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# Quantitative analysis of drug–drug interactions among active components of Xuebijing in inhibiting LPS-induced TLR4 signaling and NO production

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**Despite the long history of Traditional Chinese Medicine (TCM) in disease treatment, the underlying "Jun–Chen–Zuo–Shi" principle remains largely unexplored. To address this gap, it is essential to elucidate the interactions between active substances in TCM through quantitative molecular and cellular pharmacology. The Chou–Talalay method is particularly effective for investigating drug combinations, making it highly relevant for TCM formulas. This study employed the Chou–Talalay method to explore the drug–drug interactions in Xuebijing (XBJ), a TCM formula used for treating sepsis. The aim was to elucidate the "Jun–Chen–Zuo–Shi" principle by investigating the interactions of the main active substances in XBJ: danshensu and salvianolic acid B (from *Radix Salviae Miltiorrhizae*), senkyunolide A (from *Rhizoma Chuanxiong*), ligustilide (from *Radix Angelicae Sinensis*), safflower yellow and hydroxysafflor yellow A (from *Flos Carthami*), and paeoniflorin (from *Radix Paeoniae Rubra*). We quantitatively analyzed their TLR4 antagonistic activities and used the combination index (CI) to quantify their interactions, revealing synergism (CI < 1), additive effects (CI = 1), and antagonism (CI > 1). The results show these agents inhibit nitric oxide (NO) production, with some combinations demonstrating synergistic effects at certain concentrations, while others present antagonistic effects. Understanding these interactions provides a scientific foundation for optimizing TCM formulations, enhancing quality control, efficacy, and safety.**

**Keywords** Xuebijing, Traditional Chinese medicine, Toll-like receptor 4, Chou–Talalay method, Drug combination, Nitric oxide

Traditional Chinese Medicine (TCM) is grounded in holistic principles that emphasize the balance of the body's vital energies (Qi), the duality of Yin and Yang, and the Five Elements theory[1]. Qi is regarded as the fundamental life force or energy that flows throughout the body, sustaining physiological functions. Yin and Yang represent opposing yet complementary forces that must be in harmony for health; Yin is associated with coolness, rest, and substance, while Yang is linked to warmth, activity, and function. The Five Elements—Wood, Fire, Earth, Metal, and Water—are connected to various organs, tissues, and physiological processes within the body, forming a dynamic system that underpins both health and disease[2].

A central concept in TCM is the Jun–Chen–Zuo–Shi theory, which outlines the strategic roles of herbs within a TCM formulation[3]. The Jun (sovereign) herb is the primary ingredient responsible for addressing the main disease or symptoms. The Chen (minister) herb supports the Jun herb by enhancing its therapeutic effects or addressing additional symptoms. The Zuo (assistant) herb aids the actions of both the Jun and Chen herbs or targets secondary symptoms. The Shi (courier) herb guides or directs the formula's effects to specific areas of the body or harmonizes the actions of the other herbs[4]. However, the scientific basis underlying these combinations remains largely unexplored. To address this gap, it is crucial to elucidate the interactions between the active components of TCM formulations through quantitative molecular and cellular pharmacology. This approach involves studying the interactions of various components and their relationships with specific targets to determine whether they exhibit antagonistic, additive, or synergistic effects.

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Xuebijing (XBJ) is a TCM that has a well-defined active component, which consists of extracts from five Chinese herbs: *Flos Carthami*, *Radix Paeoniae Rubra*, *Rhizoma Chuanxiong*, *Radix Salviae Miltiorrhizae*, and *Radix Angelicae Sinensis*[5]. Approved by the China Food and Drug Administration in 2004, XBJ is used to treat sepsis, a leading cause of death in intensive care units. Clinical investigations have shown that XBJ, either alone or in combination with conventional therapy, improves organ function, shortens intensive care unit stays, and reduces sepsis mortality[6]. Adverse events are rare, occurring in only 0.3% of cases. In addition, XBJ has been applied in the treatment of acute respiratory distress syndrome (ARDS), trauma, and post-surgical infections, where systemic inflammation and immune dysregulation play significant roles in disease progression[7–9]. Its ability to modulate immune responses and reduce excessive inflammation makes it particularly beneficial in these critical conditions.

Despite its clinical success, the detailed scientific basis of the "Jun–Chen–Zuo–Shi" theory underlying the XBJ formula remains unclear. Toll-like receptor 4 (TLR4) is an innate immune receptor that detects the endotoxin lipopolysaccharide (LPS) from gram-negative bacteria and plays a crucial role in the pathogenesis of sepsis[10–12]. XBJ has been shown to modulate various immune responses, and one of its key mechanisms involves the TLR4 signaling pathway[13,14]. To better understand how the components of XBJ interact with TLR4 signaling pathway and contribute to the treatment of sepsis, this study aims to quantitatively analyze the drug–drug interactions among the main active ingredients of XBJ in inhibiting LPS-induced TLR4 signaling using the Chou–Talalay method. This method is one of the most widely used for quantifying drug–drug interactions in drug combinations, providing insights into the synergistic, additive, or antagonistic effects of the components. By applying the Chou–Talalay method, we aim to uncover the molecular mechanisms underlying the effects of XBJ's components on TLR4 signaling.

## Results

LPS, a bacterial endotoxin, is known to compromise blood-brain barrier (BBB) integrity and activate microglia, contributing to the neuroinflammatory response and behavioral alterations observed in systemic infections[15,16]. In sepsis, the production of nitric oxide (NO) is a key pro-inflammatory mediator, playing a pivotal role in the generation of reactive intermediates and the development of organ dysfunction[17]. Therefore, inhibiting NO production is an important therapeutic strategy in managing sepsis-induced inflammation and tissue damage. The active components of Xuebijing (XBJ) are derived from five Chinese herbs, each contributing distinct bioactive molecules. Among these, we focused on the following representative compounds: danshensu (Fig. 1A) and salvianolic acid B (Fig. 1B) from *Radix Salviae Miltiorrhizae*, ligustilide (Fig. 1C) from *Radix Angelicae Sinensis*, senkyunolide A (Fig. 1D) from *Rhizoma Chuanxiong*, hydroxysafflor yellow A (Fig. 1E) and safflower yellow (Fig. 1F) from *Flos Carthami*, and paeoniflorin (Fig. 1G) from *Radix Paeoniae Rubra*[18–23].

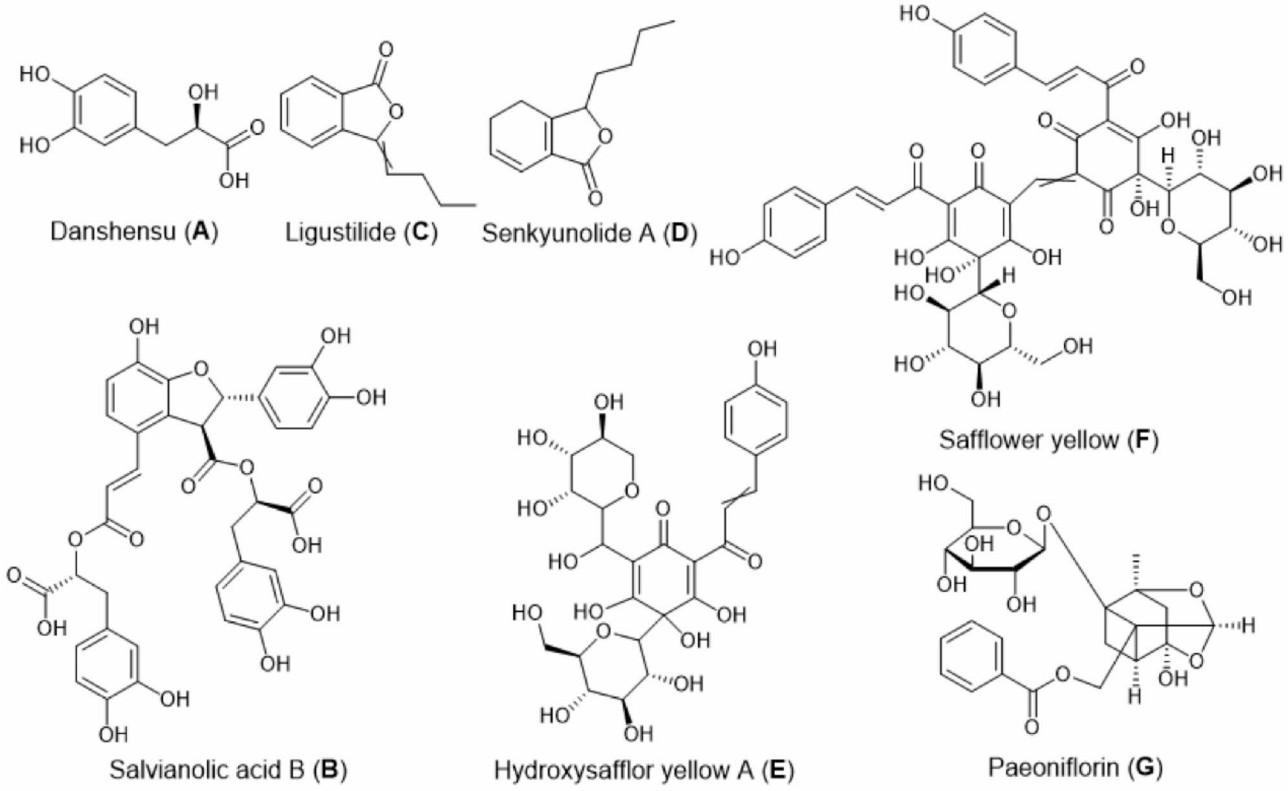


**Fig. 1**. Chemical structures of XBJ active compound danshensu (**A**), salvianolic acid B (**B**), ligustilide (**C**), senkyunolide A (**D**), hydroxysafflor yellow A (**E**), safflower yellow (**F**), and paeoniflorin (**G**).

To investigate the drug–drug interactions among the main active ingredients of XBJ, we quantitatively assessed the effects of seven representative components of XBJ on LPS-induced NO production in BV-2 cells, a widely used model for studying neuroinflammation. (Supplementary Fig. S1). Our results revealed that danshensu, salvianolic acid B, ligustilide, safflower yellow, hydroxysafflor yellow A, and senkyunolide A effectively inhibited LPS-induced NO production in BV-2 cells in a concentration-dependent manner, with $IC_{50}$ values of 68.0 ± 5.1 μM (Fig. 2A), 30.9 ± 4.0 μM (Fig. 2B), 9.4 ± 0.6 μM (Fig. 2C), 26.9 ± 2.4 μM (Fig. 2D), 76.2 ± 6.5 μM (Fig. 2E), and 30.9 ± 4.0 μM (Fig. 2F), respectively. Notably, these compounds did not exhibit significant cytotoxicity at the tested concentrations, which is consistent with the low clinical toxicity profile of XBJ observed in clinical settings[24]. However, paeoniflorin failed to inhibit NO production at concentrations as high as 200 μM (Supplementary Fig. S2), suggesting a limited role in this particular inflammatory pathway.

To better understand the interactions between these active components, we employed the Chou–Talalay method to analyze the drug–drug interactions in binary combinations. Synergistic effects were observed for the combinations of ligustilide and danshensu (Fig. 3A), ligustilide and senkyunolide A (Fig. 3B), and safflower yellow and hydroxysafflor yellow A (Fig. 3C). These combinations showed enhanced inhibition of NO production, suggesting that the combined actions of these compounds may work together to exert a more potent therapeutic effect.

Conversely, antagonistic interactions were identified in the following combinations of salvianolic acid B and danshensu (Fig. 4A), salvianolic acid B and safflower yellow (Fig. 4B), salvianolic acid B and hydroxysafflor yellow A (Fig. 4C), and hydroxysafflor yellow A and senkyunolide A (Fig. 4D). These combinations exhibited diminished efficacy, which may be due to competitive interactions or opposing effects on the same molecular targets or signaling pathways. Notably, the remaining binary combinations exhibited additive effects in inhibiting LPS-induced NO production (Fig. 5 and Supplementary Fig. S3), implying that these combinations work in a complementary manner to exert a balanced anti-inflammatory action.

## Discussion

TCM is increasingly recognized for its ability to address complex diseases by integrating multiple bioactive components that target various molecular pathways[25,26].

However, despite this promise, the detailed mechanisms underlying the interactions between TCM compounds remain poorly understood, particularly in the context of system pharmacology. This study provides a quantitative analysis of the drug–drug interactions among the seven key active components of XBJ, focusing on their effects on LPS-induced TLR4 signaling and nitric oxide (NO) production. While this study offers valuable insights into the interactions between the active components of XBJ, several limitations must be addressed in future research. First, our investigation was confined to the in vitro effects of XBJ's components on LPS-induced TLR4 signaling and NO production in BV-2 cells. These findings may not fully capture the complexity of in vivo systems, where factors such as pharmacokinetics, metabolism, and tissue distribution could significantly influence the outcomes. Additionally, the model used in this study focused on BV-2 cells, which may not comprehensively represent the broader systemic effects of XBJ in sepsis. Further studies are needed to explore these interactions in more clinically relevant in vivo models.

Furthermore, previous research on compound compatibility in TCM has primarily focused on empirical and theoretical models, often lacking robust experimental validation. In contrast, our study systematically evaluated 21 binary drug–drug combinations, identifying three synergistic combinations, four antagonistic combinations, and the remaining combinations exhibited additive effects. These results suggest that while some components of XBJ work synergistically, others may interfere with one another's actions, underscoring the importance of understanding the full spectrum of interactions when formulating effective TCM therapies. Furthermore, although we observed synergistic, antagonistic, and additive effects in the drug combinations, the underlying molecular mechanisms of these interactions remain incompletely understood. Finally, the limited effect of paeoniflorin on inhibiting NO production implies that its role in inflammation may be more intricate or context-dependent than previously thought.

Recent studies have increasingly embraced integrated multidisciplinary approaches to unravel the mechanisms underlying multi-component formulations such as XBJ. For instance, network pharmacology and molecular docking have been utilized to identify the key active components of XBJ and their potential anti-inflammatory effects through the regulation of signaling pathways related to immune responses[27]. Their findings provided qualitative insights into the pharmacological actions of XBJ but lacked a systematic quantitative analysis of drug–drug interactions. In contrast, our study utilized the Chou–Talalay method, a well-established approach for quantifying combination drug effects, to delineate the synergistic, additive, and antagonistic interactions among XBJ's primary active ingredients. This method enabled a more precise characterization of the cooperative and competitive relationships among the components within the TLR4 signaling pathway.

Our findings highlight the complexity of TCM formulations and emphasize the necessity of employing systematic pharmacology approaches to unravel the interactions between individual components. By characterizing these interactions, we gain valuable insights into how XBJ may modulate immune responses in sepsis. This study also provides a foundation for further investigation into the mechanisms of action of TCM formulations, paving the way for more rational and targeted therapeutic strategies. Additionally, expanding the scope of molecular targets and signaling pathways involved in sepsis and inflammation will offer a more comprehensive understanding of the mechanisms driving the observed interactions. Furthermore, exploring the clinical implications of these synergistic, antagonistic, and additive effects will aid in the rational design of future TCM-based therapies, optimizing the therapeutic potential of XBJ.

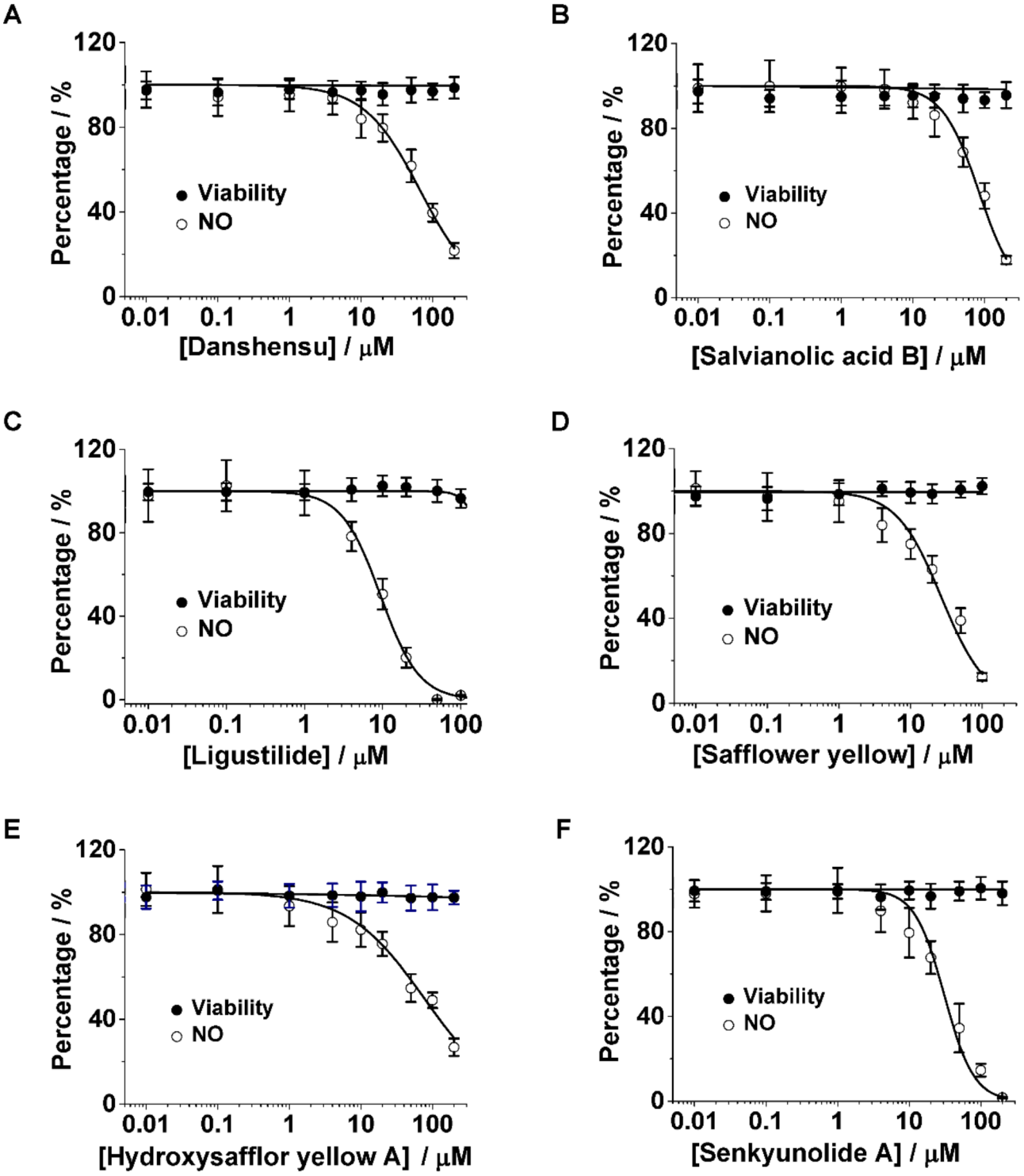


**Fig. 2**. Effects of XBJ active compounds danshensu (**A**), salvianolic acid B (**B**), ligustilide (**C**), safflower yellow (**D**), hydroxysafflor yellow A (**E**) and senkyunolide A (**F**) on the LPS induced NO overproduction. BV-2 cells were treated with LPS (200 ng/mL) and indicated concentrations of XBJ active substances for 24 h. NO in the supernatant was detected by the 2,3-diaminonaphthalene assay. The NO in the LPS (200 ng/mL) group was set as 100%. It should be noted that the effect of XBJ active compounds on BV-2 cell viability was measured by crystal violet staining.

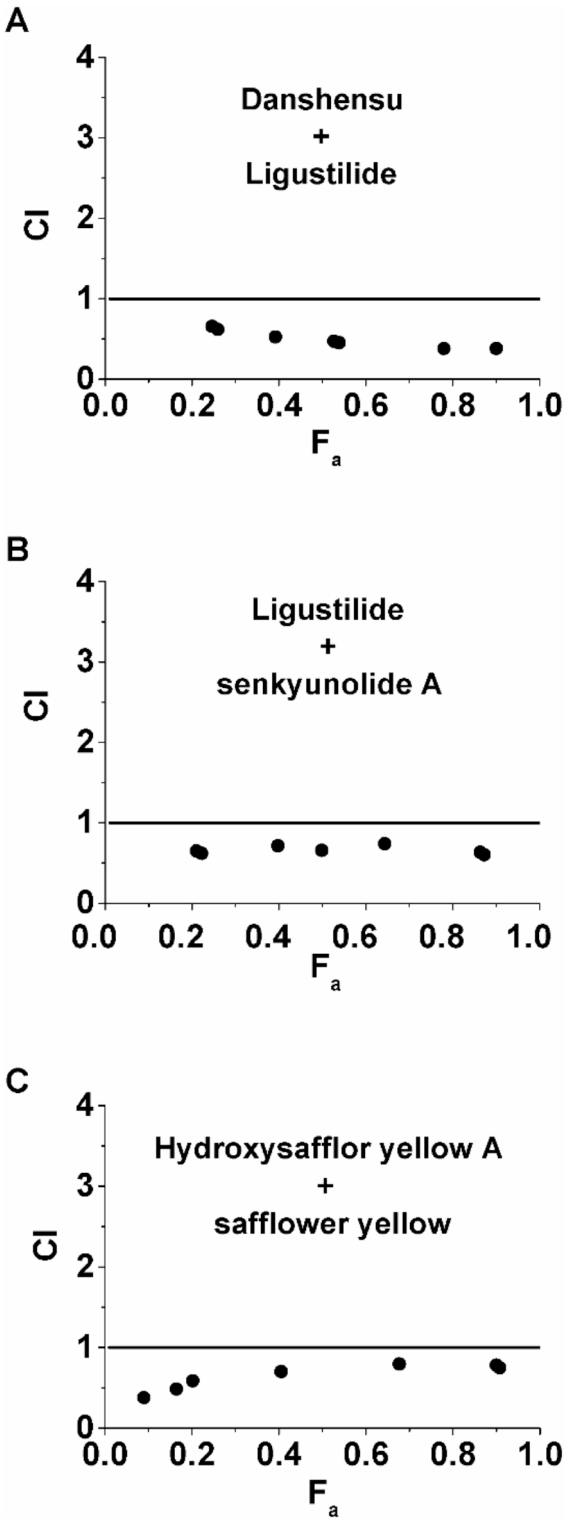


**Fig. 3**. Synergistic effects of the drug combination of ligustilide and danshensu (**A**), the combination of ligustilide and senkyunolide A (**B**), as well as the combination of safflower yellow and hydroxysafflor yellow A(**C**) in inhibiting LPS-induced NO. BV-2 cells were treated with LPS (200 ng/mL) and different combinations of XBJ active compounds for 24 h. When preparing a mixture, compounds were 2-fold diluted with a constant ratio of $(IC_{50})_1/(IC_{50})_2$). NO in the supernatant was measured by 2,3-diaminonaphthalene assay. Drug–drug interactions within the binary combinations are analyzed using the Chou–Talalay method, and the representative combinations are displayed as a plot of *CI* versus $f_a$. *CI*, combination index. $f_a$, fraction affected, that is, fraction of LPS-induced NO being inhibited by the combinations of XBJ active compounds.

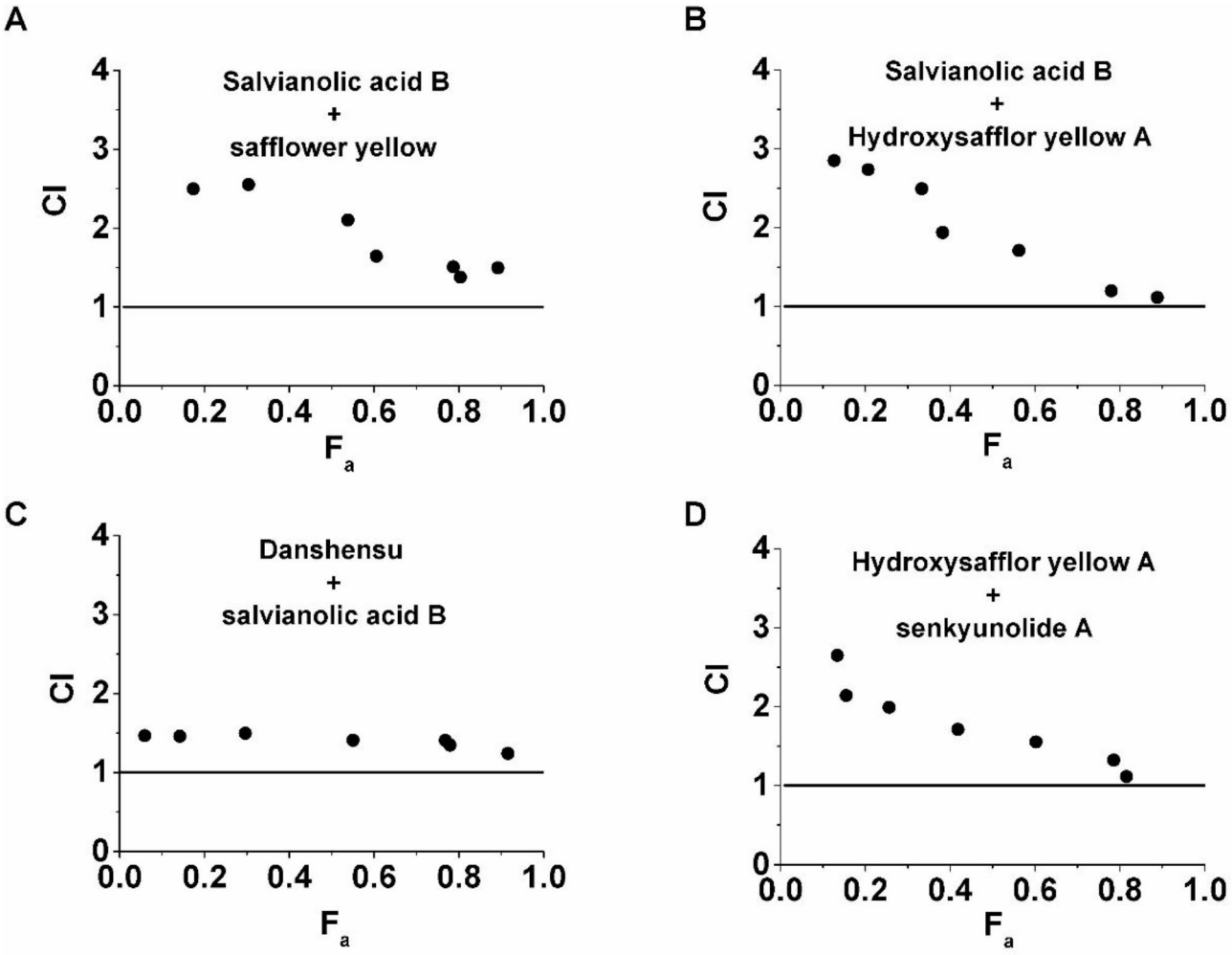


**Fig. 4**. Antagonistic effects of the drug combination of salvianolic acid B and danshensu (**A**), the combination of salvianolic acid B and safflower yellow (**B**), the combination of salvianolic acid B and hydroxysafflor yellow A (**C**), as well as the combination of hydroxysafflor yellow A and senkyunolide A (**D**) in inhibiting LPS-induced NO. BV-2 cells were treated with LPS (200 ng/mL) and different combinations of XBJ active compounds for 24 h. When preparing a mixture, compounds were 2-fold diluted with a constant ratio of $(IC_{50})_1/(IC_{50})_2$). NO in the supernatant was measured by 2,3-diaminonaphthalene assay. Drug–drug interactions within the binary combinations are analyzed using the Chou–Talalay method, and the representative combinations are displayed as a plot of *CI* versus $f_a$. *CI*, combination index. $f_a$, fraction affected, that is, fraction of LPS-induced NO being inhibited by the combinations of XBJ active compounds.

## Conclusions

In this work, we quantitatively investigated the drug–drug interactions among seven main active compounds from XBJ in inhibiting LPS-induced TLR4 downstream NO production. Three of the twenty-one two-drug combinations showed synergistic effect; four of the binary drug–drug combinations showed antagonistic effect. Additive effects were observed in two thirds (2/3) of the binary drug–drug combinations (Table 1). The main aim of drug combinations is to achieve synergistic therapeutic effects, targeting multiple pathways or mechanisms to enhance efficacy. This strategy has become the leading choice for treating severe diseases. Through a systematic pharmacology study, we dissected the combination principle, shedding light on the mechanism of action of a representative TCM formula.

## Materials and methods

### Materials

Danshensu, salvianolic acid B, ligustilide, safflower yellow, hydroxysafflor yellow A, and paeoniflorin were acquired from Energy Chemical (Shanghai, China). Senkyunolide A was obtained from Gelipu Biotech. Co. (Chengdu, China). The purities of these active compounds from XBJ were over 98%. Ultrapure lipopolysaccharide (LPS) was sourced from Invivogen (San Diego, CA, USA). Crystal violet and 2,3-diaminonaphthalene were obtained from Sigma-Aldrich (St. Louis, MO, USA).

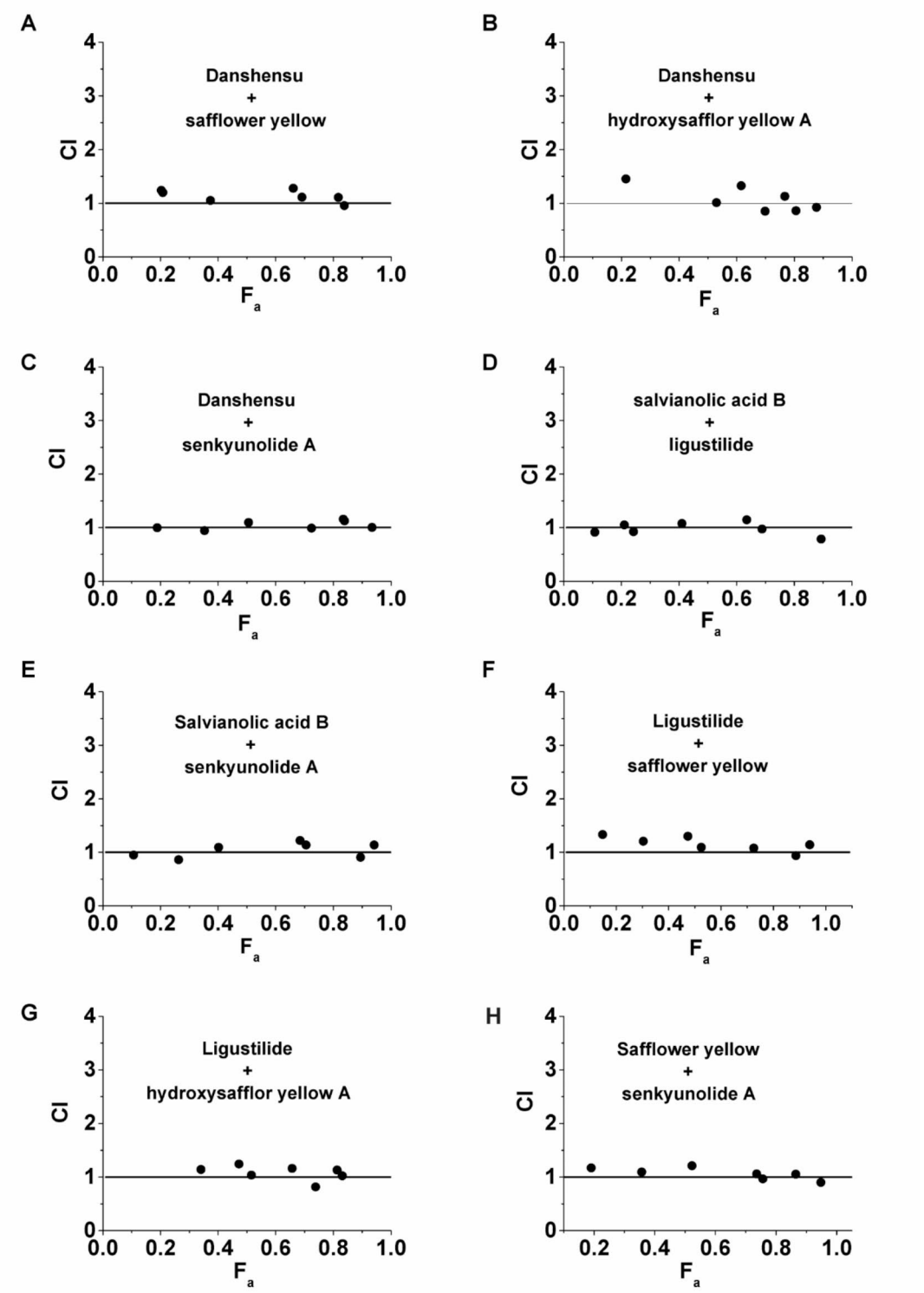


**Fig. 5**. Additive effects of the drug combination of salvianolic acid B and ligustilide (**A**), the combination of safflower yellow and danshensu (**B**), the combination of safflower yellow and ligustilide (**C**), the combination of hydroxysafflor yellow A and danshensu (**D**), the combination of hydroxysafflor yellow A and ligustilide (**E**), the combination of senkyunolide A and danshensu (**F**), the combination of senkyunolide A and salvianolic acid B (**G**), as well as the drug combination of senkyunolide A and safflower yellow (**H**) in inhibiting LPS-induced NO. BV-2 cells were treated with LPS (200 ng/mL) and different combinations of XBJ active compounds for 24 h. When preparing a mixture, compounds were 2-fold diluted with a constant ratio of $(IC_{50})_1/(IC_{50})_2$. NO in the supernatant was measured by 2,3-diaminonaphthalene assay. Drug–drug interactions within the binary combinations are analyzed using the Chou–Talalay method, and the representative combinations are displayed as a plot of *CI* versus $f_a$. *CI*, combination index. $f_a$, fraction affected, that is, fraction of LPS-induced NO being inhibited by the combinations of XBJ active compounds.

| | DSS | SAB | LIG | SY | HSYA | SenA | PF |
|---|---|---|---|---|---|---|---|
| DSS | | −− | ++ | ± | ± | ± | ± |
| SAB | | | ± | −− | −− | ± | ± |
| LIG | | | | ± | ± | ++ | ± |
| SY | | | | | ++ | ± | ± |
| HSYA | | | | | | −− | ± |
| SenA | | | | | | | ± |
| PF | | | | | | | |

**Table 1**. Summary of the binary drug–drug interactions among the main active ingredients of XBJ in inhibiting LPS-induced NO. *DSS* Danshensu, *SAB* salvianolic acid B, *LIG* ligustilide, *SY* safflower yellow, *HSYA* hydroxysafflor yellow A, *SenA* senkyunolide A, *PF* paeoniflorin. The symbols of "++", "±" and "−−" indicate synergism, additive effect, and antagonism, respectively.

## BV-2 cell culture

The mouse microglial cell line BV-2 was obtained from the China Center for Type Culture Collection (CCTCC). Authentication of the BV-2 cell line was carried out using the Short Tandem Repeat (STR) method. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin, and incubated at 37 °C with 5% $CO_2$. Prior to experiments, cells were tested for mycoplasma to ensure the absence of contamination.

## Nitric oxide (NO) assay

BV-2 cells were plated at a density of $8 \times 10^4$ cells/well in 96-well plates. After overnight incubation, media was replaced with DMEM media without FBS. LPS (200 ng/mL) and different concentrations of the main active compounds from XBJ were added. After treatment, NO assay was performed as described previously[28].

## Cell viability assay

BV-2 cells were cultured and treated as indicated in the NO assay. Cell viability was assessed using crystal violet staining as described previously[28].

## Data analysis

Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for statistical analysis. Data were expressed as mean ± standard error of the mean (SEM) from at least 5 independent experiments. Non-linear logistic regression was employed to plot and analyze concentration-response curves and determine the $IC_{50}$ values. The effects of different drug combinations on the inhibition of LPS-induced NO were analyzed using the method described by Chou[29]. The shape of the concentration-dependent curve ($m$, the coefficient signifying the shape of the concentration-effect relationship) and the median-effect dose ($D_m$, i.e. $IC_{50}$) were determined according to the median-effect equation: $\lg(f_a/(1\text{-}f_a)) = m \lg (D) - m \lg (D_m)$, where $D$ is the dose or concentration of a drug, $f_a$ is the fraction affected by $D$ (i.e. percentage inhibition/100) and $f_u$ is the fraction unaffected ($f_u = 1\text{-}f_a$). The combination index ($CI$) of two drugs was calculated according to equation:

$$\mathrm{CI} = \frac{(D)_1}{(D_m)_1[f_a/(1-f_a)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[f_a/(1-f_a)]^{1/m_2}}$$

where CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively.

## Data availability

All data generated or analyzed in this study are included in this article. Further inquiries can be directed to the corresponding author.

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## Author contributions

X.W. conceptualized and designed the study. T.Z. and H.L. conducted the experiments and analyzed the data. X.W. and C.L. wrote, reviewed, and edited the manuscript. All authors approved the final manuscript.

## Declarations

## Competing interests

The authors declare no competing interests.

## Ethical statements

This article does not contain any study with human participants or animals performed by any of the authors.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-95994-9.

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