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Inhibitory capacity of xanthine oxidase, and anticancer activity of compounds from *Sarcandra glabra* (Thunb.) Nakai flower

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Abstract

The flower of *Sarcandra glabra* (Thunb.) Nakai (FSN) has been considered an important supplementation material in natural flower-flavoured tea products in Vietnam. The present work has considerable significance for adding pharmacological value of FSN. Two compounds, namely emodin and methyl rosmarinate, were identified for the first time from a methanolic extract of FSN from Vietnam while analysing the inhibitory capacity of xanthine oxidase and cytotoxic activities. High levels of xanthine oxidase inhibiting capacity and cytotoxicity activity against HepG2 and A549 cancer cell lines were detected from emodin, with IC_{50} of 4.88 ± 0.42, 13.72 ± 0.48, and 18.33 ± 0.10 µg/mL, respectively. Emodin also activated the apoptotic factors of caspase-9, Bax, and PARP in HepG2, and caspase-3/9 and p53 in A549. Our results revealed for the first time the xanthine oxidase inhibitory effect of M70 with IC_{50} of 34.15 ± 1.33 µg/mL, which would shed light on its potential application for developing anti-hyperuricemia agents. The present work suggested that using flower-flavoured tea product of FSN could have many health benefits for gout patients.

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Introduction

Xanthine oxidase (XO) is an essential enzyme catalysing the oxidation of hypoxanthine to xanthine, and the metabolism of xanthine to uric acid (Dong *et al*., 2016; Liu *et al*., 2017). These two reactions occur in the final stage of purine metabolism in the body. Therefore, XO inhibitors reducing the biosynthesis of uric acid have been applied to prevent and treat gout. In the past two decades, some works indicated that the use of XO inhibitors from medicinal plants was one of effective therapeutic approaches for the treatment of gout (Kong *et al*., 2000; Umamaheswari *et al*., 2007; Vanyolos *et al*., 2014; Orban-Gyapai *et al*., 2015).

Many new anti hyperuricemic drugs have been synthesised and invented, recently. However, some uric acid-lowering drugs have toxic side effects (Mockenhaupt *et al*., 2008; Lin *et al*., 2005; Yaylacı *et al*., 2012). Medicinal plants, with their secondary metabolites called phytocompounds, are the primary source of drug agents, and being increasingly demonstrated as beneficial complementary treatments of gout (Nooreen *et al*., 2019). Previous works also reported that secondary metabolites from herbal products are potential candidates with safe, effective, and therapeutic properties, with low toxicity (Nooreen *et al*., 2019; Ye *et al*., 2020; Atalar *et al*., 2023). Therefore, development and clinical use of natural products for the treatment of gout could be beneficial.

Sarcandra glabra (Thunb.) Nakai is a perennial herb that belongs to family Chloranthaceae, and widely distributed throughout Southeast Asia (Xu *et al*., 2011). This herb, locally called "*soi rung*", has been long used in Vietnamese traditional medicine. Its flowers (FSN) are processed into natural flower-flavoured tea products. The FSN-marinated tea is locally known as "*tra hoa soi*", and widely available in the market. After soaking its leaves in hot water, it is also consumed as an aromatic and delicious tea (Yang, 1992; Han *et al*., 2013). This tea is not only served when receiving guests, but also brings great benefits to health, such as antioxidant, reducing joint pain and arthritis, and lowering uric acid. Former works revealed that this herb has potential

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pharmacological properties including antibacterial, antiviral, anti-inflammatory, anti-tumour, antioxidant, and anti-thrombocytopenic effects (Zeng *et al*., 2021).

Although FSN has been consumed as both a herbal drink and a natural flower-flavoured tea in supplemental diets of gout and hepatocellular carcinoma patients, until now, its effects on gout treatment, XO inhibitory capacity, HepG2 and A549 cell growth inhibition, and its chemical compounds have not been thoroughly investigated. Therefore, the present work was undertaken to study the chemical constituents, and evaluate the XO inhibitory capacity and anticancer effect of SR-1B1 and SR-1E1 from FSN extracts.

Materials and methods

Chemicals, reagents, and instruments

The main chemicals and reagents were xanthine oxidase, xanthine (> 99%), allopurinol, phosphate buffer, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), tris-HCl, NaCl, Tween 20, non-fat dry milk, natrium dihydrophosphate, dinatrium hydrophosphate, hydrochloric acid, natrium hydroxide, Dulbecco's Modified Eagle Medium (DMEM), Kaighn's modification of Ham's F12 medium (KMH F12), foetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin G-streptomycin, dimethyl sulfoxide (DMSO), and antibodies.

The main instruments were vacuum evaporator, ELISA reader (multiwell spectrophotometer), CO_2 incubator (humidified, set at 37°C, and 5% CO_2), Mini Protean 3 Cell (Bio-Rad), nitrocellulose filters (Scheicher and Schnell BioScience, Dassell, Germany), and LAS-3000 luminescent image analyser (Fuji Photo Film Co. Ltd., Kanagawa, Japan).

Plant material

The FSN was gathered from Cao Bang province, Vietnam in September 2021, and identified by Dr. Le Quang Ung and the Classification and Identification Committee of Faculty of Agronomy, Thai Nguyen University of Agriculture and Forestry. The committee was composed of nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. A voucher specimen (no. TUAF. CT2020-2021A) was thereafter deposited.

Extraction and isolation

Four grams of the FSN dry powder were extracted two times with 70% methanol (v/v) (M70) by ultrasonic, and then the supernatant was filtered to combine filtrates that were condensed in a vacuum evaporator at 45°C. The solvent-free extracts were dried to measure the yield of the dehydrated fractionation, then stabilised in DMSO for subsequent analyses.

Four kilograms of the FSN powder were extracted three times with methanol by ultrasonic (each 10 L, 60 min at 40°C), and then the filtrates were condensed in a vacuum evaporator to obtain a dark MeOH crude extract (487.0 g). The MeOH extract was suspended in water (2 L), and successively extracted with ethyl acetate to give ethyl acetate extract (EtOAc, 176.7 g), chromatographed on a silica gel column, and eluted with methylene chloride/methanol (MC:M) (9:1 - 0:1, v/v) to obtain six fractions SR-1 → SR-6. The SR-4 fraction (50.6 g) was chromatographed on a silica gel column, and eluted with methanol/water (M:W) (1:1.5 - 1:0, v/v) to obtain six fractions (SR-4A→SR-4F). The SR-4F fraction (5.0 g) was chromatographed on RP-C18 column, and eluted with methylene chloride/methanol (30:1 - 1:1, v/v) to obtain eight fractions (SR-1A–SR-1H). The SR-1B fraction (350.0 mg) and SR-1E fraction (405.0 mg) were chromatographed on a silica gel column to yield respective compounds SR-1B1 (50.0 mg, emodin) and SR-1E1 (200.0 mg, methyl rosmarinate). The procedure of SR-1B1 and SR-1E1 compound isolation is shown in Figure 1.

Xanthine oxidase inhibition assay

The XO inhibition effect of SR-1B and SR-1E1 compounds, and M70 was measured according to Chen *et al*. (2010). Serial concentrations of compounds and M70 solution were stabilised in 1% DMSO. The XO inhibitory activity was formed by mixing 40 µL of 1% DMSO (as a blank) or compound solution with 60 µL of XO enzyme solution (0.02 U/mL XO in 50 mM PBS pH 7.5 prepared before the reaction experiment). The absorbance of the reaction mixture was measured at 295 nm after 45 min at 37°C. Allopurinol served as positive control. The XO inhibition capacity was (%) = $(A_o - A_t)/A_o \times 100\%$, where A_o and A_t were the absorbance of the blank and compound at 295 nm, respectively. IC_{50} value was calculated as the concentration of the tested sample required to inhibit XO activity by 50%.

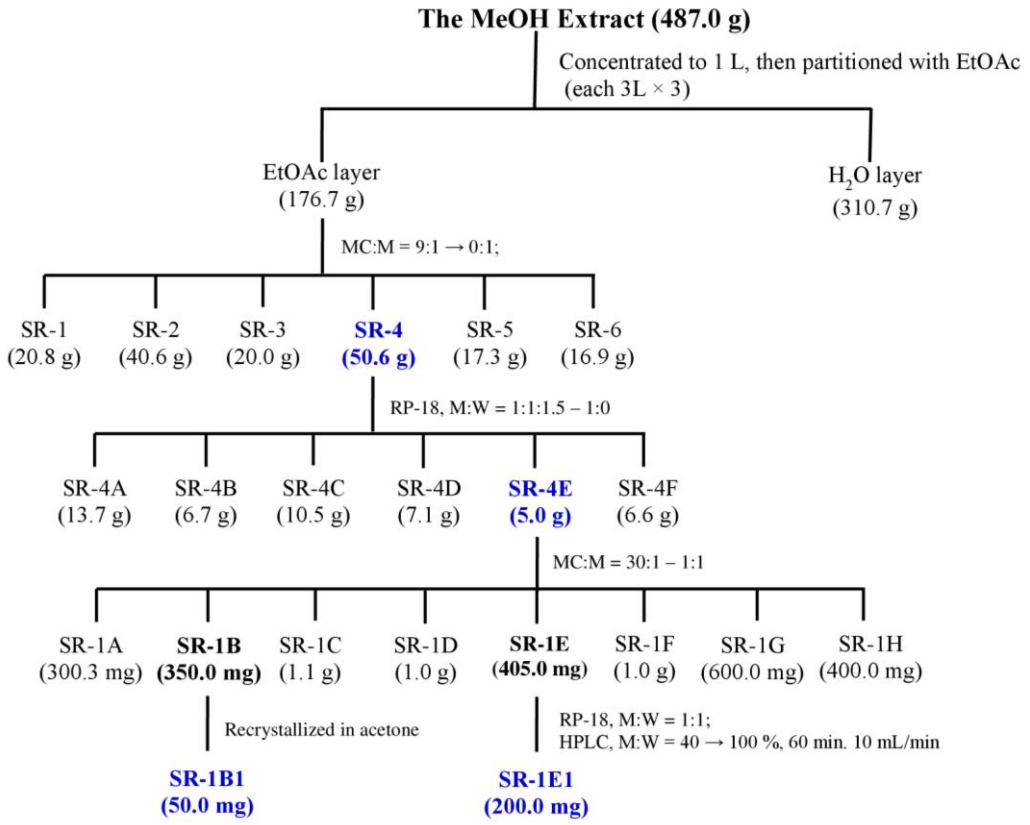

Figure 1. Schematic diagram of SR-1B1 and Sr-1E1 isolation from ethyl acetate fraction.

Cytotoxic effects of compounds SR-1B1 and SR-1E1

Cell culture

HepG2 and A549 cells were cultured in respective media of DMEM and KMH F12 added with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The media were newed every 72 h in a 5% CO_2 incubator.

Cell viability assay

$HepG_2$ or A549 cells were cultured onto 96-well plates at a density of 5×10^3 cells per well in 200 µL of medium, and repeated three times. After incubating for 24 h, cells were treated with the SR-1B1, SR-1E1, and M70 extracts at the specified concentrations in a serum-free medium for 48 h. Fluorouracil drug served as positive control. Cells treated with 0.2% DMSO served as negative control, followed by MTT assays, as described previously (Le *et al*., 2019).

Western blot analysis

The emodin with the highest anticancer capacity (Table 1) was selected to activate apoptosis in HepG2 and A549 cells. Whole cell extracts were gathered by using 1 × RIPA buffer added with complete protease inhibitor cocktail. Lysates were centrifuged to purify (12,000 *g*, 4°C, 20 min) and to give supernatants. Proteins were qualified by the Bradford assay, and then proteins were added with 2× sample buffer. The mixture was incubated at 95°C for 5 min, and run on 12% polyacrylamide gels by the Mini Protean 3 chamber. Nitrocellulose filters were used to give proteins. Blots were blocked for 2 h at room temperature in 5% non-fat milk/TBST, and then incubated at 4°C overnight with primary antibodies. After cleaning with blocking buffer three times for 30 min, membranes were probed with horseradish peroxidase-conjugated goat anti-mouse

Table 1. Xanthine oxidase enzyme inhibiting capacity of SR-1B1, SR-1E1, and M70.

Compound	IC_{50} (µg/mL)
Allopurinol*	3.42 ± 0.27^c
M70	34.15 ± 1.33^a
SR-1B1	4.88 ± 0.42^c
SR-1E1	7.31 ± 0.13^b

Values are mean ± SD of triplicates. Means followed by different lowercase superscripts are significantly different. *Positive control.

immunoglobulin G (IgG) and anti-rabbit lgG for 2 h. The membranes were cleaned three times for 1 h with a Tris-buffered saline Tween 20 solution. The bands of chemiluminescence on the polyvinylidene difluoride membrane were observed by a LAS-3000 luminescent image analyser. The antibodies were diluted for Western blots: beta-actin (1:3000); PARP and Bax (1:1000); caspase-3, caspase-9, and P53 (1:500) (Nho *et al*., 2011).

Statistical analysis

One-way ANOVA was used to analyse the data, and the level of Least Significance Difference (LSD) was calculated by Duncan's multiple range test at $p < 0.05$ for comparing means of the treatments. All analyses were processed by the SAS statistical package.

Results and discussion

SR-1B1 (compound 1) and SR-1E1 (compound 2) were firstly identified from FSN. Compound 1 was an orange-yellow amorphous powder. ^{1}H-NMR (600 MHz, acetone-d_6) and ^{13}C-NMR (150 MHz, acetone-d_6) spectra are displayed in Table 2. Compound 2 was a colourless powder. ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR (150 MHz, CD_3OD) spectra are shown in Table 3. Spectra of compounds 1 and 2 are shown in Figure 2.

In the ^{1}H NMR of compound 1, the signal of two pairs of protons meta-interacted with each other at δ_H 6.65 (1H, d, J = 2.0 Hz, H-2), 7.23 (1H, d, J = 2.0 Hz, H-4), 7.55 (1H, brs, H-5), and 7.12 (1H, brs, H-7); a methyl singlet group at δ_H 2.46 (3H, s, H-11); and two featured signals of hydroxy group at δ_H 12.06 (1H, s, 1-OH) and 12.17 (1H, s, 8-OH). The ^{13}C-NMR of compound 1 appeared to have signals of 15 carbons. There were 2 signals at δ_C 191.6 (C-9) and 182.2 (C-10), three quartic carbon signals in the aromatic ring at δ_C 149.5 (C-3), 166.2 (C-8), and 163.2 (C-1), and one methyl carbon signal was observed at δ_C 21.9 (C-11). NMR analysis revealed that compound 1 was a derivation of anthraquinon, and had high similarity with the spectrum of emodin.

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) data for SR-1E1 (acetone-d_6, δ (ppm), J (Hz)).

No.	Experimental (SR-1B1)		Literature (Emodin)	
	δ_H	δ_C	δ_H	δ_C
1		163.2		163.2
2	6.65 (1H, d, J = 2.0 Hz)	124.9	6.61 (1H, d, J = 2.4 Hz)	124.9
3		149.5		149.5
4	7.23 (1H, d, J = 2.0 Hz)	121.4	7.18 (1H, d, J =2.4Hz)	121.4
4a		134.2		134.1
5	7.55 (1H, brs)	109.7	7.48 (1H, brs)	109.7
6		166.6		166.2
7	7.12 (1H, brs)	108.8	7.07 (1H, brs)	108.5
8		166.2		166.4
8a		110.2		110.3
9		191.6		191.6
9a		114.4		114.4
10		182.2		182.1
10a		136.5		136.5
11	2.46 (3H, s)	21.9	2.43 (3H, s)	22.0
1-OH	12.06 (1H, s)		12.00 (1H, s)	
8-OH	12.17 (1H, s)		12.12 (1H, s)	

Table 3. ^{1}H (600 MHz) and ^{13}C (150 MHz) data for SR-1E1 (CD_3OD, δ (ppm), *J* (Hz)).

No.	Experimental (SR-1E1)		Literature (Methyl rosmarinate)	
	δ_H	δ_C	δ_H	δ_C
1		128.7		128.9
2	6.74 (1H, d, *J* = 1.8 Hz)	117.5	6.70 (1H, d, *J* = 2.0 Hz)	117.7
3		146.1		146.4
4		145.3		145.6
5	6.80 (1H, d, *J* = 7.8 Hz)	116.3	6.69 (1H, d, *J* =8.0 Hz)	116.5
6	6.59 (1H, d, *J* = 1.8, 7.8 Hz)	121.8	6.57 (1H, d, *J* = 2.0, 8.0 Hz)	121.9
7	3.03 (1H, dd, *J* = 7.8, 14.4 Hz) 3.07 (1H, dd, *J* = 5.4, 14.4 Hz)	37.8	3.00 (1H, dd, *J* = 5.5, 14.5 Hz) 3.06 (1H, d, *J* = 5.5, 14.3 Hz)	38.1
8	5.22 (1H, dd, *J* = 5.4, 7.8 Hz)	74.6	5.19 (1H, dd, *J* = 5.0, 7.5 Hz)	74.8
9		172.2		172.3
1’		127.6		127.7
2’	7.07 (1H, d, *J* = 2.4 Hz)	115.2	7.04 (1H, d, *J* = 2.0 Hz)	115.3
3’		146.7		147.0
4’		149.8		150.1
5’	6.72 (1H, d, *J* = 8.4 Hz)	116.5	6.78 (1H, d, *J* = 8.5 Hz)	116.7
6’	6.97 (1H, dd, *J* = 2.4, 8.4 Hz)	123.2	6.95 (1H, dd, *J* = 2.0, 8.5 Hz)	123.4
7’	7.56 (1H, d, *J* = 15.6 Hz)	147.9	7.55 (1H, d, *J* = 15.5Hz)	148.1
8’	6.28 (1H, d, *J* = 15.6 Hz)	114.1	6.26 (1H, d, *J* = 15.5 Hz)	114.2
9’		168.3		168.5
OCH_3	3.71 (3H, s)	52.7	3.70 (3H, s)	52.8

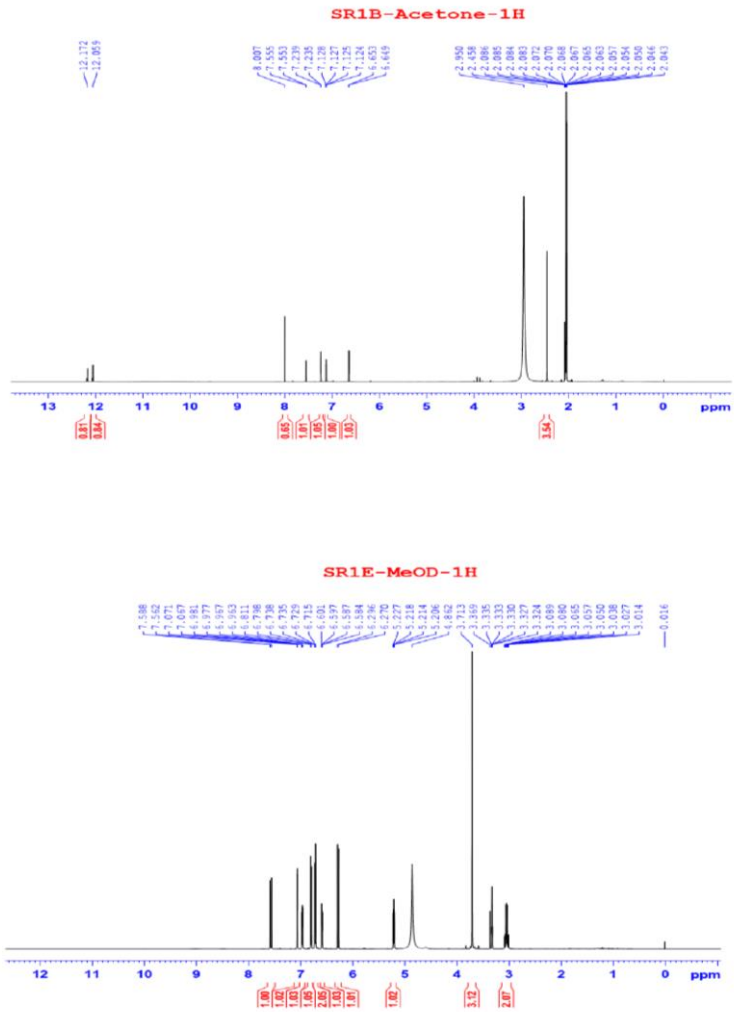

Figure 2. ^{1}H-NMR spectra of SR-1B1 (600 MHz, acetone-d_6) and SR-1E1 (600 MHz, CD_3OD).

The results of the comparison of spectral similarity of the two compounds are presented in Table 2. These results were referred to the literature values (Chao *et al*., 2010), and the structure of compound 1 was concluded to be emodin with the chemical structure displayed in Figure 3. Emodin is one of the main active components contained in various herbal plants including *Polygonum multiflorum* (Rao *et al*., 2009; Wang *et al*., 2012), *P. cuspidatum* (Lee *et al*., 2011), *Rheum palmatum* (Wang *et al*., 2011), *R. officinale* (Wei *et al*., 2011), and *Aloe vera* (Naqvi *et al*., 2010). This result could have considerable significance for adding the newly precious phytochemical compounds of FSN.

In the $_{1}$H NMR spectra of compound 2, there were six proton signals of the aromatic ring characterised for ABX-type at δ_H 6.74 (1H, d, J = 1.8 Hz, H-2), 6.80 (1H, d, J = 7.8 Hz, H-5), 6.59 (1H, dd, J = 1.8, 7.8 Hz, H-6), 7.07 (1H, d, J = 2.4 Hz, H-2'), 6.72 (1H, d, J = 8.4 Hz, H-5'), and 6.97 (1H, dd, J = 2.4, 8.4 Hz, H-6'); two olefinic proton of doublet junction with *trans* configuration at δ_H 7.56 (1H, d, J = 15.6 Hz, H-7') and 6.28 (1H, d, J = 15.6 Hz, H-8'); one oxymethine proton at δ_H 5.22 (1H, dd, J = 5.4, 7.8 Hz); two proton of methylene group at δ_H 3.03 (1H, dd, J = 7.8, 14.4 Hz, H-7) and 3.07 (1H, dd, J = 5.4, 14.4 Hz, H-7); and one methoxy group at δ_H 3.71 (3H, s). The ^{13}C NMR spectrum of compound 2 appeared to display 19 signals of carbon. There were 2 signals of carbonyl carbon, 12 carbon signals of *trans* doublet junction, one signal of carbon oxymethine, one signal of methylene group, and signal of methoxy group. The results of the comparison of spectral similarity of the two compounds are presented in Table 3. Referencing with the literature value (Woo and Piao, 2004), compound 2 was concluded to be methyl rosmarinate (Figure 3). In previous work, it was reported that emodin and methyl rosmarinate were also found from whole plant of this herb collected from China (Xu *et al*., 2008; Yu *et al*., 2012). The present work showed for the first time that emodin and methyl rosmarinate were the chemical compounds present in the flower of *Sarcandra glabra* (Thunb.) Nakai collected from Vietnam.

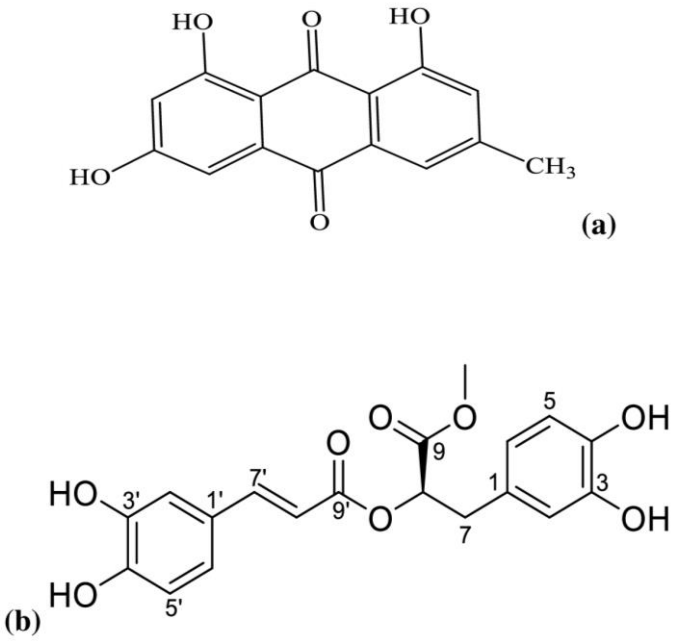

Figure 3. Chemical structure of SR-1B1 **(a)** and SE-1E1 **(b)**.

Xanthine oxidase inhibiting potency

The XO inhibiting effect *in vitro* of the M70 extract and the two compounds was evaluated by the IC_{50} values, as shown in Table 1. Results revealed that XO inhibitory capacity decreased in the following order: Allopurinol > compound 1 > compound 2 > M70. It was reported that XO inhibiting activity is not only reducing uric acid in the blood but also reducing free radical generation (Kong *et al*., 2002). Some studies proved that the activity of XO generate many free radicals. Therefore, the natural XO enzyme inhibitor supplement has both the inhibition effect of uric acid creation to prevent gout, and preventing effect of the oxidative stress disrupting the function of normal cells and tissues in the body (Cotelle, 2001; Van Hoorn *et al*., 2002). To date, extracts of FSN have been reported to exhibit various pharmacological potentials including antioxidant (Liu *et al*., 2016), anti-tumour (Zhang *et al*., 2014), antiviral (Cao *et al*., 2012), antibacterial (Jiang *et al*., 2000), anti-inflammatory (Tsai *et al*., 2017), and anti-thrombocytopenic effects (Lu *et al*., 2018). To the best of our knowledge, the XO inhibitory effect of M70 was identified for the first time in the present work. Its IC_{50} value was determined to be 34.15 ± 1.33 µg/mL. This suggested that consuming tea supplemented with FSN could bring many promising effects for gout patients.

Compound 1, compound 2, and M70 extract induced growth inhibition in HepG2 and A549 cells

Results showed that exposure to compound 1, compound 2, and M70 extracts induced their cytotoxic effects against HepG2 and A549 cells. Their IC_{50} values are displayed in Table 4. The carcinoma cell growth inhibitory capacity decreased in the following order: Flourouracil > compound 1 > compound 2 > M70. Compound 1 (emodin) had more outstanding cytotoxicity on both A549 and HepG2.

Table 4. Cytotoxicity of SR-1B1 and SR-1E1 against two cancer cell lines.

Compound	IC_{50} (µg/mL)	
	HepG2	**A549**
Flourouracil*	11.55 ± 0.15^{c}	16.50 ± 0.13^{c}
M70	140.82 ± 2.48^{a}	165.79 ± 2.73^{a}
SR-1B1	13.72 ± 0.48^{c}	18.33 ± 0.10^{c}
SR-1E1	22.54 ± 0.31^{b}	27.64 ± 0.68^{b}

Values are mean ± SD of triplicates. Means followed by different lowercase superscripts in the same column are significantly different. *Positive control.

Emodin altered expression of apoptosis-related proteins in HepG2 and A549 cells

The expression of apoptosis-related proteins in carcinoma cells was recorded by western blotting (Figure 4). Results revealed that emodin induced Bax, caspases, and PARP proteins activity in HepG2. In previous research, it was reported that emodin could induce apoptosis *via* the p53-dependent pathway in HepG2 cells (Shieh *et al*., 2004). Moreover, it was reported that emodin caused apoptosis in A549 by operating a reactive oxygen species-elicited ATM-p53-Bax signalling pathway (Lai *et al*., 2009). In the present work, results revealed that emodin activated caspase-3/9, key mediator of apoptosis in A459, and cleaved p53. This strongly demonstrated that emodin caused apoptotic cell death in HepG2 and A549 cells *via* activating apoptotic factors which are reliable evidence of the anticancer properties of the flower of *Sarcandra glabra* (Thunb.) Nakai, as well as is scientific basis to justify its utilisation in Vietnamese folk medicine. Emodin has been reported to show cytotoxic effects through the induction of apoptosis in cancer cells including human lung squamous carcinoma CH27 cell line (Lee, 2001), human promyeloleukemic HL-60 (Chen *et al*., 2002), and human cervical cancer by 25 TK (Srinivas *et al*.,

2003). In addition, emodin also inhibited cell adhesion of various human cancers (Huang *et al*., 2006).

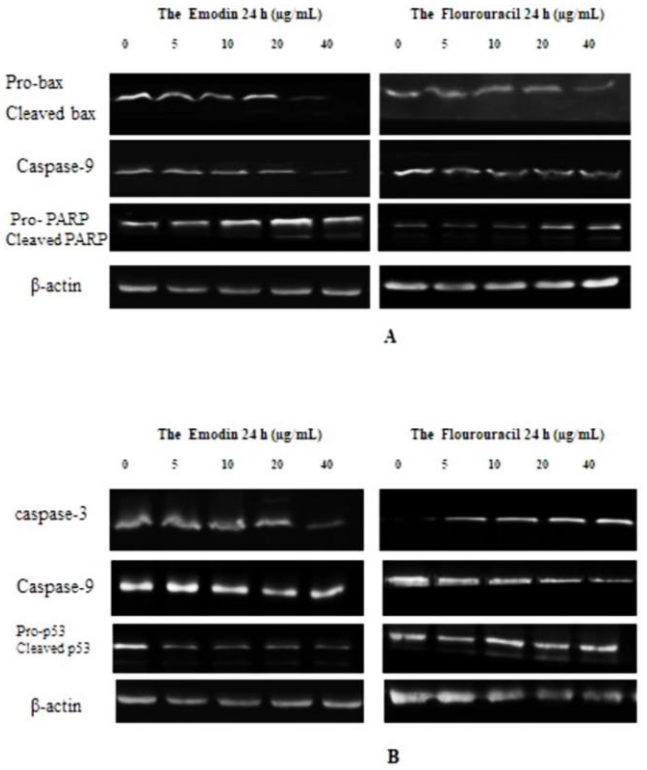

Figure 4. Emodin and Flourouracil (positive control) altered the expression of apoptosis-related proteins: Bax, caspase 9, and PARP in HepG2 **(A)** and caspase-3/9, P/53 in A549 cells **(B)** after 24 h.

Conclusion

To our knowledge, the present work would be the first to report on the chemical constituents and biological activities of FSN. Emodin and methyl rosmarinate were identified for the first time herein. Moreover, their xanthine oxidase inhibition and anticancer activities from methanolic extracts contributed strong evidence for their further clinical application to lower uric acid, and support the treatment of hepatocellular carcinoma. Thorough pharmacological mechanisms related to gout treatment effects of FSN must first be established in the near future. Additionally, when the supplementation of FSN is into tea, the synergistic effect and more clinical trials with longer research periods are required to provide strong insight and evidence for its incorporation in a healthy diet.

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