In vitro and *in vivo* inhibition of xanthine oxidase by the flowers of *Chrysanthemum morifolium* Ramat.

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Research

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Abstract

Background: Xanthine oxidase (XO) plays an important role in human's purine degradation. Excessive uric acid formation results in hyperuricemia and gout. The study aimed to determine the XO inhibitory potential of *Chrysanthemum morifolium* Ramat. dried flower and its effect on XO gene expression in animal models.

Methods: *In vitro* XO inhibitory assay was employed to investigate the XO inhibitory potential of *C. morifolium* flowers. The bioactive sub-fraction was investigated further to give additional insight on its uric acid lowering potential via animal study and XO gene expression analysis. HPLC-Q-TOF-MS/MS was utilized to identify the putative compounds in the sub-fraction.

Results: Among the fractions, EtOAc fraction exhibited the highest *in vitro* XO inhibitory potency ($51.77 \pm 0.98\%$; $IC_{50} = 10.64 \pm 0.51 \mu\text{g/mL}$) and it was further fractionated into 15 sub-fractions through open column chromatography. EtOAc F7, F8, F9, F10, and F11 possessed $>75\%$ XO inhibition. F9 and F10 exhibited high *in vitro* XO inhibitory activity, cellular pro-proliferative effect and intracellular antioxidant activity among the sub-fractions tested. These two sub-fractions were also non-cytotoxic at the concentration range of $0.1 - 10 \mu\text{g/mL}$. F10 was shown to be very effective in both serum and urine uric acid lowering properties in rats model upon oral consumption. It was subjected to further fractionation and a total of 11 sub-fractions were obtained. F10-4, F10-8, F10-9, and F10-10 possessed $>90\%$ XO inhibition. These sub-fractions were subjected to HPLC-Q-TOF-MS/MS analysis. A total of nine known compounds have been identified and 26 unknown compounds were detected.

Conclusions: The possible mechanisms contributed to the anti-hyperuricemic effect of F10 were suggested to be non-competitive inhibition of XO enzyme, XO gene expression down regulation, and enhancement of uric acid excretion. Structure elucidation of the unknown compounds and the evaluation of the XO inhibitory activity of a single or a mixture of these compounds are recommended to identify possible synergism between them.

Background

Gout is a common form of inflammatory arthritis that affects the joints and musculoskeletal system. It is induced by the deposition of monosodium urate crystals in the synovial fluid and other tissues in the body [1]. Hyperuricemia is a condition of overproduction or under-excretion of uric acid. This condition is caused by high dietary intake of foods that are rich in nucleic acids and is defined as serum uric acid level greater than 7.0 mg/mL [2]. Xanthine oxidase (XO) is the key enzyme that catalyzes the oxidation of hypoxanthine and xanthine into uric acid. During purine metabolism, XO produces reactive oxygen species (ROS) by transferring electrons to oxygen from hypoxanthine and xanthine [3]. Superoxide anion radicals generate by XO catalyzing reactions are involved in various pathological condition, such as inflammation, hepatitis, and carcinogenesis. Besides that, oxidative stress in uremia is the consequence of high ROS production and XO acts as one of the main factor of excessive ROS production [3].

Allopurinol is being used clinically as XO inhibitor for gout treatment by blocking the terminal step of uric acid biosynthesis. However, it results in many side effects like hepatitis, nephropathy, and allergic reactions. Therefore, it is necessary to search for new alternatives which confer higher therapeutic activity and lesser side effects. Natural products are more natural way of treatment and excellent sources of lead compounds in the search of new medication for clinical disorders with fewer side effects. Botanical extracts have been used to treat diseases and play a major role in primary health care in many developing countries.

Chrysanthemum morifolium, known as Hang Bai Ju in Chinese, is an edible flower that is commonly used in traditional Chinese medicine particularly for its liver balancing properties. Flower portion of *C. morifolium* has been used as an edible and medicinal material for hundreds of years as a sedative due to its cooling abilities [4]. It contains essential oils, sesquiterpenes, and phenolic compounds and has been reported to demonstrate various biological activities, such as anti-oxidative, anti-inflammatory, anti-mutagenicity, and anti-HIV [1]. Many *in vitro* studies have been performed to investigate the XO inhibitory mechanism of phenolic acids and bioactive flavonoids, such as chrysin, kaempferol and quercetin [5, 6, 7]. Numerous *in vivo* studies on the anti-hyperuricemic activity of other plant extracts have also been conducted [5, 8], but investigation on *C. morifolium* has not been well performed in a greater depth. Therefore, the present study is the first *in vitro* and *in vivo* investigation on the potential of *C. morifolium* flower sub-fractions in XO inhibition and the determination of its anti-hyperuricemic activity followed by XO mRNA gene expression study.

The main objectives of the present study were to compare the *in vitro* XO inhibitory activity, cytotoxicity and cellular antioxidant capacity of different sub-fractions of the hydromethanolic extract of *C. morifolium* Ramat. dried flower and its mode of XO inhibition. The effects of *C. morifolium* flower sub-fractions on rats' uric acid levels and XO gene expression were also determined in this study. The compounds present in the bioactive sub-fractions were identified using HPLC-Q-TOF-MS/MS.

Materials And Methods

Plant materials

Dried flowers of *C. morifolium* Ramat. originated from Hang Zhou, China was procured from a Chinese drug store (Lee Mun Onn Medicine Store) in Petaling Jaya (GPS coordinates: 3.0875° N, 101.6451° E), Selangor, Malaysia in May 2017. Authentication of the plant sample was carried out by a botanist, Dr. Mohd. Firdaus Ismail at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, and a voucher specimen (SK3139/17) was deposited at the unit's herbarium of that institute.

Chemicals and reagents

HPLC grade solvents such as methanol, petroleum ether, ethyl acetate, dimethyl sulfoxide (DMSO), hexane, chloroform, and acetonitrile as well as celite and culture Dulbecco's Modified Eagle Medium:

Nutrient Mixture F–12 (DMEM-F12) were purchased from ThermoFisher Scientific (Waltham, United States). Potassium phosphates monobasic, dibasic, xanthine, allopurinol, crystal violet, trypan blue staining solution, sodium dodecyl sulfate (SDS), 2'–7'-dichlorofluorescein diacetate (DCFH-DA), tert-butyl hydroperoxide (tBuOOH), 5-sulfosalicylic acid dihydrate (SSA), and 2-Vinylpyridine were purchased from Sigma Aldrich (St. Louis, United States). Enzyme of xanthine oxidase from cow's milk was purchased from Roche Diagnostics (Mannheim, Germany). Preparative layer plates (Silica gel 60 F254, 20 cm × 20 cm) and silica gel 60 (230–400 meshes ASTM; 0.040–0.063mm) was purchased from Merck ([New Jersey, United States](#)). Phosphate buffered saline was purchased from First Base Laboratories Sdn Bhd (Selangor, Malaysia). SYBR Green Master Mix was purchased from Bio-Rad Laboratories ([California, United States](#)) and ultrapure water from a Mili-Q® purification system was used in this research.

Plant extraction and solvent-solvent partition

Dried flowers of *C. morifolium* were powdered and approximately 500 g of the dried powder was sonicated in 80% methanol (2 L) for 2 hours at 25°C. The 80% hydromethanolic filtrate was evaporated using rotary evaporator to obtain a crude extract. The crude extract (58.86 g) was dissolved in 400 mL of methanol and then partitioned using equal volume of petroleum ether and ethyl acetate. Each fraction was then concentrated under reduced pressure to obtain petroleum ether (PE) fraction, ethyl acetate (EtOAc) fraction and residual (RS) fraction. All fractions were weighed and stored at –80°C until further use.

In vitro xanthine oxidase inhibitory activity

The XO inhibitory activity was determined based on a modified method [9]. The substrate and the enzyme solutions were prepared immediately before use. Test samples were dissolved in DMSO and diluted to different concentrations (12.5 - 400 µg/mL). The assay mixture consisted of 130 µL of 0.05 M potassium phosphate buffer (pH 7.5), 10 µL of sample, 10 µL of 0.1 unit/mL XO enzyme solution. After pre-incubation for 10 minutes at 25°C, the reaction was then initiated by the addition of 100 µL xanthine (0.15 mM) solution and incubated for 10 minutes at 37°C. The enzymatic conversion of xanthine to form uric acid was measured at absorbance of 295nm. All reactions were performed in triplicates ($n = 3$) in a 96-well UV microplate. Negative control containing the assay mixture without sample extract was also prepared. Allopurinol was used as a positive control in the assay mixture. The IC₅₀ for allopurinol and all the fractions were determined. The XO inhibitory activity was calculated as:

$$\text{XO Inhibition (\%)} = (\text{Control absorbance} - \text{Sample absorbance}) / \text{Control absorbance} \times 100\%$$

Xanthine oxidase enzyme kinetic

In order to determine the inhibitory mode of the active fractions and sub-fractions, Lineweaver-Burk plot analysis was performed. The kinetic study was carried out in the absence and presence of inhibitor with

varying concentrations (0.075–2.4 mM) of xanthine as substrate, by using the XO inhibitory activity assay. The mode of inhibition was determined from the slope of the Lineweaver-Burk plot for competitive, non-competitive and mixed-type inhibition.

Bioassay-guided fractionation using column chromatography

Approximately 2.50 g of the most bioactive fraction of *C. morifolium* was subjected to silica gel open column chromatography using silica gel 60 (230–400 meshes ASTM; 0.040–0.063mm; Merck) as stationary phase. Separation was accomplished by eluting various solvent mixtures using mobile phase of increasing polarity from 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 100:0 (v/v) of hexane: ethyl acetate. The residuals in the column were recovered by sequential elution of equal volume of methanol, then followed by equal volume of methanol: chloroform (1:1, v/v). The sub-fractions were collected and evaporated. They were then subjected to thin layer chromatography (TLC) (Silica gel 60 F254 20cm × 20cm; Merck) profiling by using hexane: ethyl acetate (1:1) as mobile phase. The sub-fractions with the same retention factor (*R_f*) were pooled together to afford 15 sub-fractions (F1–F15). All sub-fractions were subjected to XO inhibitory assay and the bioactive sub-fractions were fractionated further. Refer to Figure 1 in results for flow chart of bioassay-guided fractionation.

Cellular assays

Crystal violet assay

Crystal violet assay was performed based on a modified protocol [10]. HepG2 cell line was used in this assay. The cells were seeded in a 96-well plate and incubated for 24 hours at 37°C in CO₂ incubator. Medium was aspirated from wells, washed twice with 1× phosphate-buffered saline (PBS) solution and 100 µL/well of fresh medium containing sub-fraction (0.1–100 µg/mL) was added into the wells. Cells were incubated for 24 hours at 37°C in CO₂ incubator with the treatments condition. After 24 hours incubation, medium was aspirated and the cells were washed twice with 1× PBS solution. Next, 50 µL of 0.5% crystal violet staining solution was added into each well and incubated for 20 minutes in CO₂ incubator at 37°C. Crystal violet staining solution was removed and the plate was washed twice with milliQ water. Next, 1% of sodium dodecyl sulfate (SDS) solution was added into each well of plate and incubated for 20 minutes. The optical density of each well at 570nm (OD₅₇₀) was measured with 96-well UV microplate reader (Infinite M200Pro, Tecan Group Ltd, [Männedorf, Switzerland](#)). The percentage of viable cells were determined by comparing the average OD₅₇₀ values of extract treated cells with the OD₅₇₀ values of the non-treated cells. The means and the standard error of mean are calculated for at least three independent experiments.

Trypan blue exclusion assay

HepG2 cells were seeded and grown in a 6-well plate. Medium was washed and replaced until the cells confluency reached 70%. Cells were treated with different sub-fractions and incubated for 24 hours in CO₂ incubator at 37°C. Medium was aspirated and 0.5mL trypsin was added and incubated for 5 minutes. Next, cell suspension was collected into tube accordingly. Tubes containing HepG2 cells were re-suspended. Cells were then stained by combining 20 µL of cell sample with 20 µL of trypan blue staining solution to obtain 0.1% final concentration. Then, 20 µL of mixed cell sample was loaded into the Nexcelom Cellometer counting chamber and inserted into Auto T4 sample slot. Cells sample was analysed. Live cells as well as dead cells, total cells, percentage of viability, live cell concentration and total cell concentration were determined and calculated [11, 12].

Reactive Oxygen Species (ROS) assay

HepG2 cells were seeded and grown in a 24-well plate. Medium was washed and replaced until the cells confluency reached 70%. Medium was changed to DMEM-F12 without fetal bovine serum and incubated for 24 hours in CO₂ incubator at 37°C. Then, 10 µL of 2', 7'-Dichlorodihydrofluorescein diacetate (5 µM) reagent was added into wells and incubated in CO₂ incubator for 45 minutes. Medium was washed out using 1 mL of 1× PBS solution twice. Cells were treated with 500 µL of tert-butyl hydroperoxide (400 µM) as positive control and test samples at different concentrations (0.1–10 µg/mL). Absorbance was obtained immediately at excitation of 485 nm/± 20 and emission of 528nm/± 20 and repeated every 30 minutes for 2 hours [13].

In vivo anti-hyperuricemic effects of *C. morifolium* in potassium oxonate-induced rats

Male SD rats (7 weeks) were acclimatized to the laboratory condition on normal diet for at least one week prior to the experiment. Animals were provided with rodent diet and clean water *ad libitum*, except 1 hour prior to drug administration, whereby access to food was restricted. All the rats were randomly divided into five groups of six ($n = 6$) rats each. Experimental hyperuricemia was induced by injection of uricase inhibitor, potassium oxonate (PO). The rats were intraperitoneally (i.p.) injected with PBS containing 250 mg/kg PO (except normal control group) one hour before the administration of test samples to increase the serum uric acid (SUA) level on daily basis, for 7 days. After one hour, the rats were treated with 25 mg/kg and 50 mg/kg of *C. morifolium* EtOAc F10 via oral gavage. The extract was replaced by PBS (pH 7.4) and water in negative and normal control groups, respectively.

Allopurinol (10 mg/kg) was used as positive control in this study. Blood samples were collected by rats' tail vein bleeding on day 7. On day 6, each rat was kept separately for urine collection using single animal method [14]. On day 8, the rats were euthanized under CO₂ and blood samples were collected by cardiac puncture. Blood samples were transferred to sterile tubes and allowed to clot on ice for 30 minutes. The blood was centrifuged at 2,000g for 15 minutes at 4°C to obtain serum. The urine and serum samples

were stored at -80°C until assays. Livers were also dissected immediately on ice and homogenized at frequency of 20 kHz for 5 minutes. The homogenate was freeze in liquid nitrogen and stored at -80°C until XO activity and gene expression analysis. SUA and urine uric acid (UUA) levels were measured using Amplex® Red uric acid/uricase assay kit (ThermoFisher Scientific, United States) while liver XO activity was measured by using Amplex® Red xanthine/xanthine oxidase assay kit (ThermoFisher Scientific, United States).

qPCR analysis of XO gene expression

qPCR analysis was carried out using Bio-Rad CFX96Real-Time PCR, version 3.1 to determine the expression level of XO after drug administration. Three rats that had serum uric acid (SUA) levels close to the mean value in each group on day 7 were selected. Total RNA was extracted from their livers using NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Germany). Next, RNA quality and quantity were determined by performing agarose gel electrophoresis and Nanodrop spectrophotometry. One-step Real-Time SYBR Green qPCR was performed. The primers (forward primer: 5'-GCATGCCAGACCATACTGAA-3'; and the reverse primer: 5'-AAATCCAGTTGCGGACAAAC-3') for rat xanthine dehydrogenase (XDH; Gene ID: 497811, mRNA sequence: [NM_017154](#)) were purchased from IDT Singapore Pte Ltd, Singapore. The housekeeping gene, β -actin for rat (mRNA sequence: NM_031144) with primer sequences of 5'-ATTGTGATGGACTCCGGAGA-3' (forward) and 5'-CAGCTCATAGCTCTTCTCCA-3' (reverse) was purchased from MYTACG Bioscience Enterprise, Malaysia.

Putative identification of XO inhibitors using HPLC-Q-TOF-MS/MS

EtOAc sub-fractions were analysed by using Hypersil GOLD C_{18} column ($150 \times 4.6\text{mm}$, $5\mu\text{m}$, ThermoFisher Scientific, United States) at 25°C on Agilent 1260 Infinity HPLC with UV detector at 210 nm, 254 nm, and 280 nm wavelengths. The mobile phase consisted of water (A) and acetonitrile (B) with gradient elution (0–5 minutes, 98% A, 0.7 mL/min; 5–7 minutes, 85% A, 0.5 mL/min; 7–20 minutes, 25% A, 0.2 mL/min; 20–40 minutes, 15% A, 0.2 mL/min; 40–45 minutes, 10% A, 0.5 mL/min; 45–50 minutes, 0% A, 0.7 mL/min; 50–55 minutes, 98% A, 0.7 mL/min.). Test sample of 20 μL was injected in each run. Samples were prepared at 10 ppm in HPLC grade methanol, filtered through $0.45\mu\text{m}$ syringe filter (ThermoFisher Scientific, United States) and sent to Infra Advanced Laboratory, University of Malaya for the identification of compounds by using Agilent 6200 series TOF/6500 series Q-TOF (Santa Clara, United States) with ESI source (Santa Clara, United States). HPLC-Q-TOF-MS/MS was performed in positive mode under the following operating parameters: scanning mode, total ion current (TIC) and extracted ion chromatogram (EIC); scanning range, 10–1700 m/z ; fragmentor voltage, 175V; Gas flow, 14 L/minutes; and nebulizer, 35 psig. The mass spectrometry data were qualitatively analysed using MassHunter Workstation software (version B.05.01, Agilent Technologies) for possible markers and were compared to standard online database such as METLIN metabolite database, Human Metabolome Database (HMDB), PubChem and KEGG metabolite databases.

Statistical analysis

All data were analysed using Statistical Package for Social Sciences (SPSS) version 21.0 and expressed as mean $\pm$ standard error of mean (SEM). The data in the present study was subjected to One-way analysis of variance (ANOVA) and the significance of difference between the means was determined by post-hoc Duncan's multiple range tests at 95% least significant difference ($p \leq 0.05$).

Results

XO inhibitory activity of *C. morifolium* fractions

The yield of fractions and sub-fractions of the bioassay-guided fractionation was presented in Figure 1. All the fractions and sub-fractions were analysed for their XO inhibitory activity and the results were tabulated in Table 1. One-way ANOVA analysis demonstrated that EtOAc fraction exhibited the highest XO inhibitory activity of $51.77 \pm 0.98\%$ at 100 $\mu\text{g/mL}$ among the fractions. The activity was lower than the standard drug, allopurinol (positive control), which showed nearly 100% inhibition at the same concentration with low IC_{50} of $8.84 \pm 0.11 \mu\text{g/mL}$. The IC_{50} of the EtOAc fraction ($10.64 \pm 0.51 \mu\text{g/mL}$) was not significantly different from that of the positive control, which indicated its high potency in XO inhibition relative to the allopurinol.

Besides, the inhibitory mechanism of the *C. morifolium* crude extract and its fractions were studied by using double-reciprocal plot of enzyme kinetic. Lineweaver-Burk plots of XO activity in the presence and absence of inhibitor were shown in Figure 2. The results indicated that the mode of XO inhibition for the RS fraction was competitive, whereas the crude extract as well as the EtOAc and PE fractions were shown to be non-competitive. The most bioactive EtOAc fraction was fractionated through open column chromatography and 15 sub-fractions were obtained. The XO inhibitory activity of the 15 sub-fractions showed that EtOAc F7, F8, F9, F10 and F11 possessed the highest XO inhibitory activity ($>75\%$) at 100 $\mu\text{g/mL}$, whereas other sub-fractions exhibited low to moderate activity.

Cellular assays

Prior to the *in vivo* experiment, the non-cytotoxic concentration of F7 to F11 were determined by using crystal violet assay. Based on Figure 3, all five sub-fractions showed no toxicity to HepG2 cells up to 10 $\mu\text{g/mL}$. At concentration of 100 $\mu\text{g/mL}$, there was a decrement of cell viability (%) in all sub-fractions. Hence, the non-cytotoxic concentrations (0.1, 1 and 10 $\mu\text{g/mL}$) were selected for all the subsequent cell culture experiments. Since F9 and F10 showed pro-proliferative effect with viability two to three-fold higher than the untreated control, these sub-fractions were subjected to trypan blue exclusion assay for the confirmation of the cell viability results. Trypan blue exclusion assay results demonstrated that there was a gradual rise in cell viability as the concentration of both F9 and F10 were increased. The increase in percentage of viability becomes significant in F10 at 1 $\mu\text{g/mL}$ ($10.37 \pm 1.40\%$) and 10 $\mu\text{g/mL}$ ($11.02 \pm 1.85\%$). The increased of cell viability in F10 indicated that it is non-cytotoxic and has pro-proliferative

effects towards HepG2 cells. These data suggested that both F9 and F10 were indeed highly non-cytotoxic to human liver carcinoma (HepG2) cells. ROS assay was carried out in this study to investigate the antioxidant capacity of the EtOAc sub-fractions. Tert-Butyl hydroperoxide was used as positive control in this assay to increase the ROS level of HepG2 cells. Figure 4 showed the ROS activity of F9 and F10, respectively, at different concentrations. F10 reduced the intracellular ROS level significantly up to 60 minutes at 10 µg/mL, whereas F9 showed no significant reduction of ROS level up to 120 minutes in comparison to the normal control.

***In vivo* anti-hyperuricemic effects of *C. morifolium* in potassium oxonate-induced rats**

In vivo toxicity test was conducted to determine the non-toxic concentration and median lethal dose (LD₅₀) upon oral administration in rats based on Fixed Dose Procedure of OECD guideline 425. The non-toxic concentration range was found to be 0–50 mg/kg and the LD₅₀ was determined to be 133.33 mg/kg body weight upon oral administration in rats. Hence, daily dosage of 25 mg/kg and 50 mg/kg were suggested to be safe and were explored further for their anti-hyperuricemic activity. The results showed that the administration of potassium oxonate (250 mg/kg, i.p.) increased the SUA level of the negative control rats significantly. Oral administration of allopurinol (positive control) at 10 mg/kg resulted in significant reduction of SUA level (Table 2). The SUA level of the rats that received 25 mg/kg and 50 mg/kg of F10 were also lowered significantly as compared to the negative control. This indicated that both doses of F10 are effective in SUA level suppression. In addition to that, the SUA lowering efficacy of 50 mg/kg of F10 was not significantly different from that of the positive control throughout the study period. On the other hand, the UUA level of the rats was shown to be significantly reduced by the administration of both doses of F10. However, F10 was shown to be less effective than allopurinol, which reduced the UUA to the basal level (similar to the normal control level). To elucidate the underlying mechanisms of the anti-hyperuricemic effects of F10, its effect on XO enzyme was evaluated. The results demonstrated that the administration of F10 significantly reduced the XO level in rats (Figure 5). Besides, the administration of F10 also significantly reduced the xanthine and hypoxanthine levels relative to the negative control. However, the levels were shown to be higher than the normal control.

qPCR analysis of XO gene expression

XO gene expression in fold change after various treatments (Figure 6) were determined by obtaining the difference between the samples $\Delta\Delta C_T$ values and the ΔC_T value of housekeeping gene (β -actin). Hyperuricemic (negative) control showed three folds increase in XO gene expression as compared to the normal control group. F10 at 25 mg/kg and 50 mg/kg doses significantly down-regulated the XO gene expression relative to the negative control, with 50 mg/kg dose showed no significant difference in XO gene expression from the normal and positive control. Hence, this results suggested the ability of F10 in

down-regulating XO gene expression, with 50 mg/kg dose being more effective, comparable to the effect of allopurinol.

Putative identification of XO inhibitors using HPLC-Q-TOF-MS/MS

In view of the promising anti-hyperuricemic effect of F10, it could be inferred that the main XO inhibitory effect might be attributed to the antioxidant compounds present in the sub-fraction. Therefore, the F10 was further analysed to identify the bioactive compounds responsible for the XO inhibitory activity. In this part of our study, F10 was further purified by using preparative TLC to obtain 11 sub-fractions. It was found that the EtOAc F10–4, F10–8, F10–9 and F10–10 possessed high XO inhibitory activity of >90% inhibition, with IC_{50} lower than that of allopurinol ($8.84 \pm 0.11 \mu\text{g/mL}$). This indicated that the XO inhibitory potency of these sub-fractions is comparable to that of allopurinol. Hence, these four sub-fractions were subjected to HPLC-Q-TOF-MS/MS analysis for putative identification of XO inhibitor(s).

HPLC analysis of F10–4, F10–8, F10–9, and F10–10 revealed the presence of multiple compounds. The compounds were identified by using Q-TOF-MS/MS equipped with MassHunter Workstation software (version B.05.01, Agilent Technologies). Compounds identification were confirmed by matching the m/z of the precursor and fragment ions with standard databases, and were tabulated in Table 3. There were 35 major compounds detected with only nine known compounds identified. The known compounds identified were alkaloids (18, 32), terpenoids (7, 34), fatty acids (8), lignan (11) and flavonoids (17, 19). There were 26 unknown compounds detected and their molecular formulas were shown in this study. Positive Dragendorff test and the molecular formula of the unknown compounds suggested that most of them might be alkaloid compounds.

Discussion

XO inhibitory activity of *C. morifolium* fractions

Our study provides, for the first time, data showing that *C. morifolium* sub-fractions effective in XO inhibition *in vitro* and uric acid reduction *in vivo*. At the beginning, EtOAc fraction was shown to exhibit moderate (High: 71–100%; moderate: 41–70%; low: 0–40%) XO inhibition. This might due to its higher total flavonoid content as compared to the other fractions. *C. morifolium* EtOAc fraction has been shown to contain significantly higher total phenolic and flavonoid content than other fractions in our preliminary study [15]. This is because natural phenols possess solubility preference to solvents with intermediate polarity [16], EtOAc in this study, rather than more polar or non-polar solvent. Thus, majority of the flavonoid compounds of *C. morifolium* flower in this study might have been drawn to the EtOAc fraction during solvent-solvent partitioning, conferred to its higher bioactivity. The results are in agreement with Kennouche *et al.* (2016) in which the EtOAc extract of *Chrysanthemum segetum* showed the highest level of polyphenol and flavonoid contents [17]. Although the inhibitory effect of a complex mixture could not be directly compared to a pure compound, the moderate XO inhibitory activity of the EtOAc fraction suggested that it might serve as a potential XO inhibitor. In other words, the EtOAc fraction might exhibit

higher XO inhibition at higher concentration or purity. Therefore, it was subjected to bioassay-guided fractionation to obtain higher purity sub-fractions for further analysis.

Kinetic studies indicated that the mode of XO inhibition for the RS fraction was competitive, whereas the crude extract, EtOAc and PE fractions were shown to be non-competitive. Non-competitive inhibitor inactivating an enzyme by changes its conformation as well as its active site [18]. This indicated that the inhibitor in the crude, EtOAc and PE fractions does not compete for the XO active site with xanthine (substrate); but rather inhibiting the XO enzyme possibly by causing conformational change to the XO active site. According to Spanou (2012), pure polyphenolic compounds, such as quercetin and kaempferol derivatives possessed non-competitive XO inhibition [19]. In addition to that, previous studies have reported that flavonoids such as kaempferol and luteolin are fully reversible, and exhibited non-competitive inhibition on XO enzyme [20]. RS fraction in this study inhibited XO via competitive inhibition. This indicated that the inhibitor present in the RS fraction might resemble the structure of xanthine, thereby, competing for the same active site. Competitive inhibitor can only bind to free enzyme but not enzyme-substrate complex, thus inhibition can be overcome by increasing the concentration of substrate in the reaction [21]. According to Nguyen *et al.* (2006), most of the flavonoids, phenolic, caffeoylquinic acid derivatives and monoterpenoid glucoside compounds from *Chrysanthemum sinense* extract showed competitive inhibition on XO enzyme [22].

The increased bioactivity of F7 to F11 might be due to greater concentration of potent bioactive compounds in the sub-fractions. Fractionation is a crucial step to increase the purity of bioactive compounds and hence, the bioactivity. In some cases, mixture of compounds shows higher bioactivity than pure compound [23]. *In vitro* XO inhibitory activity of *C. morifolium* flower oil and its anti-hyperuricemic activity on male Wistar rats has been published [1], but there is limited study on the effect of a mixture of compounds in the semi-purified sub-fractions in lowering rats' uric acid level, which was investigated in this study. Before that, cytotoxic assays were carried out to determine the non-cytotoxic concentration of the sub-fractions. In this case, only the most bioactive EtOAc F7 to F11 were selected for further analysis.

Cellular assays

The present study showed that all five sub-fractions (F7–F11) were non-cytotoxic to the HepG2 cells up to 10 µg/mL but a decrement of cell viability was observed at 100 µg/mL in crystal violet assay. The net loss of cell viability at 100 µg/mL has indicated the cytotoxic effect of all five sub-fractions at this concentration towards HepG2 cells. Similar result was observed in *Chrysanthemum coronarium* extract, which possessed the inhibition of cell proliferation of six different cell lines at 100 µg/mL [24]. It was postulated that the pro-proliferative effects of F9 and F10 might be linked to the presence of antioxidant compounds, such as flavonoid, since it possessed diverse biological characteristics such as antioxidant, anti-inflammatory, anti-mutagenic, antiviral, anti-hepatotoxic, and anti-carcinogenic activities [25].

Trypan blue exclusion assay showed an increase in cell viability and pro-proliferative effect in F10 treated cells. Limited studies have been published previously revealing the influence of *C. morifolium* sub-fractions on viability of HepG2 cell lines. In literature, few plant species of the genus *Chrysanthemum* were reported to have cell proliferation in lower extract concentrations. Similar result was observed using *C. coronarium* extract which shows cell proliferative effects up to 50 µg/mL [25], while cytotoxic activity on HeLa and Velo cell lines was observed in *C. segetum* and *C. flosculosus* [26].

ROS assay demonstrated that F10 reduced the intracellular ROS level significantly. According to Zhang *et al.* (2000), high flavones content in *C. morifolium* extract possessed excellent antioxidant and oxygen radicals scavenging activity [27]. Correlation between radical scavenging activity and flavonoids compounds has been reported in *C. morifolium* [28]. Kim and Lee (2005) reported that polyphenolic compounds isolated from *C. morifolium* showed superoxide anion radical scavenging activity [29]. In addition, *C. morifolium* has been demonstrated to contain abundant phenolic and flavonoid compounds with significantly improved antioxidant activity [15]. Hence, it was suggested that the antioxidant effect of F10 might be largely attributed to the presence of flavonoid compounds.

***In vivo* anti-hyperuricemic effects of *C. morifolium* in potassium oxonate-induced rats**

Potassium oxonate served as uricase inhibitor, which caused hyperuricemia by inhibiting uric acid decomposition [1], thereby increased the uric acid level in the negative control. Our results showed that the oral administration of F10 significantly reduced the SUA level at both doses. However, the SUA level of rats treated with 25 mg/kg of the F10 was significantly higher than the normal and positive control. In contrast, the SUA level in 50 mg/kg treatment and positive control were not significantly different from the normal control (not induced to be hyperuricemic), indicated the effectiveness of the 50 mg/kg treatment ($55.58 \pm 4.81\%$ reduction) in maintaining the SUA level, comparable to the positive control, allopurinol ($50.27 \pm 5.05\%$ reduction). This is in agreement with previous reports which demonstrated the effectiveness of *Chrysanthemum* flower oil in reducing SUA levels [1]. Thus, it is noteworthy that F10 at 50 mg/kg exhibited excellent anti-hyperuricemic effects, comparable to that of allopurinol.

The oral administration of F10 lowered the rats UUA level at both doses, but not as effective as the positive control. The reduced, but higher than normal UUA level in the F10 treated rats might be due to enhanced excretion of uric acid. Honda *et al.* (2014) suggested that uric acid-lowering effect of *C. morifolium* plant is a consequence of both suppressed production and enhanced excretion of uric acid, in which the uric acid transporter genes in the kidney is reported to be up-regulated in rats treated with *Chrysanthemum* flower oil [1]. The goal of gout therapy is long-term management of hyperuricemia, which can be achieved by SUA suppression and enhanced uric acid excretion. Therefore, this study suggested that oral consumption of 50 mg/kg of F10 may be suitable to be used as a long-term serum-uric acid lowering therapy for the management of hyperuricemia and as a preventive measure for gout. It could be explore further as a potential source of natural anti-hyperuricemic and uricosuric agent.

XO is an enzyme that catalyses the oxidation of hypoxanthine and xanthine to produce uric acid in purine catabolism [1]. Since hyperuricemia is resulted from uric acid overproduction, XO inhibitors that can inhibit the production of uric acid have been considered for hyperuricemia treatment. In our study, administration of F10 significantly reduced the XO level in rats. This result suggested that one of the uric acid-lowering mechanisms associated with F10 administration involved the suppression of uric acid production via the inhibition of XO enzymes. Our results correspond to previous report depicted that *C. morifolium* exhibited significant XO inhibitory activity in a concentration-dependent manner [22]. Our results also suggested that XO inhibition may be contributed by the down regulation of XO gene expression. This agrees with the reports by Honda et al. (2014), which demonstrated that the administration of *Chrysanthemum* flower oil suppress the uric acid production by inhibition of XO in the liver [1].

According to Montalbini and Torre (1995), xanthine will be accumulated and elevated in concentration when XO enzyme is inhibited [30]. This is in agreement with our results, in which xanthine and hypoxanthine levels increased relative to the normal control after the administration of F10. Therefore, it was suggested that the suppression of uric acid production in rats was achieved by XO enzymes inhibition. However, both hypoxanthine and xanthine showed only slight increase compared to the negative control. This might be explained by the enhanced excretion or re-utilization of xanthine and hypoxanthine when XO action is inhibited. Puig *et al.* (1988) reported a direct relationship between the excretion of uric acid, xanthine and hypoxanthine [31]. To date, there is no report about the regulation of XO gene expression by plant sub-fraction. Hence, qPCR analysis of XO gene expression was conducted in this study to gain further insight into the mechanism of XO inhibition by *C. morifolium* EtOAc F10.

qPCR analysis of XO gene expression

The present study showed that both doses of F10 down-regulated the XO gene expression. This results further confirmed the ability of F10 in down-regulating XO gene expression; with 50 mg/kg dosage being more effective, comparable to that of allopurinol. Down-regulation of the XO gene expression in this study might be explained by the reduced ROS level in F10 treated rats. McNally *et al.* (2005) reported that lower level of ROS could directly down-regulate the expression of xanthine dehydrogenase (XDH) and XO [32]. They also indicated that lower hydrogen peroxide level reduces calcium ions release from endoplasmic reticulum, subsequently suppressing the conversion of XDH to XO. Hence, the XO inhibition ability of F10 is most probably attributed to the direct effect of its antioxidant activity.

In another study, *Chrysanthemum* flower oil has been shown to suppress uric acid production by inhibiting XO enzyme in the liver and accelerating renal uric acid excretion [1]. Their findings demonstrated that the increased excretion of uric acid is modulated through the up-regulation of uric acid transporter genes in the kidney. However, they did not report further on the regulation of liver XO gene expression. Pfeiffer *et al.* (1994) reported that inflammatory mediators like cytokines induced XDH/XO gene expression as well as its activity in renal epithelial cells [33]. This is also correspond to our study in

which the expression of XO was probably down-regulated due to the absence or lower concentration of inflammatory cytokines. This is because ROS act as both a signalling molecule and a mediator of inflammation, in which the increase in ROS induces inflammatory responses [34]. In the present study, the down regulation of the XO might be related to the reduced oxidative stress in the treated rats due to the high antioxidant activity of the F10.

Putative identification of XO inhibitors using HPLC-Q-TOF-MS/MS

Based on HPLC-Q-TOF-MS/MS analysis, only apigenin 7-O-glucoside (*17*) has been reported previously in *C. morifolium*, while other known compounds were reported for the first time from *C. morifolium*. Flavonoid compounds are well studied for their anti-inflammatory, antibacterial, antifungal, and anti-tumour properties. Compounds such as luteolin and apigenin isolated from *Chrysanthemum* spp. have been shown to possess good XO inhibitory activity [35]. In this study, apigenin 7-O-glucoside (*17*) and prunetin 4'-O-galactoside (*19*) were identified. These two compounds were speculated to be responsible for the high XO inhibitory activity of F10–8 in this study. Apigenin 7-O-glucoside is a stable and non-toxic natural flavonoid, with a better solubility compared to other flavonoid compounds [36]. It has been shown to have multiple significant biological activities such as anti-inflammatory, anticancer, antiviral, antibacterial, antioxidant, and pro-apoptotic effects [37, 38]. Apigenin has been shown to be the most potent inhibitor for XO, attributed to its stable interaction with the enzyme active site in a molecular modelling study [39]. Compound *19* was identified for the first time from *C. morifolium* in the present study. It has been isolated from the leaves and stems bark of *Dalbergia spinose* and has been related to anti-inflammatory activity [40]. Since current treatment of gout include the use of anti-inflammatory agent, compound *17* and *19* could be investigate further as a lead compound for gout therapy.

Majority of the compounds in the highly bioactive F10 might be alkaloids, but only two known compounds being identified in this study, namely acetylintermedine (*18*) and prosopinine (*32*).. Compound *18* is a naturally occurring pyrrolizidine alkaloid reported to be hepatotoxic. According to FDA Poisonous Plant Database, pyrrolizidine alkaloids presents in *C. morifolium* possess significant hepatotoxicity, genotoxicity, cytotoxicity, tumorigenicity and neurotoxicity on human and animals [41]. Compound *32* is a piperidine alkaloid which contain a [hydroxypiperidine](#) with a hydroxy group at position C–3, a hydroxymethyl group at C–2, and an 8-hydroxydecyl group at C–6 (PubChem CID: 42608371). Although alkaloid compounds are often toxic to human beings, they are widely used in modern medicines. For instance, colchicine is used as anti-inflammatory agent for acute gout attack treatment and prevention [42] and is currently used as a clinically effective anti-gout medicine. However, its risk to benefit ratio is considered high as gastrointestinal effects such as nausea, diarrhoea, vomiting, and stomach upset would occur in about 80% of patients [43]. In the light of that, it is necessary to search for new alternatives which confer higher therapeutic activity and lesser side effects. Since abundant of alkaloids are present in *Chrysanthemum* flowers [44] and alkaloid has been shown to exhibit XO inhibitory activity [45], it is highly recommended to isolate and elucidate the structure of the unknown compounds in the bioactive sub-fractions by other spectroscopic method including ^1H and ^{13}C NMR.

Conclusion

In a nutshell, the dried flower extract of *Chrysanthemum morifolium* was shown to be effective in XO inhibition and anti-hyperuricemic activity. Crude EtOAc fraction demonstrated moderate, non-competitive XO inhibition. Its sub-fractions, F9 and F10 exhibited high *in vitro* XO inhibitory activity, cellular proliferative effect and intracellular antioxidant activity among the sub-fractions tested. F10 was shown to be very effective in both serum and urine uric acid lowering properties in rats model upon oral consumption, comparable to that of allopurinol. Based on the results, the possible mechanisms contributed to the anti-hyperuricemic effect of F10 were suggested to be non-competitive inhibition of XO enzyme, XO gene expression down regulation, and enhancement of uric acid excretion. A total of nine known compounds have been identified as 4'-hydroxybupivacaine (1), vulgarone B (7), 4-oxo-9Z, 11Z, 13E, 15E-octadecatetraenoic acid (8), magnoshinin (11), apigenin 7-O-glucoside (17), acetylintermedine (18), prunetin 4'-O-galactoside (19), prosopinine (32), and schizonepetoside E (34). There were 26 unknown compounds detected in this study. Both known and unknown compounds found in this study could potentially serve as lead compound for the development of XO inhibitor which confers higher therapeutic activity and lesser side effects. Hence, it is highly recommended to isolate and elucidate their structure by other spectroscopic methods including NMR spectroscopy. In addition to that, the evaluation of the XO inhibitory activity of a single or a mixture of these compounds is also suggested to identify possible synergism between them.

Abbreviations

MSU: monosodium urate crystals; PE: petroleum ether; EtOAc: ethyl acetate; RS: residual; TLC: thin layer chromatography; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle Medium; SDS: sodium dodecyl sulfate; SSA: 5-sulfosalicylic acid dehydrate; DCFH-DA: 2'-7'-dichlorofluorescein diacetate; ROS: reactive oxygen species; tBuOOH: tert-butyl hydroperoxide; PO: potassium oxonate; PBS: potassium-buffered saline; XO: xanthine oxidase; SD: Sprague Dawley; SUA: serum uric acid; UUA: urine uric acid; ESI: electrospray ionization; TIC: total ion current; EIC: extracted ion chromatogram; HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes Database; ANOVA: analysis of variance.

Declarations

Ethics approval and consent to participate

All the animal experimental protocols were approved by Tunku Abdul Rahman University College Ethics Committee (approval number: TARUC/EC/2017/04-2). All efforts were made to minimize suffering and distress of rats.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NTL designed the experiments, conducted the analysis, analyzed the data and prepared the manuscript. LKE and NTL revised the manuscript. SATSW, THY and WSP reviewed the manuscript. All authors read and approved the final manuscript.

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References

1. Honda S, Kawamoto S, Tanaka H, Kishida H, Kitagawa M, Nakai Y, Abe K, Hirata D. Administered chrysanthemum flower oil attenuates hyperuricemia: mechanism of action as revealed by DNA microarray analysis. *Biosci. Biotechnol. Biochem.* 2014;78(4):655–61.
2. Loeb JN. The influence of temperature on the solubility of monosodium urate. *Arthritis Rheum.* 1972; 15:189–92.

3. Park JE, Yeom Z, Park KT, Han EH, Yu HJ, Kang HS, Lim YH. Hypouricemic effect of ethanol extract of *Aster glehni* leaves in potassium oxonate-induced hyperuricemic rats. *Clin. Nutr. Res.* 2018;7(2):126–35.
4. Chinese Pharmacopoeia Committee. *Pharmacopoeia of the People's Republic of China*, Volume 1, Chinese Medical Science and Technology Press, Beijing. 2010;292.
5. Lin L, Yang Q, Zhao K, Zhao M. Identification of the free phenolic profile of Adlay bran by UPLC-QTOF-MS/MS and inhibitory mechanisms of phenolic acids against xanthine oxidase. *Food Chem.* 2018;253:108–18.
6. Wang Y, Zhang G, Pan J, Gong D. Novel insights into the inhibitory mechanism of kaempferol on xanthine oxidase. *J. Agr. Food Chem.* 2015;63(2):526–34.
7. Zhang C, Wang R, Zhang G, Gong D. Mechanistic insights into the inhibition of quercetin on xanthine oxidase. *Int. J. Biol. Macromol.* 2018;112:405–12.
8. Hirano M, Takeda S, Hitoie S, Shimoda H. Luteolin-rich chrysanthemum flower extract suppresses baseline serum uric acid in Japanese subjects with mild hyperuricemia. *Integr. Mol. Med.* 2017;4(2):1–5.
9. Sweeney AP, Wyllie SG, Shalliker RA, Markham JL. Xanthine oxidase inhibitory activity of selected Australian native plants. *J. Ethnopharmacol.* 2001;75(2–3):273–7.
10. Feoktistova M, Geserick P, Leverkus M. Crystal violet assay for determining viability of cultured cells. *CSH Protocols.* 2016;4:343–6.
11. Chan LL, Kuksin D, Lavery DJ, Saldi S, Qiu J. Morphological observation and analysis using automated image cytometry for the comparison of trypan blue and fluorescence-based viability detection method. *Cytotechnology.* 2015;67(3):461–73.
12. Chan LL, Smith T, Kumph KA, Kuksin D, Kessel S, Dery O, Cribbes S, Lai N, Qiu J. A high-throughput AO/PI-based cell concentration and viability detection method using the Celigo image cytometry. *Cytotechnology.* 2016;68(5):2015–25.
13. Kim HS. Extracts of *Chrysanthemum zawadskii* attenuate oxidative damage to vascular endothelial cells caused by a highly reducing sugar. *Cytotechnology.* 2017;69(6):915–24.
14. Kurien BT, Everds NE, Scofield RH. Experiment animal urine collection: a review. *Lab. Anim.* 2004;38(4):333–61.
15. Chang KL, Tey MK, Chow WM, Loh KE, Intan SI. In-vitro antioxidant, xanthine oxidase inhibitory activity and antimicrobial properties of *Chrysanthemum morifolium*, *Proceedings of the 14th Symposium of Malaysian Society of Applied Biology: Bridging between Knowledge and Innovation.* 2016;161–6.
16. Galanakis CM, Goulas V, Tsakona S, Manganaris GA, Gekas V. A knowledge base for the recovery of natural phenols with different solvents. *Int. J. Food Prop.* 2013;16(2):382–96.
17. Kennouche S, Bicha S, Bentamene A, Creche J, Benayache F, Benayache S. In vitro antioxidant activity, phenolic and flavonoid contents of different polarity extracts from *Chrysanthemum segetum* L. growing in Algeria. *Int. J. Pharmacogn. Pytochem. Res.* 2016;8(9):1522–5.

18. Berg JM, Tymoczko JL, Gatto GJ Jr, Stryer L. Biochemistry. 6th editions. W. H. Freeman and Company, New York. 2007;232–4.
19. Spanou C, Veskoukis AS, Kerasioti T, Kontou M, Angelis A, Aligiannis N, Skaltsounis AL, Kouretas D. Flavonoid glycosides isolated from unique legume plant extracts as novel inhibitors of xanthine oxidase. PLoS One. 2012;7(3):e32214.
20. Akihiko N, Seki M, Kobayashi H. Inhibition of xanthine oxidase by flavonoids. Biosci, Biotechnol. Biochem. 1999;63:10:1787–90.
21. Mohan C, Long KD, Mutneja M. An introduction to inhibitors and their biological applications. First edition, Merck Millipore, EMD Millipore Corporation, Billerica MA, USA. 2013;3–41.
22. Nguyen MT, Awale S, Tezuka Y, Ueda J, Tran QI, Kadota S. Xanthine oxidase inhibitors from the flowers of *Chrysanthemum sinense*. Planta Med. 2006;72(1):46–51.
23. Nile SH, Keum YS, Nile AS, Kwon YD, Kim DH. Potential cow milk xanthine oxidase inhibitory and antioxidant activity of selected phenolic acid derivatives. J. Biochem. Mol. Toxicol. 2018;32(1):1–6.
24. Abu-rish Eman Y, Kasabri V, Hudaib MM, Mashalla SH, AlAlawi LH, Tawaha KA, Mohammad MK., Mohamed YS, Bustanji Yasser K. Evaluation of antiproliferative activity of some traditional anticancer herbal remedies from Jordan. Trop. J. Pharm. Res. 2016;15(3):469–74.
25. Xu H, Hu G, Dong J, Wei Q, Shao H, Lei M. Antioxidative activities and active compounds of extracts from catalpa plant leaves. Sci. World J. 2017;1–7.
26. Marongiu B, Piras A, Porcedda S, Tuveri E, Laconi S, Deidda D, Maxia A. Chemical and biological comparisons on supercritical extracts of *Tanacetum cinerariifolium* (Trevir) Sch. Bip. with three related species of chrysanthemums of Sardinia (Italy). Nat. Prod. Res. 2009;23(2):190–9.
27. Zhang E, Fang L, Zhang J, Yu L, Xiao X. Study on anti-oxidative activity of flos chrysanthemum extract. Food Sci. 2000;21(7):6–9.
28. Sugawara T, Igarashi K. Cultivar variation in flavonoid components and radical scavenging activity of polyphenol fractions among edible chrysanthemum flowers. J. Jpn. Soc. Food Sci. 2009;56(11):600–4.
29. Kim HJ, Lee YS. Identification of new dicaffeoylquinic acids from *Chrysanthemum morifolium* and their antioxidant activities. Planta Med. 2005;71(9):871–6.
30. Montalbini P, Torre GD. Allopurinol metabolic conversion products and xanthine accumulation in allopurinol-treated plants. Plant Sci. 1995;111(2):187–98.
31. Puig JG, Mateos FA, Jimenez ML, Ramos TH. Renal excretion of hypoxanthine and xanthine in primary gout. Am. J. Med. 1988;85(4):533–7.
32. McNally JS, Saxena A, Cai H, Dikalov S, Harrison DG. Regulation of xanthine oxidoreductase protein expression by hydrogen peroxide and calcium. Arterioscler. Thromb. Vasc. Biol. 2005; 25(8):1623–8.
33. Pfeffer KD, Huecksteadt TP, Hoidal JR. Xanthine dehydrogenase and xanthine oxidase activity and gene expression in renal epithelial cells: cytokine and steroid regulation. J. Immunol. 1994;153(4):1789–97.

34. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxid. Redox Signal.* 2014;20(7):1126–67.
35. Abu Bakar FI, Abu Bakar MF, Rahmat A, Abdullah N, Sabran SF, Endrini S. Anti-gout potential of Malaysian medicinal plants. *Front. Pharmacol.* 2018;9:261.
36. Kowalski J, Samojedny A, Paul M, Pietsz G, Wilczok T. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin–1beta and tumor necrosis factor-alpha genes in J774.2 macrophages. *Pharmacol. Rep.* 2005;57(3):390–400.
37. Kanazawa K, Uehara M, Yanagitani H, Hashimoto T. Bioavailable flavonoids to suppress the formation of 8-OHdG in HepG2 cells. *Arch. Biochem. Biophys.* 2006;455(2):197–203.
38. Cai J, Zhao XL, Liu AW, Nian H, Zhang SH. Apigenin inhibits hepatoma cell growth through alteration of gene expression patterns. *Phytomedicine.* 2011;18(5):366–73.
39. Narayanan V, Nacarajan NS. Two isoflavone galactosides from *Dalbergia spinosa*. *Phytochem.* 1988;27(7):2364–5.
40. Sarg T, Ateya AM, Abdel-Ghani Afaf, Badr W, Shams G. Phytochemical and pharmacological studies of *Dalbergia sissoo* growing in Egypt. *Pharm. Biol.* 1999;37(1):54–62.
41. Zhao XL, Chan MY, Ogle CW. The identification of pyrrolizidine alkaloid-containing plants: A study on 20 herbs of the Compositae family. *Am. J. Chin. Med.* 1989;17(1–2):71–8.
42. Hainer BL, Matheson E, Wilkes RT. Diagnosis, treatment, and prevention of gout. *Am. Fam. Physician.* 2014;90(12):831–6.
43. Burns CM, Wortmann RL. Latest evidence on gout management: what the clinician needs to know. *Ther. Adv. Chronic Dis.* 2012;3(6):271–86.
44. Husain N, Kumar A. Comparative study of phytochemical constituents in flower of *Wedelia trilobata*, *Achyranthes aspera* and *Chrysanthemum* from Durg District of Chhattisgarh, India. *Int. J. Curr. Microbiol. App. Sci.* 2015;4(4):150–6.
45. Fachiriyah E, Ghifari MA, Anam K. Isolation, identification, and xanthine oxidase inhibition activity of alkaloid compound from *Peperomia pellucida*. *IOP Conf. Ser. Mater. Sci. Eng.* 2018;349.

Tables

Table 1 Xanthine oxidase inhibitory activity and IC₅₀ of different fractions of the *C. morifolium* flower extract.

Samples	Xanthine oxidase inhibition (%)	IC ₅₀ (µg/mL)
PE	28.09 ± 2.09 ^c	40.44 ± 0.63 ^b
RS	33.86 ± 3.25 ^c	35.75 ± 3.38 ^b
EtOAc	51.77 ± 0.98 ^b	10.64 ± 0.51 ^c
Crude	11.55 ± 1.21 ^d	127.93 ± 1.68 ^a
Allopurinol	99.79 ± 0.21 ^a	8.84 ± 0.11 ^c

Values are mean ± SEM ($n = 3$). Means with different superscript letter (vertical) showed significant difference ($p \leq 0.05$). Percentage of inhibition and IC₅₀ values were measured at 100µg/mL. PE, petroleum ether; RS = residual fraction; EtOAc, ethyl acetate.

Table 2 Uric acid content in SD rats treated with *Chrysanthemum morifolium* EtOAc F10.

Treatments	Serum uric acid content (%)	Urine uric acid content (%)
Positive control	108.30 ± 5.05 ^c	98.97 ± 5.17 ^c
Negative control	217.79 ± 10.28 ^a	249.67 ± 9.93 ^a
Normal control	100.00 ± 0.00 ^c	100.00 ± 0.00 ^c
25 mg/kg EtOAc F10	134.38 ± 7.91 ^b	157.13 ± 5.71 ^b
50 mg/kg EtOAc F10	96.74 ± 4.81 ^c	143.97 ± 5.80 ^b

All values are mean ± SEM ($n = 6$). The values of normal control have been normalized to 100%. Means with different superscript letter (vertical) showed significant difference ($p \leq 0.05$).

Table 3 Assignments of putative compounds for the major peaks identified in HPLC-Q-TOF-MS/MS spectra.

Peak	t _R (min)	Chemical formula	Mass	[M-H] ⁺ (m/z)	Fragments m/z	Identification
1	13.168	C ₁₈ H ₂₈ N ₂ O ₂	304.2135	327.2030	133.0860, 177.1124, 221.1387, 283.1751, 309.1909	4'-Hydroxybupivacaine
2	13.583	C ₁₇ H ₃₀ N ₄ O ₅	370.2218	388.2558	371.2285	-
3	14.532	C ₂₃ H ₄₂ N ₄ O ₈	502.3005	520.3344	503.3070	-
4	14.828	C ₂₅ H ₄₆ N ₄ O ₉	546.3268	564.3605	525.1942, 534.3121, 547.3342	-
5	15.895	C ₃₁ H ₅₅ N ₆ O ₂	543.4393	566.4286	209.1645, 435.3317, 566.4279	-
6	16.429	C ₃₉ H ₆₄ N ₇ O ₃	678.5074	679.5138	340.2605	-
7	20.947, 22.531, 22.814	C ₁₅ H ₂₂ O	218.1682	219.1759	201.1644, 219.1751	Vulgarone B
8	24.161	C ₁₈ H ₂₆ O ₃	290.1895	291.1971	273.1855, 291.1969	4-oxo-9Z, 11Z, 13E, 15E-octadecatetraenoic acid
9	25.262	C ₁₄ H ₃₁ N O ₂	245.2366	302.2131	246.2435	-
10	28.227	C ₁₉ H ₂₅ N O	283.1951	284.2077	171.1171, 284.2017	-
11	28.462, 29.470	C ₂₄ H ₃₀ O ₆	414.2055	415.2126	119.0856, 295.1177, 415.2120	Magnoshinin
12	13.168	C ₁₅ H ₂₆ N ₄ O ₄	326.1952	327.2027	133.0859, 327.2027	-
13	14.235	C ₂₁ H ₃₈ N ₄ O ₇	458.2738	476.3079	459.2802	-

14	14.472	C ₇ H ₈ O	108.0581	109.0655	109.0647	-
15	15.184	C ₂₃ H ₄₄ N ₃ O ₇	474.3185	453.3447	475.3255	-
16	15.836	C ₂₈ H ₅₃ N ₈ O ₄	565.4191	566.4892	588.4079	-
17	17.377	C ₂₁ H ₂₀ O ₁₀	432.1066	433.1138	271.0605, 433.1131	Apigenin 7-O-glucoside
18	19.630	C ₁₇ H ₂₇ N O ₆	341.1847	342.1919	342.1914	Acetylintermedine
19	20.638	C ₂₂ H ₂₂ O ₁₀	446.1223	447.1297	285.0759, 447.1289	Prunetin 4'-O-galactoside
20	23.306	C ₁₃ H ₂₁ O	193.1589	194.1661	194.1659	-
21	25.677	C ₉ H ₁₈ N ₂ S	186.1204	187.1276	187.1273	-
22	27.515	C ₂₂ H ₂₉ N O ₆	403.2004	387.1816	105.0703, 267.1228, 387.1805, 404.2069	-
23	29.412	C ₁₂ H ₃₁ N ₁₁ O ₅	409.25	415.2127	281.1383, 415.2114, 432.2381	-
24	30.776	C ₁₆ H ₃₅ N O ₂	273.2682	274.2754	274.2750	-
25	11.136	C ₅ H ₉ N O	99.0692	100.0765	101.0793	-
26	11.611	C ₈ H ₁₄ N ₃ O ₄	216.0988	217.1061	217.1057	-
27	13.152	C ₁₅ H ₂₉ N ₅ O ₄	343.2219	327.2028	327.2021, 344.2283	-
28	13.567	C ₁₀ H ₃₀ N ₁₀ O ₃ S	370.2218	388.2559	371.2280	-
29	13.923	C ₁₉ H ₃₄ N ₄ O ₆	414.2476	432.2818	415.2538	-
30	15.820		587.4037	566.4288	588.4103	-

		$C_{30}H_{51}N_8O_4$				
31	27.499	$C_{22}H_{29}NO_6$	403.2006	387.1817	105.0703, 267.1227, 387.1807, 404.2069	-
32	28.507	$C_{16}H_{33}NO_3$	287.2468	288.2538	288.2535	Prosopinine
33	29.337	$C_{18}H_{33}N_8OS$	409.2499	415.2130	119.0854, 281.1381, 415.2113, 432.2381	-
34	13.225	$C_{16}H_{28}O_8$	348.1778	349.1850	349.1847	Schizonepetoside E
35	16.486	$C_{27}H_{58}N_{20}O$	678.5088	701.4957	114.0919, 340.2610	-

[-], unknown compounds.

Additional Files

Additional file 1 – Thin Layer Chromatography profile of *Chrysanthemum morifolium* EtOAc F1 – F10.

The additional file 1 is a Microsoft Word document.

Additional file 2 – Extracted-ion Chromatogram of EtOAc (a) F10-4, (b) F10-8, (c) F10-9, and (d) F10-10.

The additional file 1 is a Microsoft Word document.

Figures

Figure 1

Flow chart of bioassay-guided fractionation and the yield of *C. morifolium* fractions and sub-fractions. PE, petroleum ether; EtOAc, ethyl acetate; RS, residual; PO, potassium oxonate; XO, xanthine oxidase.

Figure 2

The Lineweaver-Burk plots for (a) crude extract, (b) EtOAc, (c) PE and (d) RS fractions. ♦ is in the absence of inhibitor. ■ is in the presence of inhibitor. PE, petroleum ether; RS, residual fraction; EtOAc, ethyl acetate.

(a)

(b)

Figure 3

Cell viability (%) of HepG2 cells treated with various concentrations of *Chrysanthemum morifolium* EtOAc sub-fractions. (a) Crystal violet assay (b) Trypan blue exclusion assay. Values are mean $\pm$ SEM (n = 3). Significant differences were compared between different concentrations within each sub-fraction. Similar letter showed no significant difference ($p > 0.05$).

Figure 4

Reactive oxygen species level (%) of HepG2 cells treated with Chrysanthemum morifolium EtOAc (a) F9 (b) F10. Significant differences were compared between different concentrations at each time point. Similar letter showed no significant difference ($p > 0.05$).

Figure 5

Xanthine oxidase, xanthine and hypoxanthine content in SD rats treated with *Chrysanthemum morifolium* EtOAc F10. All values are mean $\pm$ SEM ($n = 6$). The values of normal control have been normalized to 100%. Bar labelled with different letter indicate significant difference ($p \leq 0.05$) between different treatments within xanthine oxidase, xanthine and hypoxanthine group.

Figure 6

Xanthine oxidase gene expression (fold change) in SD rats treated with *Chrysanthemum morifolium* EtOAc F10. All values are mean $\pm$ SEM (n = 6). The value of normal control has been normalized. Bar labelled with different letter indicate significant difference ($p \leq 0.05$).