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Xanthine oxidase inhibitory effect of formula Er-miao tang combined with inhibitory and antioxidant activities of *Phyllanthus amarus* L. and *Orthosiphon stamineus* Benth.

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Abstract:

This study aimed to evaluate the xanthine oxidase (XO) a modified traditional formula, Er-miao tang, combined with *Phyllanthus amarus* L. and *Orthosiphon stamineus* Benth. The goal was to explore a complementary approach utilising locally available medicinal plants. XO inhibitory activity was assessed using Noro's spectrophotometric method, while antioxidant capacity was evaluated via the DPPH radical scavenging assay. Both water decoction and 40% ethanol extracts of the combined formula were tested. The combined formula showed stronger XO inhibition than Er-miao tang alone. The 40% ethanol extract exhibited the highest activity (IC_{50}=88.61 µg/ml), followed by the water decoction (IC_{50}=93.78 µg/ml), compared to Er-miao tang alone (IC_{50}=110.97 µg/ml). Both the water extract and the 40% ethanol extract of the formulation demonstrated antioxidant activity, with IC_{50} values of 65.33 and 53.25 µg/ml against DPPH radicals, respectively. The combination of Er-miao tang with *P. amarus* and *O. stamineus* enhances XO inhibitory and antioxidant activities, supporting further investigation of the formulation as a potential source of inhibitory constituents.

Keywords: **Er-miao tang, hyperuricemia, *Orthosiphon stamineus* Benth., *Phyllanthus amarus* L., xanthine oxidase inhibition.**

Classification numbers: **3.2, 3.3, 3.4**

1. Introduction

Hyperuricemia is a major risk factor for gout, leading to the deposition of monosodium urate microcrystals in tissues and recurrent acute arthritis flares [1]. Uric acid-lowering therapy, particularly through inhibition of XO, is considered a key approach in the management and prevention of gout. However, the long-term use of synthetic drugs is often associated with serious adverse effects, which has increased interest in safer natural alternatives [2]. In this context, medicinal plants and traditional herbal formulations are increasingly recognised as promising options due to their potential to effectively and safely control gout.

Er-miao tang, a classical formula composed of Cortex Phellodendri and *Atractylodes lancea*, has long been used in traditional medicine [3]. According to Dan-Xi-Xin-Fa (Zhu Danxi, Yuan dynasty), Er-miao tang was described as a treatment for acute gout that eliminates heat and excretes dampness in traditional Chinese medicine. From a modern pharmacological perspective, Er-miao tang at a 2:1 ratio of *P. amurense* to *A. lancea* has been shown to exert anti-inflammatory, xanthine oxidase (XO) inhibitory, and nephroprotective effects [3, 4].

Additionally, traditional knowledge suggests that the whole plant *Phyllanthus amarus* has been used as a medicinal agent due to its analgesic, hepatoprotective, and diuretic effects [5]. Modern pharmacological studies have further demonstrated that *P. amarus* exhibits diuretic, cardioprotective, analgesic, and anti-inflammatory activities [5]. Similarly, *Orthosiphon stamineus*, which has long been employed in folk medicine as a diuretic and for alleviating joint pain, has been shown to possess diuretic, anti-inflammatory, antioxidant, gastroprotective, and uric acid-lowering properties [6]. The methanolic extract of *O. stamineus* leaves demonstrated a dose-dependent inhibition of XO, with an IC_{50} value of 78.4 µg/ml [7]. To date, no studies have evaluated the *in vitro* XO inhibitory activity of *P. amarus* extracts. However, this medicinal plant contains a variety of bioactive constituents, including alkaloids, flavonoids, hydrolysable tannins (ellagitannins), lignans, polyphenols, triterpenes, sterols, and volatile oils [8]. The major lignans of *P. amarus* are phyllanthin and hypophyllanthin. At the same time, its flavonoid constituents, such as gallocatechin, rutin, quercetin, and kaempferol, have been reported to exhibit *in vitro* XO inhibitory effects with IC_{50} values ranging from 0.44 to >100 µM [9], or to exert uric acid-lowering activity *in vivo* [10]. Moreover, a 4:1 extract combination of *P. amarus* and *O. stamineus* has been experimentally confirmed to promote diuresis and reduce uric acid levels [11].

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To enhance damp-heat eliminating effects and support the reduction of serum uric acid levels, Er-miao tang was combined with two indigenous herbs, *P. amarus* and *O. stamineus*. The formulation consists of *P. amurense* (12 g), *A. lancea* (6 g), *P. amarus* (20 g), and *O. stamineus* (5 g), selected based on traditional herbal hierarchy and modern pharmacological evidence targeting XO inhibition. *P. amurense* serves as the principal herb due to its anti-inflammatory and uric acid-lowering properties, primarily attributed to berberine and related alkaloids [3]. *A. lancea*, as the minister herb, supports digestive function and enhances the bioavailability of active constituents [3]. *P. amarus* and *O. stamineus* act as assistant herbs, contributing XO inhibition, antioxidant activity, and uricosuric effects, respectively [11]. This composition reflects a rational integration of traditional roles with pharmacodynamic and pharmacokinetic considerations, aiming to optimise therapeutic synergy and efficacy.

2. Materials and methods

2.1. Medicinal ingredients and herbal composition in the formulation

The medicinal herbs *P. amurense* (voucher no. PA-001) and *A. lancea* (voucher no. AL-002) met the quality standards set by the Vietnamese Pharmacopoeia V. These were provided by the University Medical Centre Ho Chi Minh City, Branch 3. Additionally, the herbs *P. amarus* (voucher no. PA-003) and *O. stamineus* (voucher no. OS-004) were supplied by Hong Dai Viet Trading and Manufacturing Co. Ltd. All plant materials used in this study were deposited at the Unit of Traditional Medicine and Pharmacy, Faculty of Traditional Medicine, University of Medicine and Pharmacy at Ho Chi Minh City.

The formula of Er-miao tang was combined with *P. amarus* and *O. stamineus* in various herbal ratios as follows.

Table 1. Composition of the herbal formulations (F1-F4) and extraction solvents used in this study.

No.	Formula	Extraction solvents
F1	*Phellodendron amurense* (cortex) 12 g *Atractylodes lancea* (rhizoma) 6 g *Phyllanthus amarus* (herba) 20 g *Orthosiphon stamineus* (herba) 5 g	Water
F2	*Phellodendron amurense* (cortex) 12 g *Atractylodes lancea* (rhizoma) 6 g *Phyllanthus amarus* (herba) 20 g *Orthosiphon stamineus* (herba) 5 g	40% ethanol
F3	*Phellodendron amurense* (cortex) 12 g *Atractylodes lancea* (rhizoma) 6 g	Water
F4	*Phyllanthus amarus* (herba) 20 g *Orthosiphon stamineus* (herba) 5 g	Water

2.2. Materials and equipment

Solvents and chemicals: 40% ethanol, xanthine 0.15 mM (98%, Sigma-Aldrich, USA), xanthine oxidase enzyme 0.2 U/ml (98%, Sigma-Aldrich, USA), distilled water, NaCl 0.9%, HCl 0.5 M, DMSO (dimethyl sulfoxide) (China), phosphate buffer 50 mM (pH=7.5), allopurinol (99%, Sigma-Aldrich, USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (99%, Sigma, USA), ascorbic acid (99%, Merck, Germany)…

Instruments and Laboratory Equipment: Analytical balance (4 decimal precision, Mettler Toledo), infrared moisture analyser (Mettler Toledo), UV-Vis Spectrophotometer (SP8001, Germany).

2.3. Extraction process

The water decoction was prepared using the traditional decoction method. The herbs were sliced into moderate-sized pieces before being placed into a decoction pot. Distilled water was used as the extraction solvent at a 15:1 solvent-to-herb ratio. Each decoction was boiled for 60 minutes per session. Each herbal formula underwent two rounds of decoction, and the extracted solutions were combined. The combined decoctions were then concentrated via water bath evaporation to obtain a thick extract with a moisture content of ≤20%. The final herbal extract was stored in a refrigerated compartment (4-8°C) until further use.

To obtain the 40% ethanol extract, the herbal ingredients from the traditional formulation were finely ground and passed through a 355 µm mesh sieve to ensure a uniform, fine powder. The powdered herbs were moistened with an adequate amount of 40% ethanol, covered, and allowed to stand for 2 to 4 hours at room temperature. The moistened herbal powder was then transferred into a percolation apparatus. The mixture was macerated for 24 hours, followed by percolation at a flow rate of 20 drops/min. Ethanol 40% was used as the extracting solvent at a 10:1 solvent-to-herb ratio. The collected extract was concentrated using a rotary evaporator system to recover the solvent. The concentrate was then water-bath evaporated to obtain a thick extract with a moisture content of <20%. The final extract was stored in a refrigerated compartment (4-8°C) until use to ensure stability and preservation.

2.4. Evaluation of selected extract parameters

According to the Vietnamese Pharmacopoeia V (Appendix 1.1) [12], the standardisation of a herbal extract is based on the following criteria: Organoleptic characteristics, extraction yield, moisture content, and total ash content.

2.5. Qualitative analysis of herbal extracts using thin-layer chromatography

The thin-layer chromatography (TLC) method, as specified in the Vietnamese Pharmacopoeia V (Appendix 5.4) [12], was employed to identify the presence of characteristic chemical components found in standard medicinal herbs present in the herbal extract samples under investigation. Standard solutions were prepared by weighing approximately 2 g of each reference herb into an Erlenmeyer flask, extracting with 30 ml of methanol using ultrasonic treatment for 30 minutes, and filtering to obtain the filtrate. The filtrate was then evaporated to dryness, and the residue was reconstituted in 1 ml of methanol to obtain the standard solution.

Based on screening results and IC_{50} testing of potential extracts for *in vitro* XO inhibition, extract F2 was selected for TLC-based qualitative analysis.

TLC was employed for the qualitative analysis of Cortex Phellodendri using a mobile phase consisting of n-butanol, acetic acid, and water in a 7:1:2 ratio. Visualisation was performed under ultraviolet light at wavelengths of 254 and 365 nm.

TLC analysed the extract of Rhizoma Atractylodis with a solvent system comprising toluene, ethyl acetate, and formic acid in a 5:4:1 proportion. Detection involved UV inspection at 254 and 365 nm, followed by spraying the plate with vanillin/sulfuric acid reagent and heating it at 105°C until distinct spots were visible.

For Herba Orthosiphonis, TLC was conducted using a toluene-acetone mobile phase in a 7:3 ratio. The plate was treated with 10% sulfuric acid, heated at 105°C for several minutes, and observed under UV light at 365 nm to detect the presence of characteristic compounds.

TLC analysis of Herba Phyllanthi amari was performed using a mobile phase of chloroform and ethyl acetate (9:1). The plate was stained with 10% sulfuric acid, heated to 105°C, and examined under UV light at 365 nm to reveal the relevant chromatographic spots.

2.6. Method for evaluating xanthine oxidase inhibitory activity

The spectrophotometric method developed by T. Noro, et al. (1983) [13] was used as the model to assess *in vitro* XO inhibition in this study.

Plant extracts were dissolved in dimethyl sulfoxide (DMSO) to form a stock solution with a concentration of 2 mg/ml. The final concentration of DMSO in all solutions was less than or equal to 1%. The stock was then diluted with phosphate buffer (50 mM, pH=7.5) to a working concentration of 200 µg/ml.

The reaction mixture consisted of 100 µl of test sample solution, 300 µl of phosphate buffer (50 mM, pH=7.5), 100 µl of XO enzyme solution (0.2 U/ml in phosphate buffer), and 100 µl of distilled water. The mixture was incubated at 37°C for 15 minutes, then 200 µl of xanthine 0.15 mM in phosphate buffer was added. Incubation continued for another 30 minutes. The reaction was stopped by adding 200 µl of HCl 0.5 M.

For each test sample, a blank control was prepared by replacing the enzyme with phosphate buffer and adding HCl immediately without incubation. A negative control (with no test extract) was also conducted by replacing the sample with buffer. Each experiment was repeated three times to ensure accuracy. Allopurinol was used as the positive control and tested at concentrations of 1.7, 3.4, 5.1, 6.81, and 10.21 µg/ml.

Absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 295 nm. The percentage of XO inhibition was determined using the following equation:

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

In this formula, $Abs_{control}$ represents the absorbance of the control reaction without any inhibitor, while Abs_{sample} is the absorbance measured in the presence of the test sample.

2.7. DPPH free radical scavenging assay

The antioxidant activity of the aqueous and 40% ethanol extracts of the formulation was evaluated using a modified DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, as described by O.P. Sharma, et al. (2009) [14]. Briefly, 0.5 ml of each test sample at varying concentrations was mixed with 0.5 ml of 0.8 mM DPPH solution prepared in methanol. The mixture was incubated at room temperature for 30 minutes, and absorbance was measured at 517 nm.

The percentage of radical scavenging activity was calculated using the following formula:

$$\text{Scavenging activity } (\%) = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

where $OD_{control}$ is the absorbance of the DPPH solution without sample, and OD_{sample} is the absorbance of the test sample.

All measurements were performed in triplicate, and results were expressed as mean values. Ascorbic acid (Merck, Germany) served as the positive control.

2.8. Data analysis

The results are expressed as X±SD, where *X* represents the mean value, and *SD* denotes the standard deviation.

The IC_{50} value was determined using a graphical method and the regression equation representing the relationship between concentration (C) and the corresponding inhibition percentage (I%). The analysis was conducted using Microsoft Excel software.

3. Results

3.1. Quality assessment results of herbal extracts

Four polyherbal formulations were extracted using water and 40% ethanol as solvents, following the extraction method described above. The resulting extracts-aqueous and ethanolic-were evaluated based on organoleptic properties, extraction yield, moisture content, and total ash content (Table 2). The F1 aqueous extract contained essential oils, alkaloids, flavonoids, polyphenols, organic acids, and saponins, while the F2 40% ethanol extract contained essential oils, alkaloids, flavonoids, polyphenols, and organic acids.

Table 2. Key quality indicators of extract samples.

Extract sample	Extraction yield (%)	Moisture content (%±SD)	Total ash content (%±SD)
F1	11.29±0.26	15.54±0.10	13.00±0.52
F2	25.65±0.42	17.55±0.07	13.65±0.39
F3	10.15±0.22	17.53±0.19	8.21±0.83
F4	12.87±0.23	18.25±0.12	19.37±0.28

The highest extraction yield was observed in F2 (25.65%), and the lowest in F3 (10.15%). In terms of organoleptic properties, all extracts were semi-solid, soft, and uniformly dark brown with a pleasant aroma and bittersweet taste. F1 and F3 had a visible thin surface film, while F2 was more refined and consistent than the others.

All extracts had moisture content below 20%, which meets the standard moisture limit for soft extracts as per Appendix 1.1 of the Vietnamese Pharmacopoeia V. Total ash content was highest in F4 and lowest in F3, indicating possible differences in inorganic residue or mineral content.

3.2. Qualitative results of herbal extracts using thin-layer chromatography

Based on the preliminary screening and IC_{50} determination tests, the F2 extract exhibited the most promising results in XO inhibition. Therefore, F2 was selected for qualitative analysis via TLC, along with individual herbal extracts, to confirm the presence of constituent compounds from each herb in the combined extract.

The qualitative phytochemical composition of the F2 extract is presented in Fig. 1.

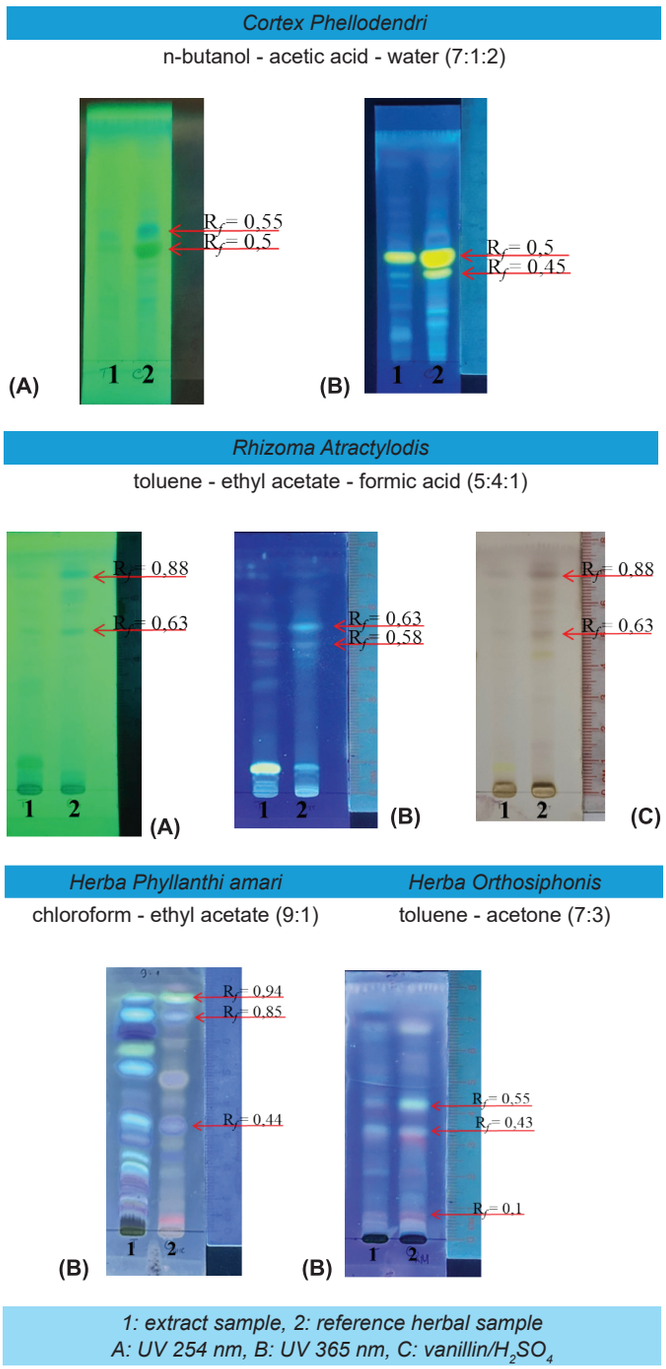

Fig. 1. Qualitative analysis of herbal constituents in F2 extract.

Cortex Phellodendri detection: When observed under UV light at 254 nm, both the F2 extract and the phellodendron reference sample displayed green and yellow bands with R_f values of 0.55 and 0.5, respectively. Under 365 nm UV light, both samples revealed two yellow, fluorescent bands at R_f values of 0.5 and 0.45, further confirming the presence of Cortex Phellodendri constituents in the F2 extract.

Rhizoma Atractylodis detection: Under UV light at 254 nm, both the F2 extract and the *Atractylodes* reference sample exhibited two green, fluorescent bands. After applying vanillin/H_2SO_4 reagent, two pink spots appeared at R_f values of 0.88 and 0.63, matching those of the reference. Under UV 365 nm, the F2 extract showed two green, fluorescent bands at R_f values of 0.63 and 0.58, confirming the presence of *Rhizoma Atractylodis* compounds.

Herba Phyllanthi amari detection: Under 365 nm UV light, the F2 extract displayed three green, fluorescent bands at R_f values of 0.94, 0.85, and 0.44. These values were consistent with the standard *Phyllanthus amarus* extract, confirming the presence of *Herba Phyllanthi amari*.

Herba Orthosiphonis detection: When examined under UV light at 365 nm, the F2 extract revealed two yellow, fluorescent bands at R_f values of 0.55 and 0.43, as well as one red fluorescent band at R_f values of 0.1. These findings matched the reference extract of *Orthosiphon stamineus*, thereby confirming the presence of *Herba Orthosiphonis* in the F2 extract.

3.3. In vitro xanthine oxidase inhibitory activity

The herbal extracts from four formulas were evaluated for XO-inhibitory activity at a concentration of 200 µg/ml. The preliminary results revealed that extracts with over 50% inhibition included F1, F2, and F3. Among the tested samples, F4 exhibited the lowest XO inhibition activity with only 4.15±3.37%.

The extracts F1, F2, and F3 showed promising XO inhibition and were thus selected for IC_{50} determination. F4, having inhibition below 50%, was considered ineffective and excluded from further IC_{50} analysis.

IC_{50} determination results of promising extracts F1, F2, F3

Based on the results, the *in vitro* XO inhibition capabilities of extracts F1, F2, and F3 were dose-dependent. The IC_{50} values (half-maximal inhibitory concentration) for these extracts were calculated as follows in Table 3. Among the extracts tested, F3 had the highest IC_{50}, thus the lowest XO inhibitory potential. Extracts F1 and F2, with lower IC_{50} values, demonstrated promising XO inhibition and are potential candidates for gout management through natural therapies.

For comparison, the positive control, allopurinol, exhibited a significantly lower IC_{50} of 2.42 µg/ml, indicating much higher potency in inhibiting XO (Table 3).

Table 3. IC_{50} values of xanthine oxidase inhibitory activity and DPPH radical scavenging activity of extracts and the positive controls.

Extract	XO inhibitory activity IC_{50} (µg/ml)	DPPH radical scavenging activity IC_{50} (µg/ml)
F1	93.78	65.33
F2	88.61	53.25
F3	110.97	
Allopurinol	2.42	
Ascorbic acid		4.97

Table 3 summarises both the xanthine oxidase (XO) inhibitory activity and the DPPH radical-scavenging activity of extracts F1, F2, and F3 in comparison with standard reference compounds. F2 exhibited the strongest XO inhibitory effect among the extracts, with an IC_{50} of 88.61 µg/ml, although allopurinol remained significantly more potent. For antioxidant activity, F2 also demonstrated the highest DPPH scavenging capacity (IC_{50}=53.25 µg/ml), while ascorbic acid displayed the strongest activity overall. Extract F3 showed weaker XO inhibition and was not assessed for DPPH activity. These results indicate that extracts F1 and F2 possess measurable antioxidant and XO inhibitory properties, with F2 performing consistently better in both assays.

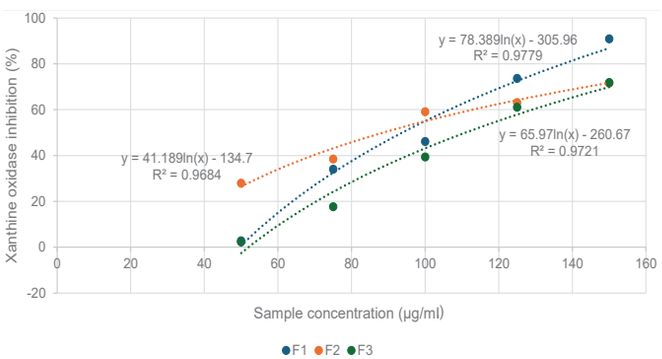

Fig. 2. XO inhibitory activity of extracts F1, F2, and F3.

3.4. Antioxidant activity of the combined formulation

The findings demonstrated that both the aqueous and 40% ethanol extracts of the combined formulation possessed antioxidant activity, with IC_{50} values of 65.33 and 53.25 µg/ml, respectively. These values indicate a lower DPPH radical scavenging capacity compared to the standard antioxidant, ascorbic acid, which exhibited an IC_{50} of 4.97 µg/ml (Table 3).

4. Discussion

The organoleptic characteristics, moisture content, total ash content of the herbal extracts were evaluated. The average moisture content remained below 20%,

meeting the standard limit for soft extracts per Appendix 1.1 of the Vietnamese Pharmacopoeia V [12]. The total ash content was also below 20% for all samples. F3 had the lowest ash content (8.21%), while F4 had the highest (19.37%). According to the Pharmacopoeia, *P. amarus* and *O. stamineus* should have ash contents below 20 and 12%, respectively [12]. Thus, the combination of these herbs in F4 likely caused its higher ash content. Extraction yields were generally low. F3 had the lowest yield (10.15%), possibly due to the dense nature of *P. amurense* bark and *A. lancea* rhizome. These materials increase solution viscosity during decoction, hindering solvent penetration. Additionally, *A. lancea* contains starch that gelatinises at high temperatures, further thickening the solution. Water-soluble gums and mucilage also swell and form colloids, obstructing extraction [15]. As a result, aqueous extracts (F1, F3, F4) had lower yields than the 40% ethanol extract (F2). These findings are also consistent with the ash content reported for the 70% ethanol extract of *O. stamineus*, which was 7.30±0.04%, with an extraction yield of 15.55% [16].

In the XO inhibitory activity screening, F2 showed the strongest inhibition and was selected for TLC analysis. Its chromatographic spots matched those of the reference herbs in colour, shape, and R_f value, confirming the presence of the constituent herbs. The TLC analysis demonstrated a clear correspondence between the extract sample and the reference herbal materials across all four investigated crude drugs. Characteristic bands of alkaloids in Cortex Phellodendri, sesquiterpene lactones in Rhizoma Atractylodis, lignans and flavonoids in Herba Phyllanthi amari, and polymethoxyflavones in Herba Orthosiphonis were consistently observed in both samples, indicating that the extraction procedure effectively preserved the key phytochemical groups of each herb. Although the intensity of several spots in the extract was lower, particularly for Rhizoma Atractylodis, the matching R_f values and colour reactions confirm the presence of the major bioactive constituents after extraction. Importantly, no additional or unexpected spots were detected in the extract, supporting the specificity of the methodology. The TLC patterns observed in this study are also consistent with previously published TLC profiles of the individual herbal components, including Cortex Phellodendri [17], Rhizoma Atractylodis [18], Herba Phyllanthi amari [19], and Herba Orthosiphonis [20]. Overall, the TLC results suggest that the chemical profile of the combined extract is consistent with that of the raw herbal materials. These findings verify the adequacy of the extraction process and support the reliability of the extract for subsequent pharmacological evaluation.

XO inhibition was measured using Noro's spectrophotometric method [11, 13, 21]. As shown in Fig. 2, inhibition increased with concentration. F1, F2, and F3 achieved over 50% inhibition at 200 µg/ml, while F4 remained below 50%. This difference may stem from variations in extraction methods and herb ratios, which can impact *in vitro* activity. F4 was prepared via traditional decoction, which may not efficiently extract flavonoids and polyphenols, key compounds for XO inhibition. In contrast, hydro-ethanolic systems used in previous studies showed higher activity. For example, T.N.Q. Do, et al. (2014) [11] demonstrated that a 4:1 combination ratio of *P. amarus* and *O. stamineus* yielded an IC50 value of 43.83 µg/ml, whereas the *P. amarus* extract alone exhibited an IC_{50} value of 84.46 µg/ml. There is still no study on the xanthine oxidase inhibitory activity of *P. amarus*; however, according to V. Murugaiyah, et al. (2009) [22], an IC_{50} of 39.39 µg/ml was found for a methanol extract of *P. urinaria*. The methanolic leaf extract of *O. stamineus* inhibited xanthine oxidase activity with an IC_{50} value of 78.4 µg/ml [7]. Moreover, the hexane extract derived from a dual herbal combination of *Carica papaya* L. and *Piper lolot* C. DC leaves demonstrated *in vitro* XO inhibitory activity, with an IC_{50} value of 27.39±0.32 µg/ml. In contrast, the crude ethanol extract of the same combination exhibited significantly lower activity, with an IC_{50} value exceeding 100 µg/ml [21]. These results highlight the importance of solvent choice and formulation ratio in enhancing extraction and synergistic effects. This variation in activity may be attributed to the structural characteristics of flavonoids in the extract, particularly the number and position of glycosyl groups. These modifications can increase molecular size and hydrophilicity, potentially reducing the compounds' ability to inhibit XO [7]. Molecular docking analyses revealed that forty-six bioactive compounds in Er-miao tang, including quercetin, wogonin, and β-sitosterol-exhibited strong binding affinity to key protein receptors. These findings suggest that Er-miao tang may exert anti-inflammatory, antioxidant, and anti-apoptotic effects, as well as reduce uric acid levels, primarily through core targets such as AKT1, IL-1β, PTGS2, and JUN, and via major signalling pathways including NF-κB, IL-17, and TNF in the management of hyperuricemia and gout [22]. *In vivo* experiments demonstrated that Er-miao tang mitigated joint damage by modulating the PI3K/AKT/mTOR/HIF-1α signalling axis [23]. Complementary *in vitro* studies indicated that TNF-α stimulation upregulated Glut1 and HK2 expression, enhanced glycolytic activity, and promoted migration and invasion of rheumatoid arthritis-FLS cells, contributing to pathological progression. Conversely, Er-miao tang regulated glycolysis-related proteins (HK2 and Glut1) via the

PI3K/AKT/*m*TOR/HIF-1α pathway, thereby suppressing inflammation, cellular migration, and invasion in rheumatoid arthritis-FLS cells [23]. Besides that, lignans in *Phyllanthus* species reduced plasma uric acid levels in hyperuricemic rats [10]. Among these, phyllanthin demonstrated the most potent activity, restoring plasma uric acid to near-normal levels comparable to standard clinical agents such as allopurinol, benzbromarone, and probenecid. At a relatively low dose of 10 mg/kg, hypophyllanthin achieved a 64.47% reduction in plasma uric acid ($p<0.01$). Moreover, hypophyllanthin significantly increased uric acid clearance (0.19±0.05 ml/(kg·hr)) compared to normal (0.13±0.04 ml/(kg·hr)) and hyperuricemic controls (0.03±0.04 ml/(kg·hr)). These findings suggest that hypophyllanthin holds strong therapeutic potential for gout and hyperuricemia-related disorders, likely through a uricosuric mechanism involving xanthine oxidase inhibition [10]. Flavonoids from this genus have been reported to exhibit xanthine oxidase inhibitory activity, including quercetin, kaempferol, rutin, apigenin, luteolin, myricetin, catechin, epicatechin, and epigallocatechin, with IC_{50} values ranging from 0.44 to >100 µM [9]. Methanol extracts of *O. stamineus* leaves exhibited notable XO inhibitory activity, ranging from 48.3 to 64.8%. This activity is likely attributable, at least in part, to the apigenin and luteolin derivatives present in the leaves, which are recognised as potent inhibitors of XO. XO is a major biological source of superoxide radicals, which act as precursors for reactive oxygen species such as singlet oxygen and hydroxyl radicals, thereby initiating lipid peroxidation directly or indirectly. Inhibition of XO is therefore considered an effective therapeutic strategy for hyperuricemia, gout, and kidney stone formation. Phenolic compounds not only inhibit XO but also exhibit superoxide scavenging properties, potentially reducing both uric acid and oxidative stress in human tissues [7].

L.D. Kong, et al. (2000) [24] reported that Cortex Phellodendri and Rhizoma Atractylodis showed weak XO inhibition *in vitro*. However, the in vivo studies of L.D. Kong, et al. (2004) [25] showed that they could lower uric acid levels comparably to allopurinol. Although Rhizoma Atractylodis lacks strong XO inhibition, it may act synergistically with Cortex Phellodendri. The traditional formula Er-miao tang not only inhibits XO but also provides analgesic and anti-inflammatory effects. Berberine, the main compound in Cortex Phellodendri, suppresses inflammation by inhibiting NO production, though it dissolves better in methanol than water [26]. These findings suggest multiple mechanisms contribute to the uric acid-lowering effects of Er-miao tang.

In this study, the 40% ethanol extract (F2) showed the best *in vitro* XO inhibition, but further *in vivo* studies are needed to compare F1 and F2. Since the samples were herbal mixtures, not pure compounds like allopurinol, their activity was weaker (allopurinol IC_{50}=2.42 µg/ml).

Despite this, the extracts may still have therapeutic potential. Plant-based formulations often have IC_{50} values 60-600 times higher than allopurinol [27-29]. Herbal remedies typically act via multiple pathways-inhibiting uric acid synthesis, promoting excretion, and reducing inflammation-rather than a single mechanism. This multi-target approach aligns with traditional medicine, where Er-miao tang, *P. amarus*, and *O. stamineus* are used to clear damp-heat, strengthen the spleen, and promote diuresis. Recent pharmacological investigations have confirmed that *P. amarus* possesses analgesic, anti-inflammatory, and diuretic effects [5]. Moreover, *O. stamineus* has been reported to exhibit diuretic, anti-inflammatory, antioxidant, gastroprotective, and uric acid-reducing activities [6].

Numerous techniques have been utilised to assess the antioxidant potential of herbal extracts. Among these, the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay stands out as one of the most traditional and widely accepted methods for evaluating antioxidant activity [14]. This assay operates on the principle that antioxidants donate a hydrogen atom to DPPH radicals, reducing them to their non-radical form, DPPH-H [14]. In this study, both the aqueous and 40% ethanol extracts demonstrated antioxidant activity, with IC_{50} values of 65.33 and 53.25 µg/ml, respectively. These results align with previously reported antioxidant properties of the individual herbal components in the formulation, suggesting that their combined effect may be due to synergistic interactions. Cortex Phellodendri exhibited notable DPPH radical scavenging activity, with an IC_{50} of 81.48±0.80 µg/ml, contributing significantly to the overall antioxidant effect [30]. Rhizoma Atractylodis showed a high total phenolic content (36.08 mg GAE/g), along with a DPPH scavenging activity of 77.85±0.64% and a FRAP value of 560.26 µmol Fe(II)/g, despite its relatively low flavonoid content [31]. The 96% ethanol extract of *P. amarus* exhibited notable antioxidant activity, as indicated by its DPPH free radical scavenging capacity, with an EC_{50} value of 129.74±3.05 µg/ml [32]. *O. stamineus* has demonstrated strong antioxidant activity, as evidenced by its ability to scavenge DPPH radicals [33]. The aqueous extract exhibited a lower IC50 value (9.6±0.021 µg/ml) compared to the ethanol extract (21.4±0.104 µg/ml), indicating higher free radical scavenging capacity. However, both extracts remained less potent than the

reference standard, ascorbic acid (IC50=4.6±0.006 µg/ml). This pronounced antioxidant activity is closely linked to the high concentration of phenolic compounds present in *O. stamineus*, which contribute significantly to its radical scavenging potential [33]. Phenolic compounds are widely recognised for their antioxidant properties, primarily due to their ability to donate electrons and chelate metal ions. The antioxidant activity of flavonoids is significantly influenced by their structural features, particularly the degree of hydroxylation and the nature of substituent groups [34]. Flavonoids exhibit strong antioxidant potential through hydrogen atom transfer mechanisms, especially when they possess ortho-dihydroxyl groups at the C3′ and C4′ positions, a double bond between C2 and C3, a hydroxyl group at C3, and a carbonyl group at C4. Among these, the ortho-dihydroxyl configuration at C3′-C4′ contributes most significantly to antioxidant activity. Flavonoids with hydroxyl substitutions on both A and B rings are classified as phenolic compounds [35]. High antioxidant capacity in flavonoids is associated with the presence of hydroxyl groups at C3′-C4′, a hydroxyl group at C3, a carbonyl function at C4, and a C2-C3 double bond [36]. Several phenolic and flavonoid compounds, including apigenin, luteolin, quercetin, myricetin, morin, and kaempferol have demonstrated xanthine oxidase inhibitory activity [37]. Furthermore, the antioxidant capacity was also influenced by the extraction solvent, with the 40% ethanol extract showing a lower IC_{50} than the aqueous extract of the combined formula in this study, likely due to the enhanced solubility of antioxidant compounds in ethanol.

5. Conclusions

The herbal extracts combining Er-miao tang with *Phyllanthus amarus* and *Orthosiphon stamineus* demonstrated inhibitory activity against xanthine oxidase, with the 40% ethanol extract exhibiting the strongest effect (IC_{50}=88.61 µg/ml). These findings suggest the potential of this herbal formulation as an adjunct therapy for gout management. However, further *in vivo* and clinical studies are required to identify the active compounds, clarify synergistic mechanisms, and comprehensively evaluate safety and long-term efficacy.

CRediT author statement

Hoang Oanh Hua: Conceptualisation, Methodology, Project administration, Writing, Editing; Thi Lan Huong Tran: Investigation, Formal analysis, Writing original draft.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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