doi.org/10.36721/PJPS.2025.38.1.REG.269-278.1

Exploring the active components and mechanisms of chang-yan-ning granule in inflammation relief: Insights from serum pharmacochemistry and network pharmacology

Ziqi Sun[1], Mingyuan Yuan[1], Xiaoli Wang[1], Minjie Li[1], Xueying Wang[1], Yaqing Li[1], Tiejun Zhang[2], Quanguo Liu[3] and Xiaoshu Zhang[1]*

[1]School of Functional Food and Wine, Shenyang Pharmaceutical University, Shenyang, People's Republic of China.
[2]Tianjin Key Laboratory of Quality Markers for Traditional Chinese Medicine, Tianjin Institute of Pharmaceutical Research, Tianjin, People's Republic of China.
[3]Hainan Huluwa Pharmaceutical Group Co., Ltd, Hainan, People's Republic of China.

Abstract: Chang-Yan-Ning granule (CYNG) is a traditional Chinese patent medicine used to treat ulcerative colitis (UC). In this study, we investigated the effect of CYNG on UC and its mechanism. In this study, the active components in CYNG drug serum were characterized by serum pharmacochemistry and network pharmacology was used to predict key targets. To elucidate the potential anti-inflammatory mechanism of CYNG in UC patients, *in vitro* experiments were performed to evaluate the effect of different concentrations of CYNG on lipopolysaccharide (LPS) -induced RAW 264.7. The results showed that the serum pharmacochemical study revealed 15 compounds, which were subjected to network pharmacology analysis. Integrating these results identified the key signaling pathway (MAPK signaling pathway). Western blot and ELISA further demonstrated that CYNG might also regulate p38/MAPK pathway through down-regulating the expression of p-p38 and p-JNK proteins and exerting anti-inflammatory effects. This study explains the anti-inflammatory mechanism of CYNG and provides beneficial support for the treatment of UC with CYNG.

Keywords: Chang-Yan-Ning granule, ulcerative colitis, serum pharmacochemistry, network pharmacology, MAPK pathway.

Submitted on 13-09-2024 – Revised on 18-11-2024 – Accepted on 22-11-2024

INTRODUCTION

Ulcerative colitis (UC) is widely regarded as a chronic persistent inflammation and barrier function impairment that can cause various human health problems (Ordas *et al*., 2012). In clinical practice, due to current drug treatments' limited efficacy for UC, traditional Chinese medicine (TCM) has attracted attention because of its multicomponent and multitarget characteristics. Thus, researchers have gradually focused on TCM as a potential alternative treatment option (Liu *et al*., 2022).

Chang-Yan-Ning granule (CYNG) contains five kinds of herbs, namely *Euphorbia humifusa* L., *Exallage chrysotricha* (Palib.) Neupane & N. Wikstr., leaf of *Liquidambar formosana Hance*, the root of *Camphora officinarum Nees*, and *Elsholtzia ciliata* (Thunb.) Hyl, were currently used to treat acute and chronic gastroenteritis and diarrhea. These medicines were also traditionally used to treat acute and chronic enteritis (Luyen *et al*., 20148). CYNG is a TCM preparation developed according to classical clinical experience. Some studies have shown that CYN is related to the regulation of immune function and the repair of colon mucosa in the treatment of UC (Yu *et al*., 2022). However, specialized scientific data on its mechanism of action were still limited. Through our in-depth study, the mechanism of CYNG action can be better understood to improve the therapeutic efficacy and safety. The pathogenesis of UC is complex and CYNG contain many ingredients; therefore, its mechanism in the treatment of UC requires clarification.

Serum pharmacology was widely used to explain the mechanism and basis of various TCM preparations against different diseases by identifying, analyzing and identifying the components of TCM transferred to the blood (Ma *et al*., 2017). Network pharmacology can efficiently and accurately improve the success rate of drug research (Bian *et al*., 2019). Therefore, our combination of serum pharmacochemistry and network pharmacology was an effective method to predict the therapeutic targets of CYNG that play a key role in UC and to screen the effective components quickly. Accordingly, by UPLC-Q-TOF/MS, the main components of CYNG and CYNG drug serum were identified and a network pharmacological approach was used to predict targets underlying the action of CYNG to exert anti-inflammatory effects. Based on these findings, we performed validation experiments to validate potential targets and signaling pathways of CYNG. The prediction was confirmed by exploring the mode of action of CYNG *in vitro*. Herein, CYNG was investigated using lipopolysaccharide (LPS)-treated RAW 264.7. Finally, the anti-inflammatory mode of CYNG was explained.

**Corresponding author:* e-mail: xiaoshu2397@163.com

MATERIALS AND METHODS

Preparation of drugs

CYNG was obtained from Hainan Huluwa Pharmaceutical Group Co., Ltd. (Hainan, China). SCC was purchased from Qiangji Pharmaceutical Co., Ltd., Guangdong (Guangzhou, China). SB203580 (SB) was obtained from Sparkjade (Shandong, China). Diluted these drugs with water to working concentrations before use. All reagents used were of at least analytical purity.

Experimental animals

Sprague-Dawley rats (male, n=12; 3-month-old; 200-220 g; license number SYPU-IACUC-S2023-0302-202) were purchased from Liaoning Changsheng biotechnology and kept them under specific pathogen-free conditions at 21°C-24°C, with 55%-65% humidity and a daily cycle of light and dark for 12h was used as the feeding condition. All mice received a standard diet with free access to water.

The rats were randomly divided into a blank control group and a CYNG group. Rats in the CYNG group received 40mg/kg CYNG. Then, blood was collected from the orbital venous plexus of the rats after 0 min, 30 min, 1h and 2h.

UPLC-Q-TOF/MS analysis

The UPLC-Q-TOF/MS was used to identify the main chemical constituents of CYNG and CYNG drug serum. An Agilent 1260 HPLC system equipped with an Agilent ZORBAX bsC18 column at a column temperature of 30°C was employed. The sampler temperature was 4°C, the flow rate was 1mL/min, and the sample volume was 1μL. The mobile phase comprised A (methanol) and mobile phase B (water containing 0.1% phosphoric acid). The gradient elution procedure was performed as follows: 0-5 min, 2% A; 5-10 min, 5% A; 10-40 min, 50% A; and 40-60 min, 95% A. Mass spectra were obtained using an Agilent 6530 Q-TOF/MS mass spectrometer in the positive and negative ion modes alongside electrospray ionization (ESI). Nitrogen was used as an auxiliary and atomizing gas. Multivariate data analysis was performed using Agilent Mass Hunter (B.06.00).

Network pharmacology

Compound SMILES ID were retrieved from the PubChem database, target genes were identified using the Swiss Target Prediction database, then, the compound-target network was built using Cytoscape 3.9.0. UC-related targets were screened from the Gene Cards and OMIM database using the keyword “Ulcer Colitis,” and then duplicate genes were eliminated. The protein-protein interaction (PPI) network diagram was obtained using the STRING database. Common targets were analyzed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, followed through microscopic letter visualization biological processes and KEGG pathways.

The RAW 264.7 macrophages were cultured

In response to CYNG activity, mouse adherent macrophage RAW 264.7 was used to investigate the changes of inflammatory markers *in vitro*. LPS (1μg/mL) was used to induce the inflammatory response of RAW 264.7 (Suzhou Meilun Biotechnology Co., LTD.). Cell counting kit-8 (CCK8, Cat: C0038, Beyotime, China) was used to evaluate the effect of CYNG on the viability of RAW 264.7.

RAW 264.7 was treated with CN (normal control) or LPS (1mg/L) for 24h. LPS-treated cells were treated with CYNG (0.2 or 2μg/mL), SB (0.75mg/mL, fig. S1A), or SCC (10μg/mL, fig. S1B) for 8h. RAW 264.7 macrophages were collected for western blotting. The effects of CYNG on NO release and inflammatory factors in RAW 264.7 were assessed through the Griess and Enzyme-linked immunosorbent assay (ELISA), respectively.

ELISA analysis

10% homogenate was prepared by accurately weighing 100 mg colon samples, adding 9 times the volume of ice-cold 0.9% normal saline and then the samples were mechanically homogenized. Next, the samples were centrifuged at 12000 rpm for 10 min and supernatants were collected for analysis. Interferon IL-1β and TNF-α were quantified using ELISA kits (Servicebio, Wuhan, China). NO and CCK-8 were quantified using ELISA kits (Beyotime, Shanghai, China).

Western Blot (WB) analysis

Cells were homogenized and centrifuged and protein concentrations in cell extracts were determined. Proteins were separated and transferred to the nitrocellulose membrane (G6014, Servicebio, Wuhan, China). Then, cell membranes were incubated with the membranes at 4°C overnight with primary antibodies and then at room temperature for 1h with the HRP-conjugated secondary antibody. After washing the film, we revealed the bands using a high-sensitivity chemiluminescent substrate kit (ED0015, Sparkjade, Shandong, China). The density of the bands was evaluated by using gray-scale analysis in Image J. Detailed antibody information: p38 (AF1111, 1: 1000), phospho-p38 (AF5887, 1: 2000) and JNK (AF1048, 1: 1000), all purchased from Beyotime Biotech Inc. Phospho-JNK and (AF3318, 1: 1000, affinity, USA), ERK1/2 (WL01770, 1: 500, Wanleibio, China), phospho-ERK1/2 (WLP1512 1: 1000, Wanleibio, China). Monoclonal mouse anti-β-Actin (GB12001, 1: 1000 Servicebio, Wuhan, China).

Ethical approval

This study was approved by the Laboratory Animal Center of Shengyang Pharmaceutical University, China vide reference SYPU-IACUC-S2023-0302-202.

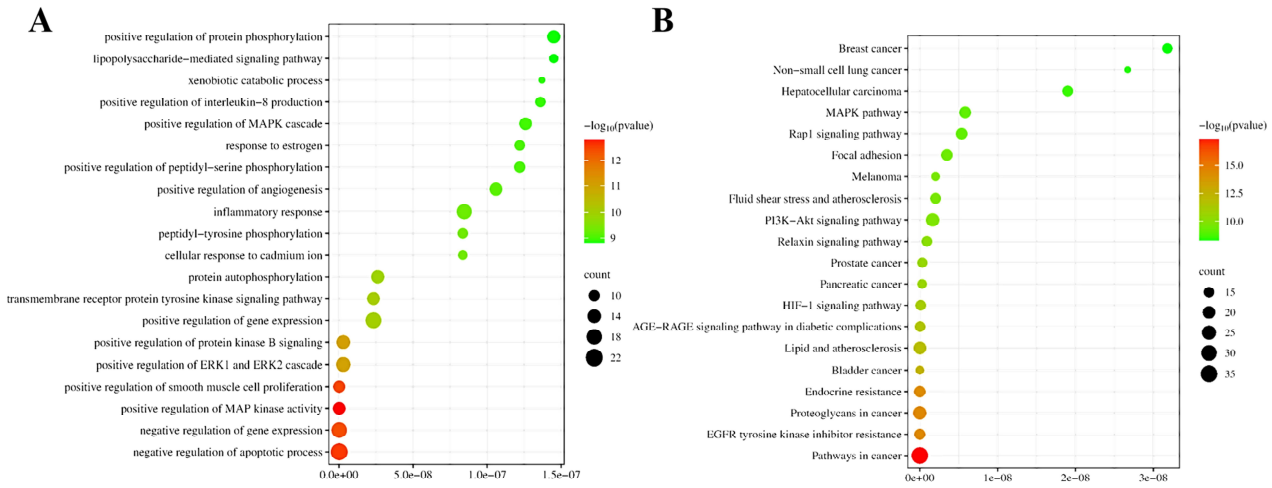

Fig. 1: Network pharmacological and molecular docking analysis of CYNG. (A) BP enrichment analysis and (B) KEGG pathway enrichment analysis.

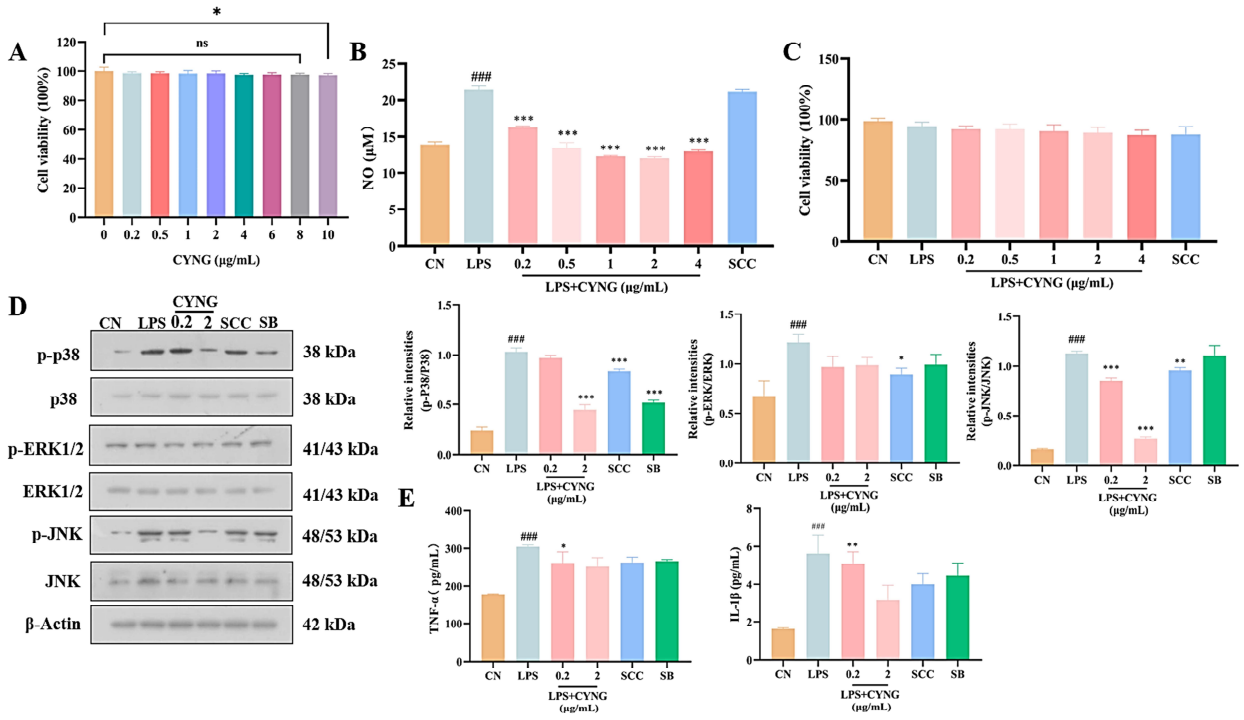

Fig. 2: CYNG suppresses the inflammatory response *in vitro*. (A) Effect of CYNG alone on cell viability. Effect of combined CYNG and LPS on (B) NO and (C) cell viability. (D) The protein expression of p38, p-p38, ERK1/2, p-ERK1/2, JNK, p-JNK and β-Actin was analyzed by western blot analysis. (E) Effect of CYNG on the level of IL-1β and TNF-α. n = 3. Data were shown as mean ± SD. #p<0.05, ##p<0.01, ###p<0.001 vs the CN group, *p<0.05, **p<0.01, ***p<0.001 vs the LPS group.

STATISTICAL ANALYSIS

The statistical analyses were conducted by using Graph Pad Prism 8.0. The whole results were expressed as mean ± standard deviation (SD). The values were compared useing a one-way analysis of variance and the Bonferroni post hoc test. P-values below 0.05 indicated statistical significance.

RESULTS

UPLC-Q-TOF/MS analysis of CYNG and its active components in serum

To identify the active components in CYNG, the total ion flow chromatogram of CYNG was acquired (fig. S2A and S2B). 39 compounds were identified (table S1). To clarify the contribution of these compounds in CYNG, the serum samples were collected from control mice and CYNG-

treated mice (intragastric administration) and tested in negative/positive ion mode (fig. S3A-D). Using known standards, 14 components were identified (6 in negative ion mode and 8 in positive ion mode) (table S2).

Network pharmacological analysis

14 CYNG active ingredients were identified to have 317 potential targets (fig. S4A). After deleting duplicates, 2007 disease targets were obtained. There were 132 common targets between the 317 active-component targets and 2007 colitis targets (fig. S4B). These shared targets were screened using the STRING method, and the obtained core PPI network (fig. S4C) had 99 nodes and 1297 edges. In addition, a compound with a target gene degree of >30 was considered a possible active ingredient (Fu *et al*., 2022). Therefore, as shown in table S3, it may be a key active ingredient in treating UC by CYNG. In addition, network pharmacology prediction was used to evaluate the enrichment of biological processes and KEGG pathways and found that the active ingredients in CYNG were associated with “positive regulation of MAPK cascade” and “positive regulation of MAP kinase activity” (fig. 1A), as well as MAPK pathways (fig. 1B).

CYNG suppressed LPS-induced inflammation and activates MAPK signaling in vitro

The cellular toxicity of CYNG to RAW 264.7 was assessed by CCK-8 assay. Treatment of RAW 264.7 with different concentrations of CYNG (0, 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0μg/mL) did not show any effect on cell proliferation and growth (fig. 2A). LPS-induced RAW264.7 macrophages were used to construct an inflammatory model. At doses ranging from 0.2-4μg/mL, CYNG dose-dependently inhibited LPS-induced NO production without significant cytotoxicity (fig. 2B and 2C). Based on these results, CYNG doses of 0.2 and 2 μg/mL, which maintained good cell viability while effectively suppressing the inflammatory response, were selected for subsequent experiments.

SB was a selective ATP-competitive p38 MAPK inhibitor that does not inhibit JNK. CYNG was detected to reduce inflammation by inhibiting p38 phosphorylation by western blot analysis. LPS-treated cells expressed significantly higher p-38 levels than control. In comparison, with the LPS group, CYNG (both doses), SCC and SB significantly decreased the protein expression levels of p-p38 without any significant difference in protein expression between the SB group and CN group, suggesting that p38 was a CYNG target. Moreover, CYNG effectively inhibited LPS-induced JNK activation in a dose-dependent manner (fig. 2D). In addition, The proinflammatory cytokines IL-1β and TNF-α were quantitatively analyzed in RAW 264.7 and it was found that LPS stimulation led to an upregulation of expression of IL-1β and TNF-α. However, the high CYNG dose significantly inhibited the expression of these proinflammatory cytokines (fig. 2E). Interestingly, adding SB (a p38 MAPK antagonist) or SCC (a positive control) to the culture medium did not significantly affect the levels of IL-1β and TNF-α secreted by LPS-stimulated RAW 264.7, indicating that the inhibitory effect of CYNG on the expression levels of IL-1β and TNF-α was caused by a general inhibition of p38 MAPK activity.

Overall, the results indicate that CYNG inhibited LPS-induced inflammation in RAW 264.7 by targeting specific signaling pathways involved in the inflammatory response.

DISCUSSION

UC was an inflammatory gastrointestinal disease with chronic, recurrent and non-specific characteristics, and existing UC treatments were unable to maintain lasting remission. TCM composition was complex and diverse. Only by studying the key active substances and mechanism of action of TCM can it be better developed and conducive to the modernization and internationalization of TCM (Tang *et al*., 2022). Serum pharmacochemistry was used to identify potentially active substances in TCM. Prototype compounds and their metabolites were considered potentially active compounds with therapeutic effects. In particular, when drug components are detected in the blood, these components may be the ultimate "biomarkers" (Shao *et al*., 2022; Zhang *et al*., 2013).

UC treatment includes using 5-aminosalicylic acid (5-ASA), corticosteroids, biologics, and various immunosuppressive drugs. However, the efficacy of these treatments is often constrained by their side effects, such as potential adverse reactions to 5-ASA, which can include hypertension, opportunistic infections, and nephrotoxicity (Barberio *et al*., 2021). In contrast, our research focuses on CYNG, a TCM formulation that may offer a different therapeutic mechanism compared to conventional drugs and potentially better safety and tolerability profiles. CYNG had been shown to alleviate gastrointestinal discomfort associated with disorders such as diarrhea. Recent studies had shown that CYNG ameliorates diarrhea caused by castor oil and Senna leaf extract by inhibiting gastrointestinal propulsion (Wang *et al*., 2017). Suggests that CYNG could provide an alternative approach to managing UC symptoms with potentially fewer side effects than conventional pharmaceuticals. Understanding the chemical basis of CYNG was important to facilitate the understanding of the mechanism of CYNG in the treatment of UC. Since the unclear mechanism of CYNG for treating UC was primarily due to its uncertain chemical composition, the chemical components of CYNG were characterized by HPLC-Q-TOF-MS. Among the 39 components in CYNG, 15 were natural compounds that were absorbed into the

blood of rats. The combined results of network pharmacology and HPLC-Q-TOF/MS predict that 12-hydroxyjasmonic acid, aesculetin, caffeic acid, cis-4-coumaric acid, 1-(1-Benzothien-3-yl) ethenone, methyl syringin, ethyl gallate, levoglucosan, and ethyl gallate were the key active ingredients of CYNG. Their anti-inflammatory properties were extensively studied. Aesculetin and ethyl gallate improve DSS-induced colitis by inhibiting the secretion of TNF-α and IL-6 and activation of the NF-κB and MAPK signaling pathways (Correa *et al*., 2020; Wang, S.K. *et al*., 2022; Wang, Z. *et al*., 2022). Besides, CYNG contains ethyl gallate, which can also treat UC mice through the Nrf2 and NF-κB pathways (Wang, Z. *et al*., 2022). Caffeic acid, cis-4-coumaric acid, and levoglucosan exert anti-inflammatory effects by regulating cytokine IL-1β and IL-10 levels (Bal *et al*., 2022; Corsini *et al*., 2017; Wan *et al*., 2021).

Network pharmacology was used to predict the target, active ingredient and action mechanism of TCM. Further study of CYNG based on serum pharmacochemistry combined with network pharmacology was beneficial in determining the bioactive components of the drug and predicting its mechanism of action. A total of 15 CYNG components were detected in this study. In addition, by analyzing the compound-target network, combined with GO functional enrichment and KEGG pathway analysis, the network pharmacology results suggest that CYNG may activate the MAPK signaling pathway, which controls a variety of biological activities, including cell survival, proliferation, growth and metabolism(Khan *et al*., 2024; Liu and Luo, 2024; Yan *et al*., 2024). Studies have shown that TCM preparations effectively regulated MAPK signaling pathways for UC (Mok *et al*., 2024). Validated by *in vitro* experiments and consistent with our findings, these findings suggest that CYNG could alleviate inflammation by modulating p38 MAPK signaling.

CONCLUSION

In summary, through serum pharmacochemistry, network pharmacology and experimental verification, the material basis and mechanism of CYNG alleviating inflammation were initially elucidated, which laid an experimental foundation for the clinical development and preparation of CYNG for the treatment of UC.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (grant No. 81703389), Postdoctoral project of Hainan Province and Youth Development Support Plan of Shenyang Pharmaceutical University (ZQN2021010).

REFERENCES

Bal SS, Leishangthem GD, Sethi RS and Singh A (2022). P-coumaric acid ameliorates fipronil induced liver injury in mice through attenuation of structural changes, oxidative stress and inflammation. *Pestic. Biochem. Physiol.,* **180**: 104997.

Barberio B, Segal JP, Quraishi MN, Black CJ, Savarino EV and Ford AC (2021). Efficacy of oral, topical, or combined oral and topical 5-Aminosalicylates, in ulcerative colitis: Systematic review and network meta-analysis. *J. Crohns. Colitis.,* **15**(7): 1184-1196.

Bian YM, He XB, Jing YK, Wang LR, Wang JM and Xie XQ (2019). Computational systems pharmacology analysis of cannabidiol: A combination of chemogenomics-knowledgebase network analysis and integrated in silico modeling and simulation. *Acta Pharmacol. Sin.,* **40**(3): 374-386.

Correa LB, Seito LN, Manchope MF, Verri WA, Jr, Cunha TM, Henriques MG and Rosas EC (2020). Methyl gallate attenuates inflammation induced by Toll-like receptor ligands by inhibiting MAPK and NF-Kb signaling pathways. *Inflamm. Res.,* **69**(12): 1257-1270.

Corsini E, Ozgen S, Papale A, Galbiati V, Lonati G, Fermo P, Corbella L, Valli G, Bernardoni V, Dell'Acqua M, Becagli S, Caruso D, Vecchi R, Galli CL and Marinovich M (2017). Insights on wood combustion generated proinflammatory ultrafine particles (UFP). *Toxicol. lett.,* **266**: 74-84.

Fu JS, Jiang Q, Xia CM, Dan WC, Gong X, Yue M and Tang XP (2022). Study on the mechanism of Sishen decoction in the treatment of rheumatoid arthritis based on network pharmacology. *Acta Chin. Med.,* **37**(01): 156-164.

Khan F, Elsori D, Verma M, Pandey S, Obaidur Rab S, Siddiqui S, Alabdallah NM, Saeed M and Pandey P (2024). Unraveling the intricate relationship between lipid metabolism and oncogenic signaling pathways. *Front. Cell. Dev. Biol.,* **12**: 1399065.

Liu Y, Li BG, Su YH, Zhao RX, Song P, Li H, Cui XH, Gao HM, Zhai RX, Fu XJ and Ren X (2022). Potential activity of traditional Chinese medicine against Ulcerative colitis: A review. *J. Ethnopharmacol.,* **289**: 115084.

Liu Y and Luo Z (2024). Repurposing anticancer drugs targeting the MAPK/ERK signaling pathway for the treatment of respiratory virus infections. *Int. J. Mol. Sci.,* **25**(13): 6946.

Luyen BT, Tai BH, Thao NP, Eun KJ, Cha JY, Xin MJ, Lee YM and Kim YH (2014). Anti-inflammatory components of Euphorbia humifusa Willd. *Bioorganic. Med. Chem. Lett.,* **24**(8): 1895-1900.

Ma FX, Xue PF, Wang YY, Wang YN and Xue SY (2017). Research progress of serum pharmacochemistry of traditional Chinese medicine. *China J. Chin. Mater. Med.,* **42**(7): 1265-1270.

Mok HL, Cheng KW, Xu Y, Huang C, Lyu C, Xu J, Hu D,

Zhu L, Lin C, Tan HY and Bian Z (2024). Modified Zhenwu decoction suppresses chronic colitis via targeting macrophage CCR2/Fyn/p38 MAPK signaling axis. *Phytomedicine.,* **129**: 155694.

Ordás I, Eckmann L, Talamini M, Baumgart DC and Sandborn WJ (2012). Ulcerative colitis. *Lancet.,* **380**(9853): 1606-1619.

Shao D, Liu X, Wu J, Zhang A, Bai Y, Zhao P and Li J (2022). Identification of the active compounds and functional mechanisms of Jinshui Huanxian formula in pulmonary fibrosis by integrating serum pharmacochemistry with network pharmacology. *Phytomedicine.,* **102**: 154177.

Tang YP, Xu DQ, Yue SJ, Chen YY, Fu RJ and Bai X (2022). Modern research thoughts and methods on bio-active components of TCM formulae. *Chin. J. Nat. Med.,* **20**(7): 481-493.

Wan F, Zhong R, Wang M, Zhou Y, Chen Y, Yi B, Hou F, Liu L, Zhao Y, Chen L and Zhang H (2021). Caffeic acid supplement alleviates colonic inflammation and oxidative stress potentially through improved gut microbiota community in mice. *Front. Microbiol.,* **12**: 784211.

Wang H, Zhu Z, Fu H, Huo X, Liu Q, Shen X and Hu J (2017). Anti-diarrheal and anti-inflammatory effect and mechanism of ChangyanningGranule in weaning mice. *Drug Eval. Res.,* **40**(12): 1701-1705.

Wang SK, Chen TX, Wang W, Xu LL, Zhang YQ, Jin Z, Liu YB and Tang YZ (2022). Aesculetin exhibited anti-inflammatory activities through inhibiting NF-κB and MAPKs pathway *in vitro* and *in vivo*. *J. Ethnopharmacol.,* **296**: 115489.

Wang Z, Xue R, Lv M, Qi Y, Yu W, Xie Z, Chen W, Wang X, Tian X and Han B (2022). Stepwise tracking strategy to screen ingredient from Galla Chinensis based on the mass spectrometry guided preparative chromatography coupled with systems pharmacology. *J. Ethnopharmacol.,* **284**: 114533.

Yan Y, Dai T, Guo M, Zhao X, Chen C, Zhou Y, Qin M, Xu L and Zhao J (2024). A review of non-classical MAPK family member, MAPK4: A pivotal player in cancer development and therapeutic intervention. *Int. J Biol Macromol.,* **271**(Pt 2): 132686.

Yu W, Zhang Y, Kang C, Zheng Y, Liu X, Liang Z and Yan J (2022). The pharmacological evidence of the chang-yan-ning formula in the treatment of colitis. *Front. Pharmacol.,* **13**: 1029088.

Zhang AH, Sun H, Yan GL, Han Y and Wang XJ (2013). Establishment of three-dimensional integrated serum pharmacochemistry-pharmacokinetics (pharmacodynamics)-systems biology for research of traditional Chinese medicine prescription and its application in Yinchenhao Tang. *Zhongguo Zhong yao za zhi.,* **38**(21): 3786-3789.