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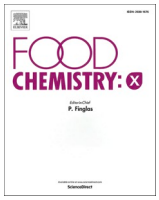

Identification of key phenolic compounds for alleviating gouty inflammation in edible chrysanthemums based on spectrum-effect relationship analyses

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ABSTRACT

Edible chrysanthemum is a common food resource for tea and functional foods with potential benefits for human health. Studies have indicated that chrysanthemum has the potential effect on inflammatory diseases, while the effects on gouty inflammation remain underexplored. The present study aimed to investigate the anti-gout activity and characterize the active ingredients of chrysanthemums by using metabolite profiles, *in vitro* experiments, and spectrum-effect analysis. Results showed that '*Boju*' (BJ), '*Hangbaiju*' (HBJ), and '*Huaiju*' (HJ) exhibited regulatory effects on monosodium urate (MSU)-induced inflammation. At the dose of 50 μg/mL, the inhibitory rates of IL-1β secretion were 24.53 %, 14.36 %, and 38.10 %, respectively. A total of 32 phenolic compounds were identified or preliminarily assigned in UPLC-Q/TOF-MS analysis. And seven phenolics related to anti-gout activity were identified by spectrum-effect relationships. According to ADME (absorption, distribution, metabolism, excretion) evaluation and experiments verification, luteolin, acacetin-7-O-glucoside, and apigenin-7-O-glucoside were critical constituents potentially associated with the reduction of inflammation in gout. Additionally, these phenolics might be suitable as quality control indicators. This study clarified the anti-gout properties of different cultivars of chrysanthemums and active compounds, providing a theoretical basis for its scientific utilization in functional foods.

1. Introduction

Edible chrysanthemum is one of the most common edible flowers worldwide (Lu, Li, & Yin, 2016). It has been cultivated as tea and functional foods for many years due to its various phytochemicals and unique flavors (Yuan, et al., 2020). Chrysanthemum possesses several health properties such as antioxidant, anticancer, anti-diabetic, and anti-inflammatory activities (Chen, et al., 2019; Li, et al., 2019; Xie, Yuan, Yang, Wang, & Wu, 2009). Among those, anti-inflammatory is one of the most extensively recognized and investigated. Research studies have demonstrated the efficacy of edible chrysanthemums in inflammation-associated diseases. For instance, it has shown beneficial effects in models of inflammation such as 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema (Yasukawa, Akihisa, Inoue, Tamura, Yamanouchi, & Takido, 1998), capsaicin-induced systemic low-grade inflammation (Yang, et al., 2023), and obesity-induced inflammation (Lee, Lee, Lee, Chang, & Kim, 2021). These studies highlight that edible chrysanthemums can serve as functional foods or dietary supplements to combat inflammation-associated diseases, based on their potent natural properties.

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Gout is a common form of inflammatory arthritis with unbearable pain, which is caused by the deposition of monosodium urate (MSU) monohydrate crystals in articular and periarticular tissues (Martinon, 2010). Nowadays, gout become a public health problem owing to the substantial rise in incidence and prevalence across the globe (Dehlin, Jacobsson, & Roddy, 2020). Alleviating gouty inflammation is essential for gout patients (Dalbeth, Gosling, Gaffo, & Abhishek, 2021). The activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome plays a major role in the pathogenesis of gouty inflammation (Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006; Wang, Mao, & Meng, 2013). It has been reported that chrysanthemums were efficient in alleviating inflammation, which was achieved through inhibiting the activation of nuclear factor-kappa B (NF-κB) (Han, et al., 2015; Li, et al., 2019). And NF-κB signaling pathways are involved in the activation of NLRP3 inflammasome (Martinon, et al., 2006). Studies have indicated that chrysanthemums have the potential effect on gouty inflammation. It is worth noting that there are multiple varieties of chrysanthemums available in the tea and herb market, and their chemical compositions can vary greatly (Li, et al., 2019; Peng, Lin, Zhao, & Sun, 2019). However, whether the differences in phytochemical constituents of different chrysanthemums result in varying anti-gout effects is unclear, which is an important issue when considering their potential use in tea, beverages, and functional foods.

According to previous studies, the phenolic compounds in chrysanthemums were likely responsible for its various bio-activities (Han, et al., 2015; Lu, et al., 2022; Peng, et al., 2019; Lin, Liu, & Zhao, 2018). For instance, chlorogenic acid, 3,5-dicaffeoylquinic acid, luteolin-7-O-glucoside, 4,5-dicaffeoylquinic acid, and kaempferol-3-O-rutinoside largely contributed to the antioxidant activity of chrysanthemums (Lu, et al., 2022; Peng, et al., 2019); chlorogenic acid, 3,5-dicaffeoylquinic acid, luteolin, and luteolin-7-Oglucoside were closely associated with anti-inflammatory activity (Han, et al., 2015); and luteolin, apigenin, and acacetin were identified as key xanthine oxidase inhibitors (Lin, et al., 2018). In recent years, several comparative studies have been conducted to analyze the phenolic compounds present in chrysanthemums. These studies have suggested that there are significant differences in the chemical profiles among different types of chrysanthemums (Li, et al., 2019; Peng, et al., 2019). However, there is limited information available on the anti-gout activities of chrysanthemums and the active profiles.

Chrysanthemum morifolium Ramat. is the main species of edible chrysanthemums in markets. In the present study, five cultivars of *Chrysanthemum morifolium* (‘*Hangbaiju*’, ‘*Gongju*’, ‘*Chuju*’, ‘*Boju*’, and ‘*Huaiju*’) were selected. The impact of chrysanthemums on gouty inflammation was measured. Then UPLC-Q/TOF-MS-based spectrum-effect relationship was established by gray relational analysis (GRA) and orthogonal partial least-squares regression analysis (OPLSR) to screen activity-related phenolics. Absorption, distribution, metabolism, and excretion (ADME) properties of effect-related compounds were also evaluated. Lastly, *in vitro* experiments were conducted to verify the related components. This study will provide active profiles basis for the application of chrysanthemums as functional products or food supplements for patients suffering from gout.

2. Materials and methods

2.1. Chemicals and reagents

The standards include 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, acacetin, gengkwanin, acacetin-7-O-glucoside, eriodictyol, and apigenin-7-O-glucoside were purchased from Biopurify Phytochemicals Ltd (Sichuan, China). Standards luteolin, quercetin-3-O-glucoside, apigenin and luteolin-7-O-glucoside were purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China).The acetonitrile and formic acid were of LC-MS ultra-quality, which were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was provided by Gibco Laboratories (Waltham, MA, USA). Phorbol 12-myristate 13-acetate (PMA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). RPMI-1640 medium was brought from Hyclone (Logan, USA). The ELISA kit products were purchased from Neobioscience Technology Company (Shenzhen, China).

2.2. Sample preparation

Five cultivars of commercial chrysanthemum products (‘*Hangbaiju*’ (HBJ), ‘*Boju*’ (BJ), ‘*Gongju*’ (GJ), ‘*Chuju*’ (CJ) and ‘*Huaiju*’ (HJ)) were procured from relevant producing areas in China. Detailed information of the samples was introduced in Supplementary data (Table S1). The extracts of chrysanthemum were prepared according to previous method (Peng, et al., 2019). After pulverization, chrysanthemum samples were passed through a 60-mesh screen. The samples (10 g) were extracted twice with 100 mL of 80 % ethanol utilizing ultrasound-assisted extraction for 60 min at 55 °C. After centrifugation, the supernatant was vacuum freeze dried to obtain chrysanthemum extracts. The above extracts were dissolved in DMSO as stock solution (100 mg/mL) and stored at − 20 °C.

2.3. Preparation of MSU crystals

MSU crystals were prepared with slight modifications according to previous studies (Chen, Li, Ou, Ren, Yang, & Zeng, 2019). Uric acid (4 g) was dissolved in 200 mL of NaOH (0.5 M, pH = 8.9) at 70 °C. 0.5 M NaOH was added to maintain the pH at 8.9. The solution was filtered slowly at room temperature for 24 h. After filtering, the crystals were harvested by centrifugation, and then dried at 42℃. The crystal shape and size were checked by polarizing light microscopy. After sterilization at 180℃ for 4 h, MSU crystals were re-suspended in PBS at a concentration of 5 mg/mL.

2.4. Cytotoxicity and ELISA assays

The THP-1 cells were provided by Stem Cell Bank, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cultures were maintained in a humidified incubator with 5 % CO_2 at 37 °C.

MTT assay was used to evaluate the cytotoxicity of samples. THP-1 cells were seeded in 96 plates with 100 ng/mL PMA. After incubation for 12 h, the medium was replaced with fresh medium for 24 h. The cells were subsequently treated with different concentrations of drugs for 12 h, followed by 200 μg/mL MSU crystals for 6 h. All groups had the same concentration of DMSO (1 ‰). MTT dissolved in serum-free medium (0.5 mg/mL) was added to each well and the mixture was incubated at 37 °C for 4 h. The absorbance was measured at 490 nm with a microplate reader.

The section of interleukin-1β (IL-1β) and TNF-α was conducted using ELISA kits. Following the stimulation with drugs and MSU, cell culture supernatants were collected. The supernatants were centrifuged at 10000 rpm for 15 min at 4℃. IL-1β and TNF-α secretions were measured using the ELISA kits according to the manufacturer’s instructions. The inhibition rates of IL-1βwere calculated according to previous studies, and presented as the percentages of the difference between model group and experimental group to model group (Cao, et al., 2022; Wang, Tu, Lian, Hung, Yen, & Wu, 2006).

2.5. Determination of total polyphenols and flavonoids

The total phenolics content (TPC) was analyzed using the Folin–Ciocalteu assay (Cheng, et al., 2020), using gallic acid as standard ($y = 0.0035x-0.0212$, $R^2 = 0.9993$). The total flavonoids content (TFC) in fractions was measured according to previous research utilizing rutin as the standard ($y = 0.0014x - 0.004$, $R^2 = 0.9987$), as reported (Kwaw,

et al., 2018).

2.6. UPLC-Q/TOF-MS analysis

Components of chrysanthemums were identified by UPLC-Q/TOF-MS analysis system (Waters, ACQUITY UPLC H-Class Xevo G2-XS). The chromatographic separation was performed on a Waters BEH-C18 column (2.1 × 100 mm, 1.7 μm). The solvent A was 0.1 % (v/v) formic acid–water, and B was acetonitrile. The gradient elution program was 0–2 min, 5 % B, 2–22 min, 5–99 % B, 22–25 min, 95–5 % B, 25–30 min, and 5 % B. The concentration of samples was 1 mg/mL, and the injection volume was 1 μL.

The electrospray ionization (ESI) source was operated in both positive and negative ion modes at m/z 100–1000. Other operation parameters were conducted as follows: capillary voltage, 2.0 kV; desolvation temperature, 450℃; source temperature:120℃; cone gas flow, 50.0 L/h; low collision energy:10 V; high collision energy: 30 V.

2.7. Spectrum-effect relationship analysis

Correlation analysis (GRA) and regression analysis (OPLSR) were used to screen anti-gout activity related phenolics. The ion of each phenolic compound was selected as the extraction ion. The peak area of the extracted ion was calculated. In the spectrum-efficacy model, the relative peak areas were set as predictive variable X, biological activities were set as dependent variables Y. IL-1β has been recognized as a key aspect of gouty inflammation (Dalbeth, et al.,2021). Thus, its inhibition rates of chrysanthemums in 50 μg/mL compared with the model were selected as the dependent variables.

2.8. ADME evaluation

The oral bioavailability and drug-likeness of effect-related compounds were obtained from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (http://tcmspw.com/tcmsp.php). Likewise, gastrointestinal absorption (GI absorption) and Lipinski's rule were evaluated by SwissADME online

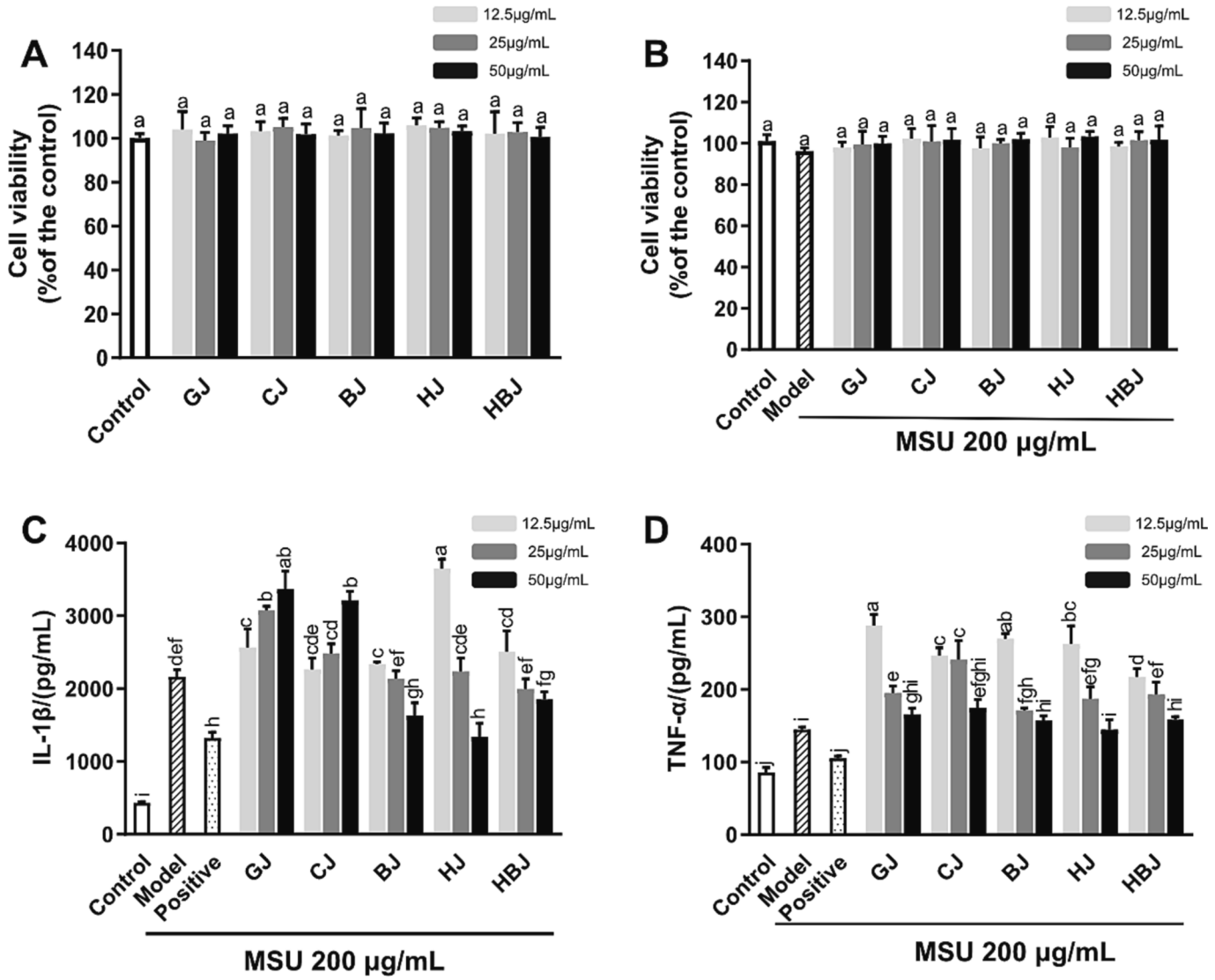

Fig. 1. Effects of chrysanthemum extracts on the production of inflammatory cytokines in MSU-stimulated THP-1 macrophages. Cytotoxic activity of chrysanthemum extracts against THP-1 macrophages without MSU (A) or with MSU (B). IL-1β (C) and TNF-α (D) levels were evaluated in THP-1 macrophages pretreated with chrysanthemum extracts (12.5, 25, 50 μg/mL). The results represented the mean ± SD (n = 5 (Fig. 1A and B); n = 3 (Fig. 1C and D)). HBJ represents '*Hangbaiju*'; BJ represents '*Boju*'; GJ represents '*Gongju*'; CJ represents '*Chuju*'; HJ represents '*Huaiju*'. Different letters represented a significant difference at $p < 0$ 0.05 among values of different groups, all experiments were carried out in triplicate.

database (http://www.swissadme.ch/).

2.9. Statistical analysis

All the experimental data were expressed as means ± standard deviations (SD) of three repeated measurements. Statistical significance was calculated by Duncan's multiple-range test ($p < 0.05$). SIMCA (14.1 version, Umetrics AB, Umea, Sweden) was used for OPLSR analysis. Duncan's multiple-range test and GRA were performed with SPSS 26.0 software (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Effects of chrysanthemums on the secretion of inflammatory cytokines

The cytotoxicity of chrysanthemum extracts on THP-1 cells was evaluated using the MTT method. Curcumin was the positive group, which was reported to have an inhibitory effect on attenuating gouty inflammation *in vivo* and *in vitro* (Chen, et al., 2019). As shown in Fig. 1A and B, all samples did not show cytotoxicity up to 50 μg/mL in THP-1 cells with or without MSU. Afterward, 12.5, 25, and 50 μg/mL of chrysanthemum extracts were used for subsequent experiments.

Gout is a form of inflammatory arthritis caused by MSU (Martinon, 2010). MSU-induced inflammation in THP-1 macrophages is a typical model of gouty inflammation *in vitro* (Chen, et al., 2019; Martinon, et al., 2006; Zhang, et al., 2019). To compare the effects of different chrysanthemum species on gouty inflammation, the levels of IL-1β and TNF-α in the cell supernatants of MSU-induced THP-1 macrophages were examined. Results (Fig. 1C and D) showed that treatment of THP-1 macrophages with MSU significantly increased the levels of IL-1β and TNF-α ($p < 0.05$) in the model group compared to the control group. The positive (curcumin, 12.5 μM) significantly decreased IL-1β and TNF-α ($p < 0.05$) levels, and the inhibition rates of IL-1β and TNF-α were 38.94 % and 27.39 %, respectively. After intervention with different varieties of chrysanthemum extracts, only BJ, HBJ, and HJ at a dose of 50 μg/mL significantly decreased IL-1β level compared to the model group ($p < 0.05$) (Fig. 1C). The inhibition rate of IL-1β of HJ (50 μg/mL) was 38.10 %, which was comparable to the positive curcumin (38.94 %). And BJ and HBJ possessed a weaker effect on attenuating gouty inflammation, the inhibition rates of IL-1β were 24.53 % and 14.36 %. We observed elevated IL-1β levels in cells-exposed MSU and treated with GJ and CJ at 12.2, 25 and 50 μg/mL. In Fig. 1D, the concentration of 12.5 and 25 μg/mL of GJ, CJ, BJ, HJ, and HBJ augmented TNF-α level in cells-exposed MSU compared to the model cells ($p < 0.05$). However, at the dose of 50 g/mL, no difference in TNF-α level was found between the model group and the five chrysanthemum-treated groups. Similarly, studies have shown that some 75 % methanol chrysanthemum extracts can inhibit LPS-induced inflammation, while some chrysanthemum extracts stimulated inflammatory cytokines (Li, et al., 2019). Han, et al. (2015) studied the inhibitory effects of different varieties of chrysanthemums on NF-κB, and results showed that only some of the samples inhibited NF-κB production. The results might be due to the difference of compound profiles in different chrysanthemum species (Han, et al., 2015; Lu, et al., 2022; Peng, et al., 2019; Lin, et al., 2018). Compared with other chrysanthemums, GJ and CJ contain relatively less phenolics and a few flavones and flavanone aglycones (Peng, et al., 2019). The results from the present study showed that different cultivars of chrysanthemums exhibit varying effects on gout inflammation, possibly due to differences in their chemical composition.

Traditionally, chrysanthemum was regarded as a safe resource in functional food. According to previous research, no acute or long-term toxicity was observed in SD rats following oral administration of chrysanthemum ethanol extract at a dose of 15 g/kg and up to 1280 mg/kg/d for a duration of 26 weeks (Li, Gu, Chen, Wang, Ye, & Jiang, 2010). Our results indicated that BJ, HBJ, and HJ extracts had an inhibitory effect on IL-1β secretion, which might be a promising source in the development of functional foods for their anti-gout benefits and safety.

3.2. TPC and TFC in chrysanthemums

According to previous studies, the phenolics in chrysanthemum were likely responsible for its various bio-activities (Chen, et al., 2019; Han, et al., 2015; Li, et al., 2019). Thus, total phenolics content (TPC) and total flavonoids content (TFC) in chrysanthemum extracts were detected. As shown in Table S2, BJ had the highest content of TPC (43.52 mg gallic acid/g) in all species of chrysanthemums; HBJ and GJ possessed higher TPC (37.70 mg gallic acid/g and 36.82 mg gallic acid/g, respectively). Moreover, the TPC values in the present study were greater than that of 1.06–12.72 mg gallic acid/g in hot-water extracts of chrysanthemum observed by Li et al. (2019). This finding suggests that ethanol is more effective than hot water in extracting higher phenolic content from chrysanthemums. And there were no significant differences ($p > 0.05$) in TFC among all samples (164.39–187.76 mg rutin/g). The results indicated that BJ, HBJ, and GJ were excellent resources for supplementing phenolics; different cultivars of chrysanthemums differ in their total phenolic content, which might result the various anti-gout effects.

3.3. Identification of phenolic compounds in chrysanthemum samples

The composition of phenolic compounds in chrysanthemums was detected by UPLC/Q-TOF-MS. A total of 32 phenolic compounds were identified or preliminarily assigned, comprising 8 caffeoylquinic acids, 20 flavones, 2 flavonols, 1 flavanone, and 1unkonw compound (Table 1 and Fig. S1). The MS^2 spectrum of each compound are available in the supplementary material (Fig. S2-6). In addition to comparing with relevant databases PubChem, the Human Metabolome Database and available references for retention time, accurate molecular ions and major MS^2 fragment ions, 11 compounds were further compared with authentic standards. These compounds include 5-caffeoylquinic acid (C2), 3,5-dicaffeoylquinic acid (C11), quercetin 3-O-glucoside (C7), luteolin-7-O-β-d-glucoside (C8), luteolin (C23), apigenin-7-O-glucoside (C12), eriodictyol (C21), acacetin-7-O-glucoside (C26), apigenin (C28), acacetin (C32), gengkwanin (C30).

3.3.1. Caffeoylquinic acids

Among all samples, 8 caffeoylquinic acid derivatives including 3 mono-caffeoylquinic acids, 3 di-caffeoylquinic acids, and 2 tri-caffeoylquinic acids were detected. 3 caffeoylquinic acids (C1, C2, C3) were identified with the same molecular formula ($[M-H]^-$) at m/z 353, and the characteristic fragment at m/z 191[M-H-caffeoyl]$^-$. C2 was 5-caffeoylquinic acid (5-CQA) by comparison with an authentic standard. C1 and C3 exhibit base peaks at [quinic acid-H]$^-$ and [quinic acid-H_2O-H]$^-$, respectively, in the MS^2 spectrum. These results suggested that C1 and C3 correspond to 3-CQA and 4-CQA, respectively (Gouveia & Castilho, 2012). C10, C11, C14 showed the same formula $C_{25}H_{24}O_{12}$ and the major fragments at 353[M-H-caffeoyl]$^-$, 191[M-H-2 caffeoyl]$^-$, 179 [caffeic acid-H]$^-$, indicating that they were di-caffeoylquinic acid isomers. Based on the authentic reference standard, C11 was identified as 3,5-dicaffeoylquinic (3,5-diCQA). C10 and C14, identified as 3,4-diCQA and 4,5-diCQA respectively, produced well-defined and relatively intense MS^2 fragments at m/z 335 and 353/173, based on previous studies of di-CQA's elution order and main MS^2 ions (Gouveia, et al., 2012; Lin & Harnly, 2008). C5 and C24 were tentatively identified as tri-caffeoylquinic acids with the molecular formula ($[M-H]^-$) at m/z 677, and the characteristic fragments at m/z 515 and 353 (Lin & Harnly, 2010).

3.3.2. Flavonoids

In the present study, 23 flavonoids were identified, including 20 flavones, 2 flavonols, and 1 flavanone. C4, C12, C15, C16, C17, C19,

Table 1
Identification of phenolic compounds in chrysanthemum extracts by UPLC-Q/TOF-MS.

No.	t_R (min)	Ion adduction	Compounds	Parent ion (*m/z*)	Error (ppm)	Fragmentation profile (*m/z*)	Molecular formula	Subclass	Occurrence
C1	3.93	[M-H]-	3-Caffeoylquinic acid	353.0858	1.3	191.0532,179.0330,135,0433	$C_{16}H_{18}O_9$	Phenolic acid	HBJ GJ CJ HJ
C2[a]	5.24	[M-H]-	5-Caffeoylquinic acid	353.0854	−5.4	191.0534,179.0328,135.0431	$C_{16}H_{18}O_9$	Phenolic acid	HBJ BJ GJ CJ HJ
C3	5.45	[M-H]-	4-Caffeoylquinic acid	353.0856	−4.8	191.0530,179.0326,173.0434,135.0425	$C_{16}H_{18}O_9$	Phenolic acid	HBJ BJ GJ CJ HJ
C4	6.03	[M-H]-	6,8-C, C-diglucosylapigenin	593.1514	1.3	473.1085	$C_{27}H_{30}O_{15}$	Flavone	HBJ GJ CJ
C5	6.96	[M-H]-	Tri-caffeoylquinic acid	677.1716	−0.3	515.1381,353.0859,191.0532, 179.0320	$C_{31}H_{34}O_{17}$	Phenolic acid	BJ CJ
C6	7.00	[M-H]-	Luteolin-7-O-rutinoside	593.1527	3.5	285.0392	$C_{27}H_{30}O_{15}$	Flavone	HBJ
C7[a]	7.04	[M-H]-	Quercetin-3-O-glucoside	463.0868	−1.9	301.0327	$C_{21}H_{20}O_{12}$	Flavonol	BJ GJ CJ
C8[a]	7.11	[M-H]-	Luteolin-7-O-β-d-glucoside	447.0922	−1.1	285.0383	$C_{21}H_{20}O_{11}$	Flavone	HBJ BJ GJ CJ HJ
C9	7.12	[M-H]-	Luteolin-7-O-glucuronide	461.0716	−0.9	285.0393	$C_{21}H_{18}O_{12}$	Flavone	HBJ BJ GJ CJ HJ
C10	7.37	[M-H]-	3,4-Dicaffeoylquinic acid	515.1175	−2.9	353.0851,335.0756,191.0533,179.0322	$C_{25}H_{24}O_{12}$	Phenolic acid	HBJ GJ CJ HJ
C11[a]	7.48	[M-H]-	3,5-Dicaffeoylquinic acid	515.1180	−1.9	353.0835,191.0535,179.0320, 135.0424	$C_{25}H_{24}O_{12}$	Phenolic acid	HBJ BJ GJ CJ HJ
C12[a]	7.72	[M-H]-	Apigenin-7-O-glucoside	431.0968	−2.3	268.0355, 269.0419	$C_{21}H_{20}O_{10}$	Flavone	HBJ BJ GJ CJ HJ
C13	7.76	[M-H]-	Luteolin-7-O-6″malonylglucoside	533.0927	−0.8	489.1026, 285.0858	$C_{24}H_{22}O_{14}$	Flavone	HBJ BJ GJ CJ HJ
C14	7.78	[M-H]-	4,5-Dicaffeoylquinic acid	515.1186	−0.8	353.0855,191.0534,179.0325, 173.0431	$C_{25}H_{24}O_{12}$	Phenolic acid	HBJ BJ GJ CJ HJ
C15	8.01	$[M+H]^+$	Apigenin-7-O-6″-malonylgalactoside	519.1126	0.2	271.0548	$C_{24}H_{22}O_{13}$	Flavone	HBJ BJ GJ CJ HJ
C16	8.22	[M-H]-	Apigenin-7-O-acetylglucoside	473.1082	−0.4	269.0426	$C_{23}H_{23}O_{11}$	Flavone	HBJ BJ GJ HJ
No.	t_R (min)	Ion adduction	Compounds	Parent ion (*m/z*)	Error (ppm)	Fragmentation profile (*m/z*)	Molecular formula	Subclass	Occurrence
C17	8.33	[M-H]-	Apigenin-7-O-acetylglucoside isomer	473.1073	−2.3	269.0430, 270.0465	$C_{23}H_{22}O_{11}$	Flavone	HBJ BJ GJ CJ HJ
C18	8.34	$[M+H]^+$	Diosmetin-7-O-6″-malonylglucoside	549.1241	1.6	301.0649	$C_{25}H_{24}O_{14}$	Flavone	HBJ BJ GJ CJ HJ
C19	8.44	[M-H]-	Apigenin-7-O-acetylglucoside isomer	473.1083	−2.3	269.0429	$C_{23}H_{22}O_{11}$	Flavone	HBJ BJ HJ
C20	8.47	[M-H]-	Luteolin-7-O-6″-acetylglucuronide	489.1028	−1.6	285.0378,284.0302	$C_{23}H_{22}O_{12}$	Flavone	HBJ BJ GJ CJ HJ
C21[a]	8.73	[M-H]-	Eriodictyol	287.0538	−6.3	151.0013,135.0428	$C_{15}H_{12}O_6$	Flavanone	BJ
C22	8.74	$[M+H]^+$	Acacetin-7-O-glucuronidemethyl ester	475.1040	0.4	285.0749,268.0590	$C_{23}H_{22}O_{11}$	Flavone	HJ
C23[a]	8.84	[M-H]-	Luteolin	285.0378	−7.4		$C_{15}H_{10}O_6$	Flavone	HBJ BJ GJ CJ HJ
C24	9.01	[M-H]-	Tri-caffeoylquinic acid isomer	677.1512	0.9	515.1188,353.0860,191.0454,179.0334,	$C_{34}H_{30}O_{15}$	Phenolic acid	GJ
C25	9.10	[M-H]-	Apigenin-7-O-acetylglucoside isomer	473.1077	−1.5	268.0353, 269.0415	$C_{23}H_{22}O_{11}$	Flavone	HBJ BJ HJ
C26[a]	9.12	$[M+H]^+$	Acacetin-7-O-glucoside	447.1270	−4.7	285.0739	$C_{23}H_{22}O_{10}$	Flavone	HBJ BJ HJ
C27	9.34	[M-H]-	Unknow	491.1188	−0.4	283.0589, 268.0353	$C_{23}H_{24}O_{12}$	–	HBJ BJ CJ HJ
C28[a]	9.60	[M-H]-	Apigenin	269.0432	−5.6		$C_{15}H_{10}O_5$	Flavone	HBJ BJ
C29	9.65	$[M+H]^+$	Acacetin-7-O-6″-malonylglucoside	533.1290	−0.9	285.0742	$C_{25}H_{24}O_{13}$	Flavone	HBJ BJ
C30[a]	9.88	[M-H]-	Gengkwanin	283.0592	−4.9	268.0356	$C_{16}H_{12}O_5$	Flavonol	HBJ BJ CJ HJ
C31	10.11	$[M+H]^+$	Acacetin-7-O-6″-acetylglucuronide	489.1383	−2.9	285.074	$C_{24}H_{24}O_{11}$	Flavone	HBJ CJ
C32[a]	11.93	[M-H]-	Acacetin	283.0591	−5.3	268.0355	$C_{16}H_{12}O_5$	Flavone	BJ

t_R represents retention time; HBJ represents '*Hangbaiju*'; BJ represents '*Boju*'; GJ represents '*Gongju*'; CJ represents '*Chuju*'; HJ represents '*Huaiju*'.
[a] The chemical constituents were identified using the reference standards.

C25, and C28 were identified as apigenin derivates based on the base fragments at m/z 269 ($[M-H]^-$) or 271 ($[M+H]^+$). C2 and C28 were confirmed as apigenin-7-O-glucoside and apigenin by comparison with standards. Based on previous qualitative analysis of chrysanthemums and database, C16, C17, C19, and C25 were deduced as apigenin-7-O-6″-acetyl-glucoside isomers with the same molecular ion ($[M-H]^-$) at m/z 473, C4 was tentatively identified as 6,8-C, C-diglucosylapigenin (Li, et al., 2019). C15 was identified as apigenin-7-O-6″-malonylgalactoside due to molecular formula ($[M+H]^+$) at m/z 519 and a base peak ion at m/z 271 in MS^2 spectrum (Peng, et al., 2019).

Six luteolin derivates with the same characteristic fragment of at m/z 285 were identified. C8 and C23 were confirmed as luteolin-7-O-β-d-glucoside and luteolin by comparing with the authentic standards.C6 and C9 were tentatively identified as luteolin-7-O-rutinoside and luteolin-7-O-glucuronide; C32 and C44 were deduced as luteolin-7-O-6″-acetylglucuronide and luteolin 7-O-6″-malonylglucoside based on previous report (Li, et al., 2019).

C26, C30, and C32 were identified as acacetin-7-O-glucoside,

gengkwanin, and acacetin by comparing with the authentic standards. And according to the published literature, C22, C29, and C31 were deduced as acacetin-7-O-glucuronide methyl ester, acacetin-7-O-6″-malonylglucoside, and acacetin-7-O-6″-acetylglucuronide, respectively (Chen, et al., 2021; Peng, et al., 2019). C18 was confirmed as diosmetin-7-O-6″-malonylglucoside due to molecular formula ($[M + H]^+$) at m/z 549 and a base peak ion at m/z 301 in MS^2 spectrum (Chen, et al., 2021).

3.4. Screening of potential anti-gout active compounds by spectrum-effect relationship analysis

As mentioned previously, chrysanthemum extracts had different effects on gouty inflammation, various phenolic compounds might contribute to their differences. However, the main compounds of chrysanthemums responsible for the anti-gout activity remain unclear. Spectrum-effect relationship pertains to establishing a correlation between the fingerprint of natural products and pharmacodynamic data, which is useful for integrating information on the bioactive compounds and efficacies of natural products (Du, Zhu, Ge, & Li, 2021; Zhang, Chen, Yang, & Shi, 2018). To evaluate the correlation, spectrum-effect relationship analysis was performed by gray relational analysis (GRA) and orthogonal partial least-squares regression analysis (OPLSR).

3.4.1. Gray relational analysis

GRA is applied to analyze the association degree between multiple variables. As shown in Table 2, correlation values between all compounds and the inhibition rate of IL-1β were greater than 0.6. A higher correlation value indicates a strong relationship between the compound and activity. In general, there was regarded as a strong correlation between compound and pharmacological effect when the correlation value is greater than 0.8 (Chen, Gou, Shi, Xue, & Feng, 2019). As shown in Table 2, 14 compounds (C15, C26, C18, C12, C16, C30, C19, C13, C29, C22, C11, C25, C2, C8) were highly related to anti-gout activity.

3.4.2. OPLSR analysis

GRA is skilled in processing small data with insufficient information, but difficult to describe the overall contributions of the combinatorial components through pharmacodynamic indicators (Zhang, Zheng, Ni, Li, & Li, 2018). Considering the limitation of GRA, OPLSR was used in the following experiment. In OPLSR, the spectrum-effect relationship model of chrysanthemum samples on the anti-gout effects was established. Generally, the independent variable fitting index (R^2X), the dependent variable fitting index (R^2Y), and the model prediction index (Q^2) values greater than 0.5 are required. For the inhibition of IL-1β rate, R^2X, R^2Y_{cum}, and Q^2_{cum} were 0.836, 0.97 and 0.918, respectively.

The results showed the regression coefficients (b) and Variable Importance for Projection (VIP) values of anti-gout effects obtained with OPLSR (Fig. 2). Compounds with VIP > 1 and b > 0 were considered as positive correlation with pharmacodynamic activity, which implied that the activity of sample could be effective if the amount of these compounds increased. These components were highlighted in orange in Fig. 2. Conversely, compounds with VIP > 1 and b < 0 were deemed to have a negative correlation with bioactivity. As demonstrated in Fig. 2A and B, it was found that 10 phenolic compounds (C12, C15, C16, C18, C21, C22, C26, C28, C30, and C32) had a positive impact on the inhibition rates of IL-1β, while 6 phenolic compounds (C1, C3, C4, C7, C10, C24) had a negative impact. It should be noted that the components with negative correlations to the biological effects mainly belonged to phenolic acids (C1, 3-caffeoylquinic acid; C3, 4-caffeoylquinic acid; C10, 3,4-dicaffeoylquinic acid; C24, tri-caffeoylquinic acid isomer). Previous studies demonstrated the efficacy of caffeoylquinic acids in reducing gout. For instance, Meng et al. (2014) conducted a study indicating that oral administration of chlorogenic acid at a dose of 160 mg/kg can alleviate MSU-induced arthritis in rats; Jiang et al. (2017) utilized the extract of *Gnaphalium pensylvanicum* wild., which was rich in caffeoylquinic acids, to ameliorate hyperuricemia and acute gouty arthritis in an animal model. However, caffeoylquinic acids are poorly bioavailable and they are extensively metabolized *in vivo* (Clifford, Jaganath, Ludwig, & Crozier, 2017). Hence, the metabolites of caffeoylquinic acids may respond to anti-gout effects *in vivo*, instead of the parent compound. Consequently, assessing the bioavailability of polyphenols is crucial to elucidate their pharmacological effects *in vitro*.

Combining the results from GRA and OPLSR, C12 (apigenin-7-O-glucoside), C15 (apigenin-7-O-6″-malonylgalactoside), C16 (apigenin-7-O-6″-acetylglucoside), C18 (diosmetin-7-O-6″-malonylglucoside), C22 (luteolin), C26 (acacetin-7-O-glucoside) and C30 (gengkwanin) were anti-gout activity related phenolics in chrysanthemums. The predicted compounds (C12, C22, C26, C30) were used for the following ADME evaluation, which were compared with the authentic standards. The structure of other compounds (C15, C16, and C18) could not be confirmed due to the lack of accurate information.

Table 2
Correlations and grades of the GRA of chrysanthemums on anti-gout.

Compounds	Correlations	Rank	Compounds	Correlations	Rank
C1	0.731	29	C17	0.842	11
C2	0.823	15	C18	0.882	4
C3	0.770	23	C19	0.852	8
C4	0.734	28	C20	0.724	30
C5	0.769	24	C21	0.792	19
C6	0.759	27	C22	0.837	12
C7	0.697	31	C23	0.788	21
C8	0.801	16	C24	0.696	32
C9	0.767	25	C25	0.830	14
C10	0.760	26	C26	0.885	3
C11	0.834	13	C27	0.902	1
C12	0.879	5	C28	0.794	17
C13	0.850	9	C29	0.842	10
C14	0.773	22	C30	0.858	7
C15	0.889	2	C31	0.792	18
C16	0.865	6	C32	0.791	20

3.5. ADME evaluation

ADME evaluation of effect-related compounds was conducted, which is useful for evaluating the bioavailability of active components (Liu, et al., 2021; Zhang, Tang, & Qiu, 2021). As shown in Table 3, luteolin (C22), and gengkwanin (C30) were supposed to have good oral bioavailability, which met high gastrointestinal absorption (GI absorption) or oral bioavailability (OB) ≥ 30 %. Apigenin-7-O-glucoside (C12) and acacetin-7-O-glucoside (C26) were expected to have low oral bioavailability, but high drug-likeness (DL) value (DL > 0.65). A compound with DL > 0.18 is considered as good drug-likeness (Liu, Wang, Zhou, Wang, & Yang, 2013). Hence, apigenin-7-O-glucoside and acacetin-7-O-glucoside were also selected as the candidate effective compounds for further analysis.

3.6. Verification of the anti-gout related compounds by in vitro assays

The above results illustrated that apigenin-7-O-glucoside (C12), luteolin (C22), acacetin-7-O-glucoside (C26), and gengkwanin (C30) might be anti-gout related compounds in chrysanthemums. To further confirm the reliability of the results, the production of IL-1β in MSU-stimulated THP-1 macrophages was used to verify the anti-gout activities of related compounds. As illustrated in Fig. 3, luteolin, gengkwanin, acacetin-7-O-glucoside and apigenin-7-O-glucoside (25 μM) had no cytotoxicity on THP-1 macrophages without MSU (Fig. 3A) or with MSU (Fig. 3B). Therefore, the concentration of 25 μM was used in the following experiment. The result revealed that predicted compounds (except for genkwanin) significantly decreased the IL-1β secretion level compared with the model group ($p < 0.05$), which confirmed the predicted results (Fig. 3C). According to previous reports, activation of the NLRP3 inflammasome plays a major role in the pathogenesis of gouty

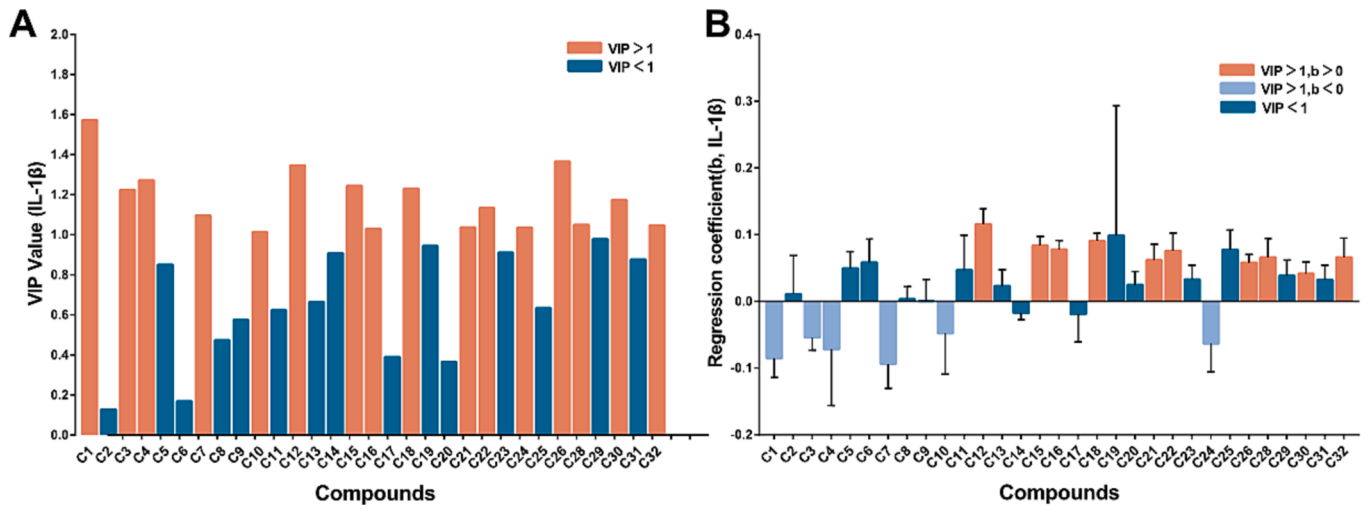

Fig. 2. The spectrum-effect relationship analysis by orthogonal partial least-squares regression analysis (OPLSR). Fig. 2A and B were VIP plot values and regression coefficients (b) of anti-gout effects (IL-1β), respectively.

Table 3
ADME properties of effect related compounds.

No.	Compounds	TCMSP			SwissADME	
		Molecule ID	OB (%)	DL	GI absorption	Lipinski's rule
C12	Apigenin-7-O-glucoside	MOL000007	9.68	0.74	Low	Yes
C22	Luteolin	MOL000006	36.16	0.25	High	Yes
C26	Acacetin-7-O-glucoside	MOL011878	19.66	0.79	Low	Yes
C30	Gengkwanin	MOL005573	37.13	0.24	High	Yes

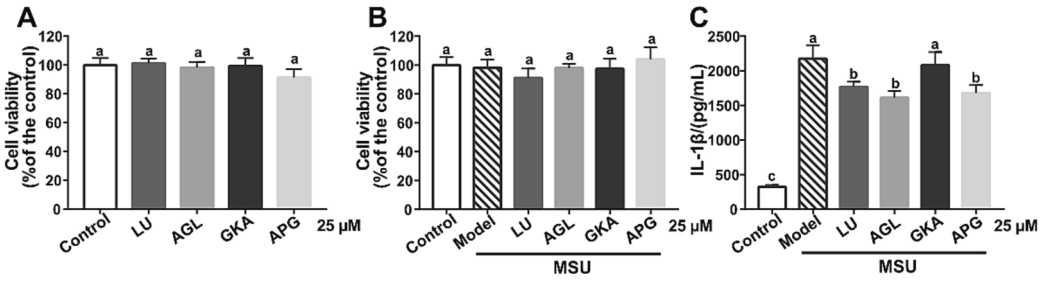

Fig. 3. Effects of anti-gout activity-related phenolics in chrysanthemum extracts on the production of IL-1β in MSU-stimulated THP-1 macrophages. Cytotoxic activity of phenolics against THP-1 cells without MSU (A) or with MSU (B). IL-1β (C) levels were evaluated in THP-1 macrophages pretreated with phenolics (25 μM). Luteolin, acacetin-7-O-glucoside, gengkwanin, and apigenin-7-O-glucoside are indicated by LU, AGL, GKA, and APG, respectively. The results represented the mean ± SD (n = 5 (Fig. 3A and B); n = 3 (Fig. 3C). Different letters (a-c) represented a significant difference at $p < 0$ 0.05 among values of different groups, all experiments were carried out in triplicate.

inflammation (Martinon, et al., 2006). Apigenin-7-O-glucoside had been reported to have a strong inhibitory effect on LPS-induced NF-κB/NLRP3/caspase-1 in RAW246.7 cells (Wang, et al., 2020). Also, it was reported that after acacetin-7-O-glucoside treatment, the levels of NLRP3 inflammasome and IL-1β were reduced in glucose-induced H9c2 cardiac cells (Yao, Li, Jin, Chen, Tian, & He, 2021). Luteolin inhibited the activation of NLRP3 inflammasome in high glucose-induced mouse podocyte cell-5 (MPC-5) (Yu, Zhang, Qian, Wen, & Wu, 2019). The results from the present study indicated that luteolin, gengkwanin, and apigenin-7-O-glucoside were essential constituents of chrysanthemums in managing gouty inflammation, possibly by inhibiting NLRP3 inflammasome pathway. Therefore, these three components in chrysanthemums could be considered as potential anti-gout components.

4. Conclusion

In this study, the effects of different chrysanthemum cultivars on gouty inflammation were examined. The results supported that BJ, HBJ, and HJ possessed dual regulatory effects on gouty inflammation. The UPLC-Q/TOF-MS-based spectrum-effect predictions and experiments confirmed that luteolin, acacetin-7-O-glucoside, and apigenin-7-O-glucoside were the major anti-gout compounds in chrysanthemums. These data suggested BJ, HBJ, and HJ might be excellent resources for chrysanthemum flower consumption due to their anti-gout benefits, supporting the development of chrysanthemums as functional foods.

CRediT authorship contribution statement

Yuting Huang: Conceptualization, Methodology, Software, Investigation, Writing – original draft. **Mingfang Tao:** Writing – review &

editing. **Rong Li:** Writing – review & editing. **Fuqiang Liang:** Writing – review & editing. **Tingting Xu:** Writing – review & editing. **Qiang Zhong:** Writing – review & editing. **Yanan Yuan:** Writing – review & editing. **Ting Wu:** Conceptualization, Supervision, Writing – review & editing. **Siyi Pan:** Project administration. **Xiaoyun Xu:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100897.

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