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ANTIOXIDANT AND XANTHINE OXIDASE INHIBITORY PROPERTIES AND LC-MS/MS IDENTIFICATION OF COMPOUNDS OF ETHANOLIC EXTRACT FROM MULBERRY LEAVES

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ABSTRACT

Background. Xanthine oxidase (XO) enzyme inhibitors are used to treat hyperuricemia. Certain natural substances have been reported to be strong inhibitors of xanthine oxidase. The purpose of this study was to evaluate the antioxidant activity and XO inhibition effect of ethanolic extract from mulberry leaves.
Material and methods. The chemical composition of Mulberry leaf ethanolic extract (MLEE) was identified by the LC-MS/MS method. Antioxidant activity was measured by three different assays (i.e. superoxide, hydrogen peroxide, and hydroxyl radicals assays). The inhibitory effect on XO and its inhibitory mechanism were investigated through the testing of inhibition kinetics and inhibition rate.
Results. MLEE showed significant antioxidant activity in the superoxide, hydrogen peroxide, and hydroxyl radicals assays (IC_{50} of 0.33 ±0.006 mg/mL, 45 ±0.8 μg/mL, and 2.54 ±0.05 mg/mL for each assay respectively). The effects of XO inhibitory activity showed that MLEE was a reversible and competitive inhibitor with IC_{50} values of 1104.76 ±7.1 μg/mL. Eleven compounds, including six flavonoids and five phenolic acids, were identified.
Conclusion. MLEE has been shown to exhibit antioxidant activity and XO inhibitory effect, which provides a new insight into the properties of mulberry leaves and their potential future applications as a natural anti-gout drug.

Keywords: mulberry leaves, antioxidant, xanthine oxidase, LC-MS/MS

INTRODUCTION

Mulberry (*Morus alba* L.), which belongs to the Moraceae family, has been widely cultivated in many Asian countries, including China and Japan, and its leaves are used in traditional Chinese herbal medicine to promote urination, lower blood pressure and other diseases (Katsube et al., 2009; Yang et al., 2014). As mulberry leaves possess many active constituents and pharmacological efficacy, they have prospective applications in the food and pharmaceutical industries (Thabti et al., 2012; Yang et al., 2017). Flavonoids and polyphenols are natural active constituents, and many important bioactive functions are related to them (Yang et al., 2014). Many experiments have shown that they demonstrate antioxidant, anti-inflammatory,

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anticancer and anti-microbial activities and can act as modulators of the activities of enzymes such as XO (Bogucka-Kocka et al., 2016; Danihelová et al., 2012). Moreover, mulberry leaves consist of rutin, isoquercetin, astragalin, which have been shown to be effective antioxidants and XO inhibitors (Chiang et al., 1994; Masuoka et al., 2012). Nevertheless, the XO inhibitory effect of ethanolic extract from mulberry leaves has not been adequately investigated.

Xanthine oxidase (XO) is a critical enzyme involved in purine metabolism, and is a terminal enzyme that catabolizes human purines, catalyzes hypoxanthine to produce xanthine, and then produces uric acid, which could also directly catalyze xanthine to produce uric acid (Jayaraj et al., 2014). Reactive oxygen (ROS), superoxide and hydrogen peroxide are produced by this oxidative reaction (Sahin, 2016). Not only is uric acid pro-inflammatory to vascular cells in the body, but sustained over-production can also cause the deposition of urate monohydrate crystals in human synovial joints and other tissues, which eventually leads to the gout (Kubota et al., 2016; Liu et al., 2016; Nile et al., 2017). Patients with gout often have other comorbidities, including hyperlipidemia, diabetes and hypertension, which have significant adverse effects on their quality of life and health (Ren et al., 2016). The clinical treatment and prevention of gout are achieved using drugs to inhibit the activity of XO and reduce the production of uric acid. For instance, allopurinol and fluorconastine are effective XO inhibitors and are used as the main drugs for the treatment of gout. However, side effects including hematological myelosuppression, liver, kidney and gastrointestinal toxicity, and allergic reactions have been frequently reported (Wang et al., 2015). Therefore, it is very important to find an effective and safe XO inhibitor for pharmaceutical applications.

Hence, the aim of this study was to prove that ethanolic extract from mulberry leaves can inhibit XO activity, and further decipher their underlying inhibitory mechanisms. In addition, chemical compositions were qualitatively determined by LC-MS/MS. Our work may promote the economic value of mulberry leaves as a potential pharmaceutical ingredient for applications to treat gout.

MATERIALS AND METHODS

Reagents

Xanthine oxidase, allopurinol and xanthine were obtained from Solarbio (Beijing, China). NBT, NADH, and PMS were obtained from Aladdin (Shanghai, China). Other solvents and chemicals were of analytical reagent grade.

Mulberry leaves were collected from the MeiLing mountain of Jiangxi, China, in April 2017. The leaves were identified by the botanist LiPing Luo from Nanchang University, China.

Preparation of plant extract

For the preparation of the plant extract, 100 g of mulberry leaves were chopped into pieces and then extracted twice by refluxing with 80% ethanol (1:30, w/v) for 70 min in a sonication extractor (200W). After filtration, the solutions were concentrated using a rotary evaporator. It was precipitated four times with 95% ethanol and purified using an AB-8 resin column. Finally, MLEE powder was obtained by freeze-drying.

Determination of total polyphenols and flavonoids content

The polyphenol content in MLEE was measured by Folin-Ciocalteau method (Simirgiotis et al., 2013). The total flavonoid content in MLEE was determined according to the previously described method with some modifications. 1.0 ml of the sample solution was added to 1 ml of $NaNO_2$ solution (1:20, w/v). After 6 minutes, 1 ml of $Al(NO_3)_3$ (1:10, w/v) was added and kept at room temperature for 6 minutes. Finally, 4 ml of NaOH (1M) was added to the solution and then made up to 10 ml with 60% ethanol. After standing for 12 minutes, absorbance was read at 510 nm (Hu et al., 2016).

LC-MS/MS conditions

LC-MS/MS analysis was conducted with a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer TripleTOF 5600+ (AB Sciex Agilent Technologies, Germany) equipped with an orthogonal electrospray ionization source (ESI) in the negative ion mode. The conditions were as following: the mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). Mass spectrometer

electrospray capillary voltage was 4.5 kV, Heat Compensation Flow nebulizer pressure was 50 psi, collision energy (the first order was 10eV, the second order was 30 ±15VeV), fragmentor voltage was 135V and the mass to charge ratio (m/z) ranged from 70–1250 m/z using 550°C drying gas (N_2) temperature of and 6 L/min drying gas flow (He et al., 2013).

Superoxide radical scavenging activity

The scavenging effect of the superoxide radical was evaluated by the NADH/PMS system according to the previously described method (Xing et al., 2006). The reaction mixture, containing various concentrations of sample, NBT (300 μmol/L), NADH (456 μmol/L), and PMS (60 μmol/L) in Tris-HCl buffer (16 mmol/L, pH 8.0), was incubated at room temperature for 5 min.

Hydrogen peroxide (H_2O_2) scavenging assay

The scavenging activity of hydrogen peroxide was determined according to the previously described method with some modifications (Liu et al., 2009). Briefly, 2 mL of different concentrations of MLEE were mixed with 2 mL of H_2O_2 (800 μmol/L) prepared in a potassium phosphate buffer (pH 7.4) and the solution was incubated at room temperature for 10 min.

Hydroxyl radical (OH) scavenging activity

Hydroxyl radical (OH) scavenging activity was determined according to the previously described method with slight modifications (Naheed et al., 2017). Briefly, 1 mL of $FeSO_4$ (9 mmol/L) was added to a microfuge tube. Then, 1 mL of different concentrations of MLEE and 1 mL salicylic acid (9 mmol/L) were added. The final volume of reaction mixture was added to 1 mL of H_2O_2 (8.8 mmol/L) solution and incubated at 37°C for 30 min.

Xanthine oxidase inhibitory assay

Xanthine oxidase inhibitory activity was determined as described previously (Jayaraj et al., 2014), with some modifications. Briefly, 1 ml of different concentrations of samples and 2.9 mL phosphate buffer (pH 7.5) and 0.1 mL (0.1 unit/mL) of bovine milk XO were mixed and reacted at 25°C for 15 minutes. Then, 2 ml (150 μmol/L) xanthine solution was added into the mixture to trigger a reaction, and incubated at 25°C for 30 minutes. 1 mL of 1N HCl was added to terminate the reaction, and the spectrophotometer was measured at 295 nm.

Analysis of inhibitory kinetics

In order to determine the inhibition type of MLEE, Lineweaver-Burk plot analysis was performed. MLEE was tested by the method of inhibiting the XO, and plots were obtained at several concentrations of MLEE using different concentrations of XO (Zhang et al., 2017).

Statistical analysis

The result were expressed as mean ±SD values ($n = 3$) and analyzed by one-way ANOVA using Origin 8.5 software. $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Phytochemical analysis of MLEE

Polyphenols and flavonoids were the main components of MLEE, the respective contents of which were 42.6% and 33.8%. Eleven compounds, including six flavonoids and five phenolic acids, were detected, and their chemical formulas and mass were summarized in Table 1. This also included retention times, $[M-H]^-$ (m/z), MS^2 ions and identified compounds. The main parameters of the identified compounds are shown in Table 1.

Antioxidant activity

Superoxide radical produced *in vivo* is a very harmful factor to cellular components, and can result in the formation of H_2O_2 *via* a dismutation reaction, which has many undesirable effects on the human body (Kumaran and Karunakaran, 2007). The data for the superoxide radical scavenging effect of MLEE and ascorbic acid is shown in Table 2. The assay results prove that MLEE has good scavenging activity against superoxide radical (IC_{50} = 0.33 ±0.006 mg/mL), which was lower than ascorbic acid (IC_{50} = 0.27 ±0.004 mg/mL) but higher than vitamin E (IC_{50} = 0.96 ±0.03 mg/mL).

Hydrogen peroxide is not a free radical, but can cross membranes and may slowly oxidize a number of biomolecules and form hydroxyl in the presence of metal ions. Hence, metal chelating and hydrogen peroxide scavenging processes are important for living organisms (Gulcin et al., 2003). The result of the superoxide radical scavenging effect of MLEE and ascorbic acid is shown in Table 2. The MLEE had better hydrogen peroxide scavenging ability exhibited

Table 1. Eleven compounds identified by LC-MS/MS

Number	Rt mi	$[M-H]^-$ m/z	Formula	MS^2 ions	Identified compounds
1	4.53	179.0353	$C_9H_8O_4$	135	caffeic acid
2	3.35	325.0933	$C_{15}H_{18}O_8$	163, 119	p-coumaric acid glycoside
3	2.11	353.0880	$C_{16}H_{18}O_9$	191, 179, 135	chlorogenic acid
4	4.65	385.1144	$C_{17}H_{22}O_{10}$	223	sinapic acid hexoside
5	1.63	515.1209	$C_{25}H_{24}O_{12}$	353, 191, 179	dicaffeoyl-quinic acid
6	9.14	447.0946	$C_{21}H_{20}O_{11}$	285, 255	astragalin
7	8.03	463.0892	$C_{21}H_{20}O_{12}$	300, 255	isoquercitrin
8	7.58	609.1486	$C_{27}H_{30}O_{16}$	300	rutin
9	8.64	593.1532	$C_{27}H_{30}O_{15}$	285	kaempferol-rhamnoside-hexoside
10	10.03	533.0959	$C_{24}H_{22}O_{14}$	489, 285	kaempferol-malonyl-hexoside
11	10.03	489.1056	$C_{23}H_{22}O_{12}$	285	kaempferol-acetyl-hexoside

Table 2. Antioxidant activity evaluation of the MLEE and ascorbic acid

	IC_{50} superoxide radical mg/mL	IC_{50} H_2O_2 μg/mL	IC_{50} hydroxyl radical mg/mL
MLEE	0.33 ±0.006	45 ±0.8	2.54 ±0.05
Ascorbic acid	0.27 ±0.004	80.46 ±1.4	0.37 ±0.02
Vitamin E	0.96 ±0.03	110.56 ±3.2	1.83 ±0.06

All values are expressed as mean ±standard deviation (n = 3).

the lowest IC_{50} value at 45 ±0.8 μg/mL, which was stronger than ascorbic acid (IC_{50} = 80.46 ±1.4 μg/mL) and vitamin E (IC_{50} = 110.56 ±3.2 mg/mL). Thus, the MLEE was shown to have potential as a kind of hydrogen peroxide scavenger. The hydroxyl radical, a highly toxic radical, can cause oxidative damage to biological macromolecules including lipids, proteins and nucleic acids (Ozyürek et al., 2008). As shown in Table 2, the hydroxyl scavenging ability of MLEE and its fractions increased in a dose-dependent manner. In addition, ascorbic acid showed excellent scavenging activity. Clearly, ascorbic acid could effectively scavenge hydroxyl radicals with an IC_{50} 0.37 ±0.02, and MLEE was also observed to have scavenging activity with an IC_{50} value of 2.54 ±0.05 mg/mL close to vitamin E.

Xanthine oxidase inhibition

As shown in Figure 1, MLEE negatively correlated with the relative activity of XO, and inhibited XO activity in a dose-dependent manner. The IC_{50} value of MLEE was 1104.76 ±7.1 μg/mL, whereas that of allopurinol was 1.19 ±0.04 μg/mL. Although allopurinol showed a stronger inhibitory rate than MLEE, many previous studies have demonstrated that allopurinol can cause many strong side effects (Wang et al., 2017). Therefore, drugs with less toxic side effects are in urgent need of development. Taking all of these factors into consideration, we can come to the conclusion that MLEE contains XO inhibitors and merits further research.

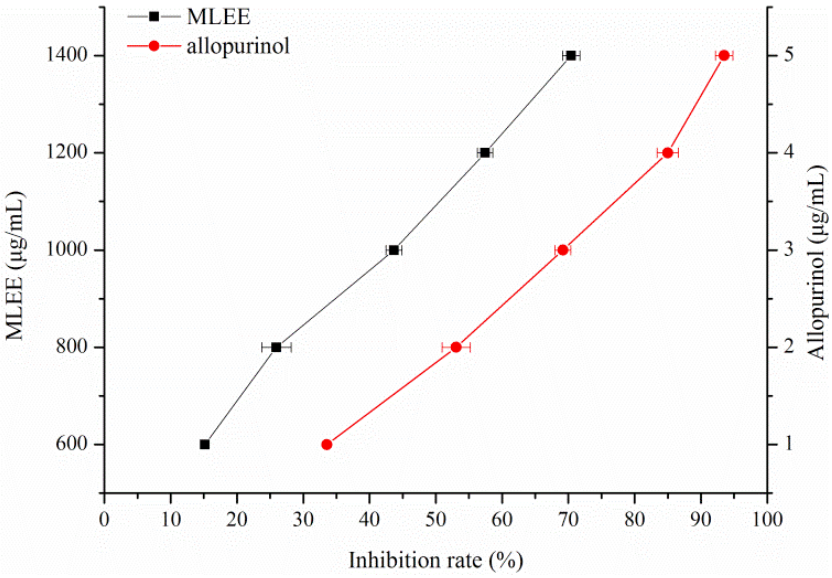

Fig. 1. XO inhibition rate by MLEE and allopurinol

Determination of inhibitory type

The inhibition mechanism of MLEE was investigated based on the Lineweaver-Burk plot (Fig. 2A). From curves 1 to 5, as the MLEE concentration increases, the slope of the straight line decreases, and all lines pass through the origin, indicating that MLEE causes the reversible inhibition of XO. The catalytic activity of XO was reduced by affecting the synthesis of the enzyme-substrate complex and the decomposition of the enzyme-product complex (Phan et al., 2013). To investigate the kinetics of the enzyme in the presence of MLEE, the Lineweaver-Burk plot equation

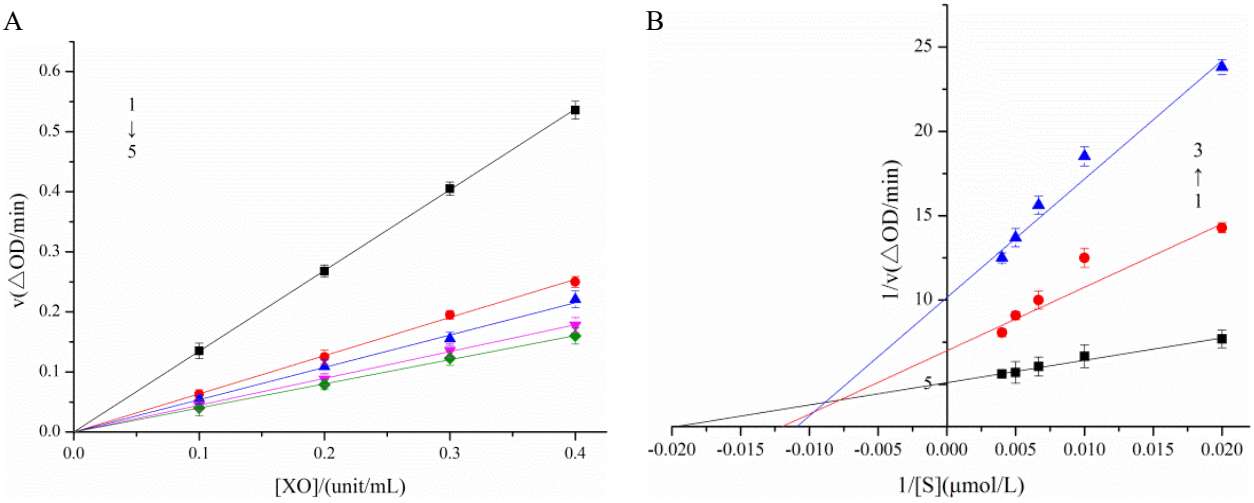

Fig. 2. (A) reversible results plots for MLEE: c(xanthine) – 150 μmol/L, c MLEE) – 0, 0.2, 0.4, 0.6, 0.8 mg/mL for curve 1–5 respectively. (B) Lineweaver-Burk double reciprocal plots for MLEE: c(XO) – 0.1 units/mL, c(MLEE) – 0, 0.5, 1 mg/mL for curve 1–3, respectively

in double reciprocal form was used and the results were displayed in Figure 2B. All of the data lines with different slopes and intercepts intersected in the second quadrant, indicating that MLEE was a mixed-type inhibitor against the formation of uric acid. Furthermore, MLEE may have a single inhibition site or a single class of inhibition sites on XO (Phan et al., 2013; Xi et al., 2016; Zhang et al., 2018).

CONCLUSIONS

In this study, we analyzed polyphenol and flavonoid content, antioxidant activity, XO inhibitory effect, and inhibitory mechanisms of MLEE. The results suggest that MLEE has significant antioxidant and XO inhibitory properties. The inhibition type of MLEE on XO was reversible and mixed-type inhibition of MLEE compounds was simultaneously determined using a validated LC-MS/MS method. This finding shows that mulberry leaves contain a variety of compounds, many of which could inhibit XO, such as rutin, isoquercetin, and astragalin. Hence, we came to the conclusion that MLEE contains bioactive compounds which could make a potential contribution to the development of new anti-gout drugs and other oxidative stress-related conditions in humans. However, more studies *in vivo* are needed to determine appropriate candidates for clinical investigation.

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