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Bioactivity-guided Isolation and Identification of Xanthine Oxidase Inhibitors from *Morus alba* Bark

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ABSTRACT

Objectives: The objective of this research was isolation and evaluation of xanthine oxidase inhibitory effects of isolated substances from mulberry bark (*Morus alba* L.). **Methods**: All four fractions: *n*-hexane, chloroform, ethyl acetate and water were evaluated for their effects on xanthine oxidase activity. The active compounds were isolated from the most potential fraction using column chromatography. The structures of these compounds were elucidated using ^{1}H and ^{13}C-NMR and their xanthine oxidase inhibitory activities were evaluated spectrophotometrically. The pharmacokinetics parameters of the most promising substances were predicted using SwissADME tool. **Results**: Three compounds (**Me01**, **Me02**, **Me04**): ursolic acid **Me01,** oxyresveratrol **Me02** and kuwanon G **Me04** were isolated from ethyl acetate fraction. The xanthine oxidase activity assay showed that two compounds **Me02** (oxyresveratrol) and **Me04** (kuwanon G) both demonstrated inhibitory effects on xanthine oxidase with the IC_{50} values of 42.08 µg/ml and 52.41 µg/ml, respectively. The predicted adsorption, distribution, metabolism and excretion (ADME) properties illustrated that oxyresveratrol (**Me02**) had high solubility in water, high gastro-intestinal (GI) absorption, no violation of Lipinski's rule of five and was not affected by P-glycoprotein, which is the cause of poor bioavailability of many drugs. **Conclusion**: Our findings suggest that oxyresveratrol is potent natural xanthine oxidase inhibitor in drug discovery and development for prevention and treatment of gout.

Keywords*: In vitro; Isolation; Morus alba; Mulberry bark; Xanthine oxidase*

INTRODUCTION

Gout is a disorder of purine metabolism that increases the level of uric acid in the body, which is characterized by severe pain and swelling. This often has serious consequences for patients such as knee damage, joint deformities, kidney failure, if not diagnosed promptly and treated properly[1].

Xanthine oxidase (XO) is an enzyme that plays an important role in the degradation of purine into uric acid in humans. XO enzyme inhibitors reduce uric acid levels in the body and are one of the drugs used to prevent and treat diseases related to hyperuricemia, including gout[1]. However, the XO inhibitors such as allopurinol and febuxostat still have some undesirable effects including skin rash, nausea, vomiting, kidney failure, Steven - Johnson syndrome[1]. Therefore, searching for herbs and natural compounds that have effect on xanthine oxidase activity with low toxicity has been recently considered as an alternative solution in the discovery and development of new drug for gout treatment.

In Vietnamese traditional medicine, mulberry (*Morus alba* L.) leaves (*tang diep*), fruits (*tang tham*), twigs (*tang chi*) and root bark (*tang bach bi*) are all valuable medicines which are used to treat many diseases such as headache, dizziness, hypertension, cough, osteoarthritis pain...[2]. Several researches have shown that mulberry twig extract reduces serum uric acid and increases uric acid excretion in white mice with hyperuricemia[3,4]. Although the trunk is one of the most similar parts to the twig in terms of its chemical composition, the relationship between the chemical composition and the *in vitro* inhibitory effect of *in vitro* xanthine oxidase of *Morus alba* L. trunk extract has not been known yet. Therefore, the study was performed with the aim of discovering potential xanthine oxidase inhibitors from mulberry tree trunks.

MATERIAL AND METHODS

Plant materials

The mulberry trunks (*Morus alba* L.) were collected in Song Mai, Bac Giang, Vietnam in August 2016. A plant sample was identified by Dr. Nghiem Duc Trong, Department of Botany, Hanoi University of Pharmacy. The voucher specimen (ID: HNIP/18484/17) was deposited at the Herbarium of Department of Botany, Hanoi University of Pharmacy. The bark and the wood were separated, dried, cut into 1-2 cm pieces and placed in plastic bags.

Chemicals

Sephadex LH-20 (Sigma-Aldrich), normal phase silica gel 60 (0,040-0,063 mm; 230 - 400 mesh; Merck), reversed-phase silica gel (30-50 µm; Fujisilisa Chemical Ltd., Japan), thin layer chromatography DC-Alufolien 60 F_{254} (Merck), reversed-phase RP-18 F_{254} (Merck), vanillin-sulphuric acid spraying reagent for seperation; DMSO (Sigma Aldrich), xanthine oxidase (Sigma Aldrich), xanthine (Sigma Aldrich), quercetin (National Institute of Drug Quality Control) for xanthine oxidase assay.

Extraction and isolation

The plant materials (500 g bark and 500 g wood) were separately ground and refluxed with methanol (the ratio of solid : solvent = 1: 6) thrice for 3 hours each. Then, the extracts were combined and evaporated to dryness under reduced pressure to afford 29.16 g and 38.84 g methanol extract from mulberry wood and bark, respectively. Next, the methanol extract was dispersed in hot water at the ratio of 1: 4, then liquid- liquid phase seperated in turn with *n*-hexane, chloroform and ethyl acetate. Each fraction was concentrated under reduced pressure and dried in order to obtain *n*-hexane (11.20 g and 1.84 g), chloroform (3.27 g and 0.63 g), ethyl acetate (4.24 g and 2.74 g) and water fractions (7.63 g and 8.32 g) from mulberry bark and wood, respectively.

The fraction that exhibited the highest *in vitro* xanthine oxidase inhibitory activity was selected for fractionation and isolation of active constituents using silica gel column chromatography (particle size: 0.04 – 0.063 mm) with gradient eluting solvent dichloromethane (DCM) and methanol (MeOH) (1:0, 1:1, 0:1, *v/v*), yielding 12 fractions (F1 to F12). **Me01** was isolated from fraction F1 by natural evaporation of solvent and recrystallization with ethanol. **Me02** was isolated from fraction F7 by Sephadex LH-20 chromatography and eluted by methanol – water (5:1, *v/v*) as pale-yellow crystal while **Me04** was obtained from fraction F10 using reversed-phase silica gel chromatography (particle size: 30 – 50 µm) with methanol – water (1:1, *v/v*) as mobile phase.

The structures of isolated compounds were elucidated based on the ^{1}H and ^{13}C-NMR spectral data which were recorded on a Bruker Avance 500 MHz for ^{1}H and 125 MHz for ^{13}C spectrometer.

Xanthine oxidase assay

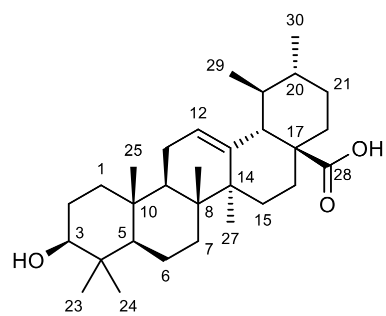
The xanthine oxidase inhibitory activity of fractions and isolated compounds were assayed spectrophotometrically using method described by Mai *et al.* with modifications[5]. The reaction mixture consisting of 50 µl of test solution at different concentrations prepared by diluting 10 mg/ml solution of crude extract in dimethyl sulfoxide (DMSO) with 70 mM phosphate buffer (pH = 7.5), 35 µl of phosphate buffer and 30 µl of enzyme solution in phosphate buffer were incubated at 25°C for 15 minutes followed by addition of 60 µl of 150 µM xanthine and incubating for 30 minutes at 25°C. 30 µl of 1N HCl was added to stop the reaction and the absorbance was measured at 290 nm. Each experiment was performed in triplicate. Quercetin was used as positive control. The half maximal inhibitory concentration (IC_{50}) values were calculated by using GraphPad Prism 8.0 software.

Molecular docking and ADME prediction

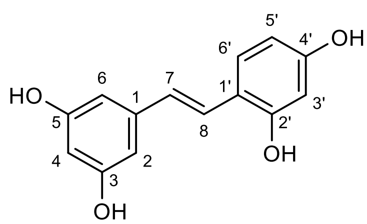
The crystal structures of xanthine oxidase (ID: 3NVY) were collected from RCSB protein data bank[6]. After removing water molecules, hydrogen atoms were added followed by protonating using Protonate3D process in MOE 2009 software. The partial charges were computed by Amber 99 forcefield. The structures of ligands were drawn using Chem Office 17.0 software then optimized and assigned atomic charges. After that, enzyme and ligands were docked, using Triangle Matcher method and London dG scoring function.

Table 1. Evaluation of *in vitro* xanthine oxidase inhibition effect of extracts and isolated compounds from mulberry trunk

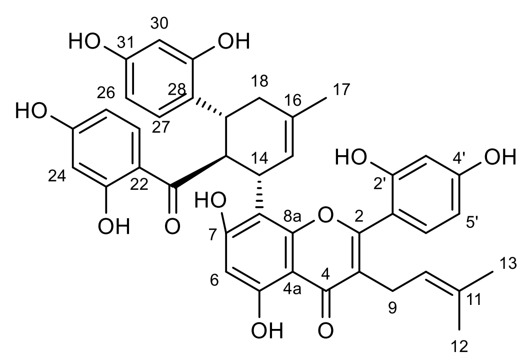
Part	Fraction/Compound	IC_{50} (µg/ml)	95% Confidence Interval (µg/ml)
	Methanol	84.94	38.17 – 189.00
	n-Hexane	145.10	58.51 – 359.70
Bark	Chloroform	151.50	92.80 – 247.20
	Ethyl acetate	18.59	10.70 – 26.28
	Water	> 300	-
	Methanol	125.40	38.78 – 405.60
	n-Hexane	> 300	-
Wood	Chloroform	47.19	41.23 – 56.04
	Ethyl acetate	33.19	21.06 – 52.31
	Water	> 300	-
Positive control	Quercetin	2.77	1.81 – 4.03
	Ursolic acid (**Me01**)	> 300	-
Isolated compound	Oxyresveratrol (**Me02**)	42.08	55.34 – 76.57
	Kuwanon G (**Me04**)	52.41	38.03 – 72.75



A. Ursolic acid



B. Oxyresveratrol



C. Kuwanon G

Figure 1. The structure of isolated compounds. **A.** Ursolic acid; **B**. Oxyresveratrol; **C**. Kuwanon G

ADME parameters of isolated compounds were predicted using Swiss ADME, a free web tool[7]. The predicted properties included water solubility, GI absorption, blood-brain barrier (BBB) permeant, interaction with P-glycoprotein (P-gp), cytochromes P450 (CYPs) inhibition and number of violations of Lipinski's rule of five of ligands.

RESULTS

Isolation and structure elucidation

The effect of methanol extracts and four fractions (*n*-hexane, chloroform, ethyl acetate and water) of mulberry bark and wood on xanthine oxidase activity were evaluated at six concentrations: 3, 10, 30, 50, 100 and 300 µg/ml. The results are presented in **Table 1**.

As shown in **Table 1**, the extract from mulberry bark had a better inhibitory effect on xanthine oxidase activity than the core extract. On the other hand, the ethyl acetate fraction showed the best inhibitory activity with IC_{50} of 18.59 µg/ml. Therefore, the ethyl acetate fraction from mulberry bark was chosen to isolate potential xanthine oxidase inhibitors.

Using silica gel column chromatography, Sephadex LH-20 chromatography and reversed-phase silica gel chromatography, three pure compounds, **Me01**, **Me02** and **Me04** were isolated. Their structures were elucidated based on the NMR spectral data.

Me01 was obtained as white amorphous powder. ^{1}H-NMR and ^{13}C-NMR spectra data of **Me01** were represented below:

^{1}H-NMR (500 MHz, $CDCl_3$ & CD_3OD), δ (ppm): 5.24 (1H, t, *J* = 7.5 Hz, H-12), 3.20 (1H, t, *J* = 16.5 Hz, H-3), 2.19 (1H, d, *J* = 11.0 Hz, H-18), 1.09 (3H, s, H-27), 0.98 (3H, s, H-26) , 0.95 (3H, s, H-25), 0.93 (3H, s, H-30), 0.86 (3H, d, *J* = 6.5 Hz, H-29), 0.81 (3H, s, H-23), 0.77 (3H, s, H-24).

^{13}C-NMR (125 MHz, $CDCl_3$ & CD_3OD), δ (ppm): 180.7 (C-28), 138.0 (C-13), 125.4 (C-12), 78.8 (C-3) , 55.1 (C-5), 52.7 (C-18), 47.7 (C-17), 47.4 (C-9), 41.9 (C-14), 39.4 (C-8), 38.9 (C-19), 38.8 (C-20), 38.6 (C-1), 38.5 (C-4), 36.8 (C-22), 36.7 (C-10), 32.9 (C-7), 30.6 (C-21), 29.6 (C-15), 27.9 (C-23), 26.7 (C -2), 24.1 (C-16), 23.4 (C-27), 23.2 (C-11), 21.0 (C-30), 18.2 (C-6), 16.9 (C-26), 16.8 (C-29), 15.5 (C-24), 15.3 (C-25).

The ^{1}H-NMR and ^{13}C-NMR spectra showed the presence of the proton of the hydroxymethine group at 3.20 ppm (1H, t, *J* = 16.5 Hz, H-3) corresponded to the signal of carbon C-3 at the δ_C of 78.8 ppm. The presence of olefinic hydrogen was confirmed by signals at 5.24 ppm (1H, t, *J* = 7.5 Hz, H-12). Based on the ^{13}C-NMR and DEPT spectra, it could be concluded that there were 30 carbons, including 7 primary carbons, 9 secondary carbons, 7 tertiary carbons and 7 quaternary carbons. By comparison with published spectral data[8], **Me01** was identified as ursolic acid (**Figure 1A**)

Compound **Me02** was obtained as needle-shaped and light greenish yellow crystal. ^{1}H-NMR and ^{13}C-NMR spectra data of **Me02** were represented below:

^{1}H-NMR (500 MHz, Acetone-d_6), δ (ppm): 6.23 (1H, t, *J* = 2 Hz, H-4), 6.37 (1H, dd, *J* = 8.5 Hz, 2.5 Hz, H-5'), 6.43 (1H , d, *J* = 2.5 Hz, H-3'), 6.51 (2H, d, *J* = 2 Hz, H-2, H-6), 6.88 (1H, *J* = 16.5 Hz, H-8), 7.32 (1H, d, *J* = 16.5 Hz, H-7), 7.39 (1H, d, *J* = 8.5 Hz, H-6′).

^{13}C-NMR (125 MHz, Acetone-d_6), δ (ppm): 159.6 (C-3, C-5), 159.1 (C-4'), 156.9 (C-2'), 141.6 (C -1), 128.2 (C-6'), 126.3 (C-8), 124.4 (C-7), 117.3 (C-1'), 108.4 (C-5'), 105.5 (C-2, C-6), 103.6 (C-3'), 102.3 (C-4)

^{13}C-NMR spectrum exhibited 14 signals corresponding to 14 carbons, including four aromatic ring carbons linked to hydroxy appearing at δ_C 159, 159.6 ppm (C-3', C-5'), 159.1 ppm (C-4) and 156.9 ppm (C-2'), 10 sp^2 hybridized carbons signals in the range of δ_C 102.3 to 141.6 ppm; The DEPT spectrum showed that there are 6 quaternary carbons and 8 primary carbons. Based on the ^{1}H-NMR, ^{13}C-NMR spectra and published data[9], the structure of **Me02** were identified as oxyresveratrol (**Figure 1B**).

Compound **Me04** is reddish-brown, amorphous powder. ^{1}H-NMR and ^{13}C-NMR spectra data of **Me04** were represented below:

^{1}H-NMR (500MHz – Acetone-d_6): 7.42 (1H, H-27), 7.25 (1H, H-6'), 6.77 (1H, d, *J* = 8.5 Hz, H-33), 6.69 (1H, brs, H-3'), 6.56 (1H, d, *J* = 8.5 Hz, H-5'), 6.21 (1H, brs, H-30), 6.07 (1H, d, *J* = 8.5Hz, H-32), 6.04 (1H, s, H-6), 5.98 (1H, brs, H-24), 5.96 (1H, d , *J* = 8.5 Hz, H-26), 5.18 (2H, m, H-10, H-15), 4.44 (1H, d, H-19), 3.32 -4.43 (2H, m, H-14, H-20), 3.21 (2H, H-9), 1.70-1.98 (2H, m, H-18), 1.61 (3H, s, H-12), 1.52 (3H, s, H-17), 1.48 (3H, s, H13),

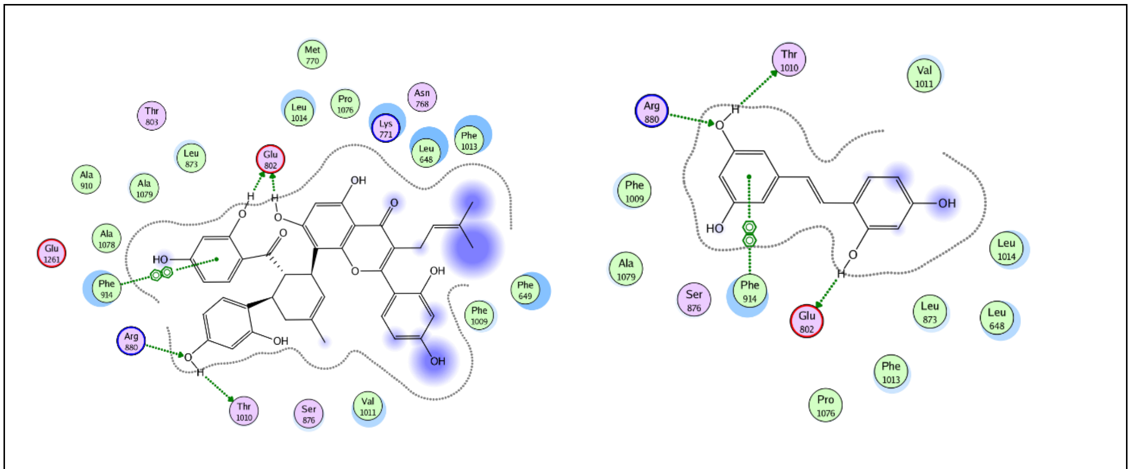
^{13}C-NMR (125MHz – Acetone-d_6): 206.6 (C-21), 183.1 (C-4), 165.6 (C-23), 164.9 (C-25), 162.1 (C -7), 161,5 (C-4'), 161.3 (C-8a), 160,8 (C-2), 157.3 (C-2'), 157.1 (C-5), 157.1 (C-29), 157.1 (C-31), 133.9 (C-27), 133.6 (C-16), 132.1 (C-6'), 132.1 (C- 33), 132.1 (C-11), 124.4 (C-15), 122.9 (C-10), 121.4 (C-28), 115.6 (C-3), 113, 2 (C-1'), 107.9 (C-5'), 107.9 (C-32), 107.9 (C-26), 107.6 (C-22), 105.4 (C-8), 103.6 (C-3'), 103.6 (C-30), 103.5 (C-4a), 102.8 (C-24), 98.3 (C-6), 47.6 (C-20), 38.6 (C-19), 25.8 (C-12), 24.5 (C-14), 24.5 (C-18), 24.5 (C-9) , 23.1 (C-17), 17.7 (C-13).

The ^{1}H-NMR (500MHz – Acetone-d_6) spectrum of **Me04** showed the presence of three ABX spin systems with δ_H (ppm): 7.25 (1H, H-6'), 6.56 (1H, d, *J* = 8.5Hz, H-5') and 6.69 (1H, brs, H-3'); 5.96 (1H, d, *J* = 8.5 Hz, H-26), 7.42 (1H, H-27) and 5.98 (1H, brs, H-24); 6.77 (1H, d, *J* = 8.5, H-33), 6.07

Table 2. Predicted ADME properties of oxyresveratrol (Me02) and kuwanon G (Me04)

Compound	Water solubility	GI absorption	BBB permeant	P-gp substrate	CYP inhibition	Lipinski's rule of five
Me02	Soluble	High	No	No	CYP1A2, CYP2C9, CYP3A4	0 violation
Me04	Poorly soluble	Low	No	No	No	3 violations

GI: Gastro-Intestinal, BBB: Blood-Brain Barrier, P-gp: P-glycoprotein, CYP: cytochrome P450



A. Kuwanon G

B. Oxyresveratrol

Figure 2. The binding mode of kuwanon G (**A**) and oxyresveratrol (**B**) with xanthine oxidase

(1H, d, J = 8.5Hz, H-32) and 6.21 (1H, brs, H-30). A prenyl group was also identified by the characteristic signals at δ_H (ppm): 5.18 (2, m, H-10), 1.61 (3H, s, H-12), 1.48 (3H, s, H-13). The ^{1}H-NMR spectrum of **Me04** also showed the signal of methylcylohexene group including 4 methine protons at δ_H: 5.18 ppm (2H, m, H-10, H-15), 4.43-4.70 ppm (2H, m, H-14, H-20) and 4.44 ppm (1H, d, J = 7.5Hz, H-19), methylene protons at δ_H: 1.70-1.98 ppm (2H, m, H- 18) and methyl proton at δ_H: 1.52 ppm (3H, s, H-17).

^{13}C-NMR and DEPT spectrum of **Me04** presented 40 signals including: 3 primary carbons, 2 secondary carbons, 15 tertiary carbons and 20 quaternary carbons. Based on the spectral data and published literature[10], the structure of **Me04** was confirmed as kuwanon G (**Figure 1C**).

Evaluation of xanthine oxidase inhibitory activity of isolated compounds

The effect of ursolic acid, oxyresveratrol and kuwanon G on xanthine oxidase activity *in vitro* was evaluated and presented in **Table 1**. The results showed that only oxyresveratrol and kuwanon G exhibited xanthine oxidase inhibitory effect with IC_{50} values of 42.08 µg/ml and 52.41 µg/ml, respectively. In order to investigate the mechanism of action of oxyresveratrol and kuwanon G, molecular docking was performed. The results were shown in **Figure 2**.

As shown in **Figure 2**, the hydrogen bonding between hydroxyl group of oxyresveratrol, kuwanon G and Glu 802, Arg880, Thr1010 residues and π-π interaction between phenyl moiety and Phe914 residue of xanthine oxidase might play key role in XO inhibitory activity. These results were consistent with researches of C. M. Lin[11] and S. Lin[12], in which, they identified that flavonoids occupied the active cavity of XO by interaction with Glu802, Arg880, Phe914 and Thr1010, four of the key residues in the active site of enzyme.

Prediction of ADME parameters is an important step in optimization of lead compound. In this study, water solubility, GI absorption, BBB permeant, interaction with P-gg, CYP inhibition and number of violations of Lipinski's rule of five of oxyresveratrol (**Me02**) and kuwanon G (**Me04**) were estimated using SwissADME tool and showed in **Table 2**.

The results in **Table 2** illustrated that oxyresveratrol (**Me02**) was a potential candidate in drug research and development of xanthine oxidase inhibitor with high solubility in water, high GI absorption and no violation of Lipinski's rule of five. The results also indicated that oxyresveratrol was not affected by P-glycoprotein, which is the cause of poor bioavailability of many drugs.

DISCUSSION

According to the results of evaluating the *in vitro* xanthine oxidase inhibitory effect of methanol extracts and fractions, the ethyl acetate fraction from mulberry trunk bark showed the strongest activity with IC_{50} of 18.59 µg/ml. Ethyl acetate is a slightly polar solvent with Polarity Index of 4.4, therefore this solvent could well dissolve most of flavonoids. It can be predicted that this class of compounds might play a key role in XO inhibitory effect of ethyl acetate extract from mulberry barks. From the ethyl acetate fraction, ursolic acid, a pentacyclic triterpenoid carboxylic acid and two other compounds with similar framework, oxyresveratrol and kuwanon G, were isolated. However, only oxyresveratrol and kuwanon G demonstrated effect on xanthine oxidase activity with IC_{50} values of 42.08 µg/ml and 52.41 µg/ml, respectively.

Oxyresveratrol is a stilbene derivative presented in many species such as *Smilax china* L. (Smilacaceae)[13], *Morus alba* L. (Moraceae)[14], *Morus nigra* L. (Moraceae)[15]. Previous studies have demonstrated that oxyresveratrol could inhibit the inflammatory response *in vitro* and *in vivo* - one of the typical symptoms of acute gout[16], prevent DNA damage more effective than both trolox and ascorbic acid[17], inhibit protein kinase C[18] or reduce the production of TNF-α necrosis factor[19]. In this study, oxyresveratrol was considered as a potential compound in prevention and treatment of gout with high affinity toward xanthine oxidase and suitable ADME properties. However, more studies are still required in order to confirm the effectiveness of oxyresveratrol clinically.

Kuwanon G is a common flavonoid Diels-Alder that could be easily found in *Morus* species[20, 21]. Recent studies have shown that kuwanon G could relieve the symptoms of asthma[22], decrease the disruption of the intestinal epithelium by improving the cell viability and tight junction activity, reduce the production of inflammatory cytokines and prevent oxidative damage[23].

This is the first study in which the *in vitro* xanthine oxidase inhibitory activity of oxyresveratrol and kuwanon G was assessed. The molecular docking results revealed that these two compounds competitively inhibited XO activity by forming the hydrogen bonding with Glu 802, Arg880, Thr1010 residues of xanthine oxidase, which were consistent with researches reported by C. M. Lin[11] and S. Lin[12]. These are remarkable features in drug discovery and development for prevention and treatment of gout.

CONCLUSION

From mulberry trunk bark (*Morus alba* L.), three compounds **Me01**, **Me02** and **Me04** were isolated. Based on the nuclear magnetic resonance spectroscopy data, it was identified that **Me01** was ursolic acid, **Me02** was oxyresveratrol and **Me04** was kuwanon G. The xanthine oxidase assay showed that two compounds **Me02** (oxyresveratrol) and **Me04** (kuwanon G) have inhibitory effects on xanthine oxidase activity with IC_{50} values of 42.08 µg/ml and 52.41 µg/ml, respectively. The predicted ADME properties suggested that oxyresveratrol was a potent lead in novel drug discovery for gout. However, more studies are needed in order to confirm the effectiveness of oxyresveratrol clinically.

Conflict of interest

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

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