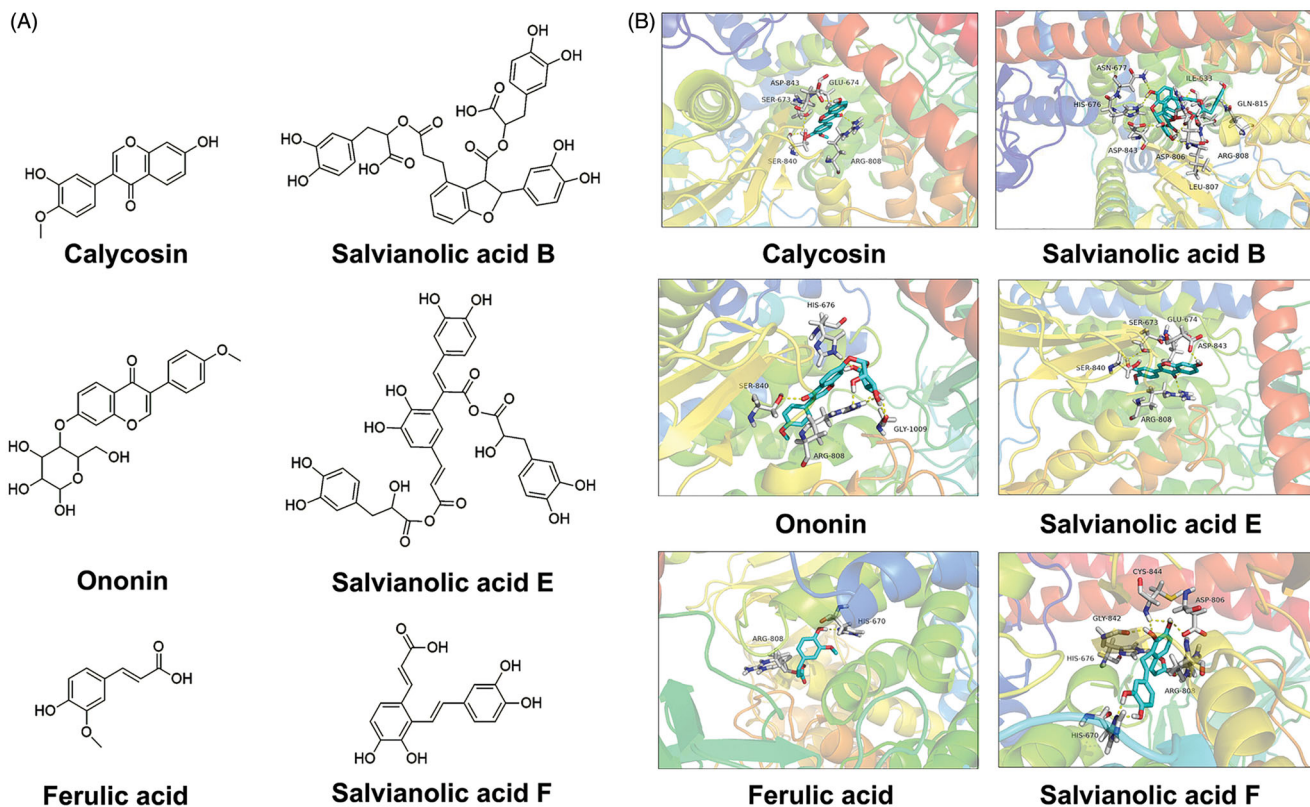


Figure 5. Molecular modelling of PI3K and the active ingredients. (A) Chemical structures of the active ingredients in NXT. (B) The 3D maps of the interaction of PI3K (PDB: 5ITD) with the active ingredients in NXT.

Table 5. The inhibition rate of the biological activity ingredients to NF- κ B, MMP-9 and NO.

NO	Identification	Inhibition rate (IR, %)		
		NF- κ B	MMP-9	NO
32	Calycosin	37.32 \pm 1.85	47.32 \pm 4.12	33.68 \pm 6.47
34	Ferulic acid	46.99 \pm 3.29	53.66 \pm 3.40	27.99 \pm 2.52
43	Salvianolic acid B	47.39 \pm 7.15	44.06 \pm 6.67	36.98 \pm 3.29
44	Ononin	32.93 \pm 4.97	39.60 \pm 1.69	36.62 \pm 0.49
46	Salvianolic acid E	36.67 \pm 4.33	46.67 \pm 6.49	37.39 \pm 7.15
66	Salvianolic acid F	40.09 \pm 5.65	42.42 \pm 1.50	50.33 \pm 0.27

Table 6. The binding energy of the biological activity ingredients and PI3K.

NO	Identification	Composition	Binding energy (kcal/mol)
32	Calycosin	C ₁₆ H ₁₂ O ₅	-6.64
34	Ferulic acid	C ₁₀ H ₁₀ O ₄	-5.51
43	Salvianolic acid B	C ₃₆ H ₃₀ O ₁₆	-4.34
44	Ononin	C ₂₂ H ₂₂ O ₉	-4.37
46	Salvianolic acid E	C ₃₆ H ₃₀ O ₁₆	-6.76
66	Salvianolic acid F	C ₁₇ H ₁₄ O ₆	-7.61

843, SER-840, ARG-808 and HIS-670. It is worth mentioning that all the ingredients could interact with ARG-808; it may be the major active site of PI3K.

Inhibition of the PI3K/AKT pathway by the biological activity ingredients in NXT

We explored the role of the biological active ingredients in NXT with the PI3K/AKT pathway under different conditions. In Figure 6(A), 293T cells were treated by TNF- α ; all the ingredients could inhibit PI3K/AKT pathway, especially ferulic acid, salvianolic acid E and salvianolic acid F. Salvianolic acid B and

salvianolic acid E were found more effective than the other ingredients in the LPS treated model to inhibited PI3K and p-AKT (Figure 6(B)). In Figure 6(C), oxidative stress model was induce by H₂O₂ and intervened using the biological active ingredients. The level of PI3K and p-AKT significantly decreased in the intervention group in comparison to the model, and calycosin, salvianolic acid B, and salvianolic acid E were much more effective than other ingredients.

Discussion

The treatment of cardiovascular disease by NXT may be related to its antioxidative and anti-inflammatory effects. According to Li et al. (2012), long-term administration of NXT can inhibit the expression of NF- κ B, decrease the content of MMP-9, in MMP-9/TIMP-1 serum may inhibit inflammation, and the incidence of critical lesions in patients with CHD could be reduced. In addition, NXT also has a protective effect on myocardial peroxidation damage caused by H₂O₂ (Zhang et al. 2013). It can pre-regulate enhanced antioxidant functions by increasing total superoxide dismutase, total antioxidative capacity, and catalase activity, reducing the production of malondialdehyde and reactive oxygen species. However, due to the large number of compositions and the complication of the interaction between various components, the basis and mechanism of the pharmacodynamics are unclear. Defining the basis of pharmacodynamics is not only a prerequisite for elucidating the mechanism of traditional Chinese medicines, but also an important basis for quality control, and provides a new material basis for the discovery of active compounds (Zhang et al. 2008).

Standard methods can screen effective ingredients by extracting, separating, purifying, and then combining *in vivo*

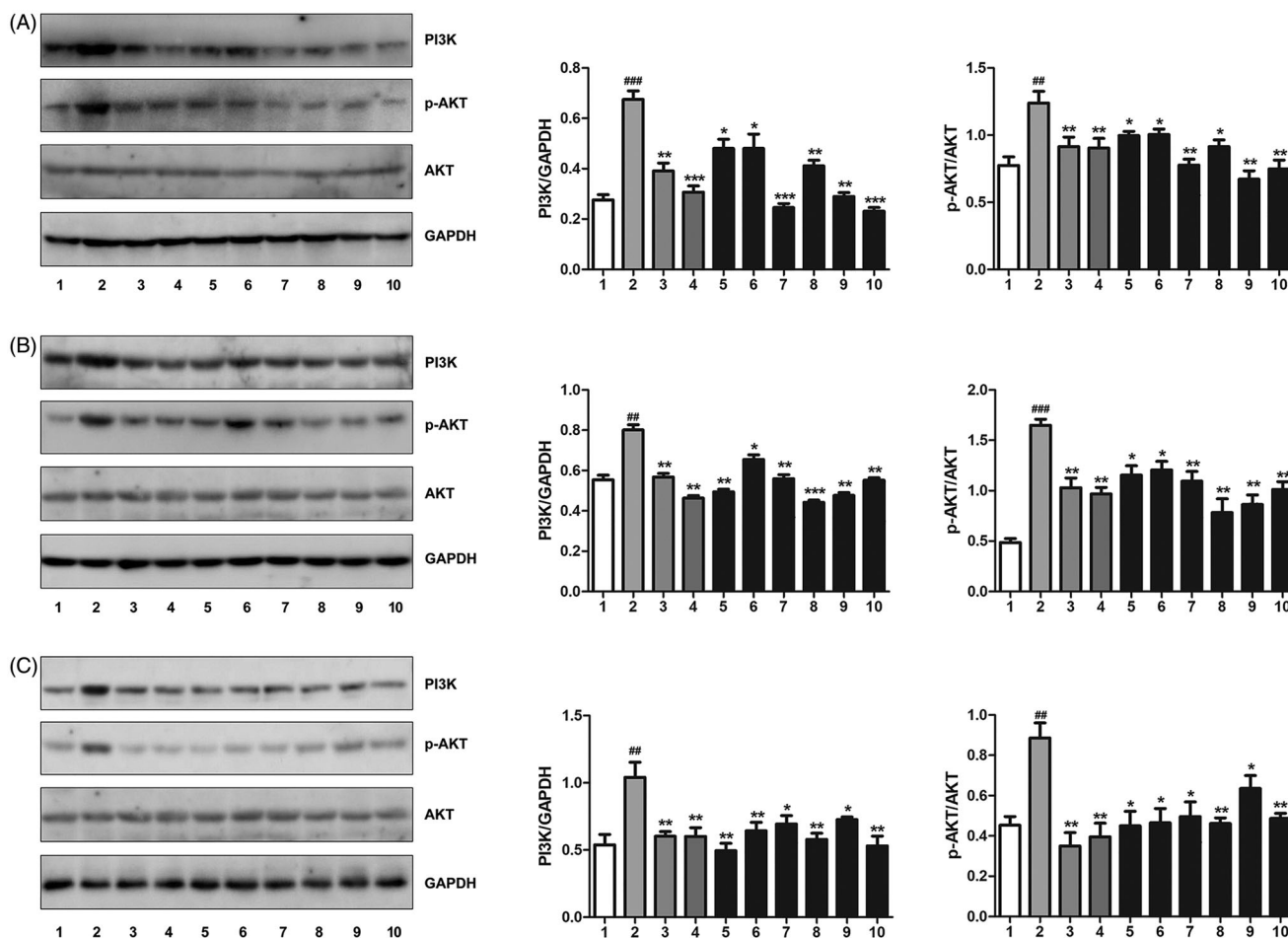


Figure 6. The active ingredients in NXT could inhibit the PI3K/AKT pathway. (A) Western blotting for the cells treated by TNF- α . (B) Western blotting for the cells treated by LPS. (C) Western blotting for the cells treated by H₂O₂. The results are the mean \pm SEM (n = 3 per group); ##*p* < 0.01, ###*p* < 0.001 versus the control group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, versus the model group. Lane 1: control, lane 2: model, lane 3: positive drugs (DEX for A, B and NIM for C), lane 4: NXT, lane 5: Calycosin, lane 6: Ononin, lane 7: Ferulic acid, lane 8: Salvianolic acid B, lane 9: Salvianolic acid E and lane 10: Salvianolic acid F.

experiments. This requires a large amount of manpower and material resources, as well as a long experiment period, and has a poor specificity. In recent years, systematic biological screening systems based on DNA, proteins, membrane receptors and cells have become an effective method for screening (Warzecha et al. 2012). UPLC/Q-TOF, combined with cell biological activity detection, has been directly applied to the screening of effective active ingredients in natural medicines (Zhou et al. 2017). Compared to the traditional screening technology, it has the characteristics of fast, trace and high specificity, and has gradually replaced the traditional screening technology. In this study, based on our previous work, we established a multi-screening method using UPLC/Q-TOF combined with high-throughput screening, and linking chemomics with systems biology to build an integrated systems biology. We have screened 21 components that could be NF- κ B inhibitors, 16 active ingredients can inhibit the expression of MMP-9 and 13 ingredients have antioxidative effects in NXT, comprehensively revealing the correlation between the chemical constituents of NXT and the response of the body's disease system.

Interestingly enough, the drug's activity in inhibiting NF- κ B, MMP-9, and NO was not dose-dependent for all doses tested. When the concentration of NXT reaches a certain level, the protective effect on cells will gradually decline. The reasons for this situation may be concerned with the following aspects: first of all, NXT has different protective mechanisms for cell damage. It

imposes a significant inhibitory effect on NF- κ B, and thus causes a relatively low effective concentration. In addition, dose-effect relationship is found between the efficacy and the dose within a certain range, but it becomes insignificant when the concentration exceeds the certain range. In addition, excessive drug concentration will give rise to cytotoxicity, causing the cell state to be affected and reducing the protective effect of drugs on cells. In subsequent studies, the optimal dose will be screened to ensure drug's cytoprotective effect at the optimal concentration.

According to our results, we found six components in NXT that inhibit NF- κ B, MMP-9, and NO (salvianolic acid B, ferulic acid, calycosin, salvianolic acid E, ononin, and salvianolic acid F), which are the main active ingredients in NXT. As a matter of record, ferulic acid has antioxidation and anti-inflammatory effects, and is widely used in the treatment of cardiovascular diseases (Mancuso and Santangelo 2014; Doss et al. 2016). Calycosin and ononin can reduce TSLP production by regulating NF- κ B activation to attenuated allergic inflammation (Shen et al. 2014). In addition, they can also inhibit the expression of MMP-9 while purifying NO, in order to protect the blood-brain barrier integrity of ischaemic brain tissue (Fu et al. 2014; Quan et al. 2015). Salvianolic acid B could inhibit NF- κ B and AP-1 DNA binding activities activated by TNF- α (Zhou et al. 2005). It also can block the activation of NF- κ B, JNK, and ERK1/2 signalling pathways to inhibit MMP-9 (Zhou et al. 2005; Ma et al. 2015), and has an antioxidative effect (Jiang YF et al. 2015). In past

studies on salvianolic acid components, the focus of attention has been on the higher content of salvianolic acid A and B (Jiang X et al. 2015), but the pharmacological activities of salvianolic acids E and F, which have analogous structures with the basal body of salvianolic acid B, are rarely reported. This study is the first report on the inhibition of NF- κ B, NO, and MMP-9 by salvianolic acid E and salvianolic acid F simultaneously. Both of them had significant anti-inflammatory and antioxidant activity, however salvianolic acid F was much better than salvianolic acid E. These ingredients are the main effective active substances in NXT, which is the main reason for treatment of cardiovascular diseases. In addition, the co-action targets of the six components are also explored in this paper. PI3K/AKT signalling pathway plays an important role in various diseases such as inflammation, cancer, and cardiovascular diseases. The active ingredients in NXT can be targeted to PI3K, reduce the over expression from inflammation and oxidative stress, thereby inhibiting the expression of p-AKT, in favour of anti-platelet aggregation, inhibit the production of unstable atherosclerotic plaques, and protect the coronary microvascular function. That may be the mechanism of NXT in the prevention and cure of CHD.

In this study, we finally established the pharmacodynamic substance basis of NXT by multiple screening, clarified the healing effect and molecular mechanism of its multi-component synergy, and explored the synergistic relationship between its active ingredients. This article provides a theoretical basis for the treatment of coronary heart disease and clinical application of NXT, which is conducive to the development of new drugs and has a great significance in promoting the modernization and internationalization of traditional Chinese medicine.

Conclusions

NXT proves effective by inhibiting NF- κ B, MMP-9, and NO which is a key in the treatment of CHD. We found six components in NXT determined to inhibit NF- κ B, MMP-9, and NO: ferulic acid, calycosin, ononin, salvianolic acid B, salvianolic acid E and salvianolic acid F, which come from the traditional Chinese medicine RA, RCX, RAS, RAB, CS, and RSM. In addition, all of the biological active ingredients in NXT could inhibit the PI3K/AKT pathway, this should be the treatment mechanism of CHD.

Disclosure statement

The authors declare that they have no competing interests.

Funding

This work was supported by the National Key R&D Program of China [2018YFC1704500] and National Science Foundation of China [81673708, 81873104].

References

- Abeyrathna P, Su Y. 2015. The critical role of Akt in cardiovascular function. *Vascul Pharmacol.* 74:38–48.
- Anderson L, Thompson DR, Oldridge N, Zwisler AD, Rees K, Martin N, Taylor RS. 2016. Exercise-based cardiac rehabilitation for coronary heart disease. *Cochrane Database Syst Rev.* 1:CD001800.
- Busti C, Falcinelli E, Momi S, Gresele P. 2010. Matrix metalloproteinases and peripheral arterial disease. *Intern Emerg Med.* 5:13–25.
- Chhibber-Goel J, Singhal V, Bhowmik D, Vivek R, Parakh N, Bhargava B, Sharma A. 2016. Linkages between oral commensal bacteria and atherosclerotic plaques in coronary artery disease patients. *NPJ Biofilms Microbiomes.* 2:7–19.
- Dilly AK, Ekambaram P, Guo Y, Cai Y, Tucker SC, Fridman R, Kandouz M, Honn KV. 2013. Platelet-type 12-lipoxygenase induces MMP9 expression and cellular invasion via activation of PI3K/Akt/NF- κ B. *Int J Cancer.* 133:1784–1791.
- Doss HM, Dey C, Sudandiradoss C, Rasool MK. 2016. Targeting inflammatory mediators with ferulic acid, a dietary polyphenol, for the suppression of monosodium urate crystal-induced inflammation in rats. *Life Sci.* 148:201–210.
- Feng H, Wang Z, Wang C, Zhu X, Liu Z, Liu H, Guo M, Hou Q, Chu Z. 2019. Effect of furostanol saponins from *Allium macrostemon* Bunge bulbs on platelet aggregation rate and PI3K/Akt pathway in the rat model of coronary heart disease. *Evid Based Complement Alternat Med.* 2019:1–7.
- Fu S, Gu Y, Jiang JQ, Chen X, Xu M, Chen X, Shen J. 2014. Calycosin-7-O- β -D-glucoside regulates nitric oxide /caveolin-1/matrix metalloproteinases pathway and protects blood-brain barrier integrity in experimental cerebral ischemia-reperfusion injury. *J Ethnopharmacol.* 155:692–701.
- Gutiérrez E, Flammer AJ, Lerman LO, Elizaga J, Lerman A, Fernández-Avilés F. 2013. Endothelial dysfunction over the course of coronary artery disease. *Eur Heart J.* 34:3175–3181.
- Han BH, Seo CS, Yoon JJ, Kim HY, Ahn YM, Eun SY, Hong MH, Lee JG, Shin HK, Lee HS, et al. 2018. The inhibitory effect of ojeoksan on early and advanced atherosclerosis. *Nutrients.* 10:1256–1279.
- Hoesel B, Schmid JA. 2013. The complexity of NF- κ B signaling in inflammation and cancer. *Mol Cancer.* 12:86–100.
- Jiang X, Lv B, Li P, Ma X, Wang T, Zhou Q, Wang X, Gao X. 2015. Bioactivity-integrated UPLC/Q-TOF-MS of Danhong injection to identify NF- κ B inhibitors and anti-inflammatory targets based on endothelial cell culture and network pharmacology. *J Ethnopharmacol.* 174:270–276.
- Jiang YF, Liu ZQ, Cui W, Zhang WT, Gong JP, Wang XM, Zhang Y, Yang MJ. 2015. Antioxidant effect of salvianolic acid B on hippocampal CA1 neurons in mice with cerebral ischemia and reperfusion injury. *Chin J Integr Med.* 21:516–522.
- Li SR, Wang TH, Zhang BJ. 2012. Effects of naoxintong capsule on the inflammation and prognosis in borderline lesion coronary heart disease patients. *Zhongguo Zhong Xi Yi Jie He Za Zhi.* 32:607–611.
- Liang Q, Cai Y, Chen R, Chen W, Chen L, Xiao Y. 2018. The effect of naoxintong capsule in the treatment of patients with cerebral infarction and carotid atherosclerosis: a systematic review and meta-analysis of randomized trials. *Evid Based Complement Alternat Med.* 2018:1–9.
- Lubos E, Handy DE, Loscalzo J. 2008. Role of oxidative stress and nitric oxide in atherothrombosis. *Front Biosci.* 13:5323–5344.
- Lv P, Tong X, Peng Q, Liu Y, Jin H, Liu R, Sun W, Pan B, Zheng L, Huang Y. 2016. Treatment with the herbal medicine, naoxintong improves the protective effect of high-density lipoproteins on endothelial function in patients with type 2 diabetes. *Mol Med Rep.* 13:2007–2016.
- Lv Y, Pan Y, Gao Y, Lu J, Li Y, Bai J, Zhai J. 2015. Effect of danhong injection combined with naoxintong tablets on prognosis and inflammatory factor expression in acute coronary syndrome patients undergoing percutaneous coronary intervention. *Acta Cardiol Sin.* 31:301–307.
- Lyu Y, Jiang X, Dai W. 2015. The roles of a novel inflammatory neopterin in subjects with coronary atherosclerotic heart disease. *Int Immunopharmacol.* 24:169–172.
- Ma L, Guan YQ, Du ZD. 2015. Salvianolic acid B down-regulates matrix metalloproteinase-9 activity and expression in tumor necrosis factor- α -induced human coronary artery endothelial cells. *Chin Med J (Engl).* 128:2658–2663.
- Ma X, Huang F, Zhang Z, Song F, Ou S. 2016a. Lung cancer resection with concurrent off-pump coronary artery bypasses: safety and efficiency. *J Thorac Dis.* 8:2038–2045.
- Ma X, Lv B, Li P, Jiang X, Zhou Q, Wang X, Gao X. 2016b. Identification of “multiple components-multiple targets-multiple pathways” associated with naoxintong capsule in the treatment of heart diseases using UPLC/Q-TOF-MS and network pharmacology. *Evid Based Complement Alternat Med.* 2016:9468087.
- Mancuso C, Santangelo R. 2014. Ferulic acid: pharmacological and toxicological aspects. *Food Chem Toxicol.* 65:185–195.
- Quan GH, Wang H, Cao J, Zhang Y, Wu D, Peng Q, Liu N, Sun WC. 2015. Calycosin suppresses RANKL-mediated osteoclastogenesis through inhibition of MAPKs and NF- κ B. *Int J Mol Sci.* 16:29496–29507.
- Shen D, Xie X, Zhu Z, Yu X, Liu H, Wang H, Fan H, Wang D, Jiang G, Hong M. 2014. Screening active components from Yu-ping-feng-san for regulating initiative key factors in allergic sensitization. *PLoS One.* 9:e107279.

- Song HK, Kim J, Lee JS, Nho KJ, Jeong HC, Kim J, Ahn Y, Park WJ, Kim DH. 2015. *Pik3ip1* modulates cardiac hypertrophy by inhibiting PI3K pathway. *PLoS One*. 10:e0122251.
- Wang R, Dong LD, Meng XB, Shi Q, Sun WY. 2015. Unique microRNA signatures associated with early coronary atherosclerotic plaques. *Biochem Biophys Res Commun*. 464:574–579.
- Wang Y, Yan X, Mi S, Li Z, Wang Y, Zhu H, Sun X, Zhao B, Zhao C, Zou Y, et al. 2017. Naoxintong attenuates ischaemia/reperfusion injury through inhibiting NLRP3 inflammasome activation. *J Cell Mol Med*. 21:4–12.
- Wang Z, Shi H, Zhao H, Dong Z, Zhao B, Weng X, Liu R, Li X, Hu K, Zou Y, et al. 2018. Naoxintong retards atherosclerosis by inhibiting foam cell formation through activating Ppar α Pathway. *Curr Mol Med*. 18:698–710.
- Warzecha CC, Hovhannisyan R, Carstens RP. 2012. Dynamic fluorescent and luminescent reporters for cell-based splicing screens. *Methods Mol Biol*. 867:273–287.
- Xu H, Jin J, Chen L, Li C, Xu Q, Shi J, Zhao B, Hou Y, Wang H. 2016. Naoxintong/PPAR α signaling inhibits H9c2 cell apoptosis and autophagy in response to oxidative stress. *Evid Based Complement Alternat Med*. 2016:4370381.
- Yang X, Li Y, Sun L, Liu Y, Ma C, Chen Y, Tan H, Li Q, Li X, Wang Y, et al. 2017. Naoxintong enhances atorvastatin-induced plaque stability while ameliorating atorvastatin-induced hepatic inflammation. *J Cardiovasc Pharmacol*. 69:55–64.
- Yuan S, Jin J, Chen L, Hou Y, Wang H. 2017. Naoxintong/PPAR γ signaling inhibits cardiac hypertrophy via activation of autophagy. *Evid Based Complement Alternat Med*. 2017:3801976.
- Zhai C, Cheng J, Mujahid H, Wang H, Kong J, Yin Y, Li J, Zhang Y, Ji X, Chen W. 2014. Selective inhibition of PI3K/Akt/mTOR signaling pathway regulates autophagy of macrophage and vulnerability of atherosclerotic plaque. *PLoS One*. 9:e90563.
- Zhang F, Huang B, Zhao Y, Tang S, Xu H, Wang L, Liang R, Yang H. 2013. BNC protects H9c2 cardiomyoblasts from H₂O₂ -induced oxidative injury through ERK1/2 signaling pathway. *Evid Based Complement Alternat Med*. 2013:802784.
- Zhang X, Qi LW, Yi L, Li P, Wen XD, Yu QT. 2008. Screening and identification of potential bioactive components in a combined prescription of Danggui Buxue decoction using cell extraction coupled with high performance liquid chromatography. *Biomed Chromatogr*. 22:157–163.
- Zhang Z, Li K. 2018. Curcumin attenuates high glucose-induced inflammatory injury through the reactive oxygen species-phosphoinositide 3-kinase/protein kinase B-nuclear factor- κ B signaling pathway in rat thoracic aorta endothelial cells. *J Diabetes Investig*. 9:731–740.
- Zhao S, Tang Y, Cai H, Liu W, Zhang L, Chen D, Chen B. 2018. Treatment of Danhong injection combined with Naoxintong capsule in acute coronary syndrome patients undergoing PCI operation: study for a randomized controlled and double-blind trial. *Evid Based Complement Alternat Med*. 2018:8485472.
- Zhong XN, Wang HH, Lu ZQ, Dai YQ, Huang JH, Qiu W, Shu YQ, Xu W, Cheng C, Hu XQ. 2013. Effects of Naoxintong on atherosclerosis and inducible nitric oxide synthase expression in atherosclerotic rabbit. *Chin Med J (Engl)*. 126:1166–1170.
- Zhou M, Ma X, Sun J, Ding G, Cui Q, Miao Y, Hou Y, Jiang M, Bai G. 2017. Active fragments-guided drug discovery and design of selective tropane alkaloids using ultra-high performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry coupled with virtual calculation and biological evaluation. *Anal Bioanal Chem*. 409:1145–1157.
- Zhou Z, Liu Y, Miao AD, Wang SQ. 2005. Salvianolic acid B attenuates plasminogen activator inhibitor type 1 production in TNF-alpha treated human umbilical vein endothelial cells. *J Cell Biochem*. 96:109–116.