Research Article

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Screening and analysis of xanthine oxidase inhibitors in jute leaves and their protective effects against hydrogen peroxide-induced oxidative stress in cells

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Abstract: Jute (*Corchorus capsularis* L.) is an annual herb of the bast fiber plant and has great potentials in food and medicinal usages because of its various bioactivities. In this study, ultrafiltration coupled with high-performance liquid chromatography-mass spectrometry was established for screening xanthine oxidase inhibitors from the jute leaves extract. Under the optimum screening conditions, three inhibitors were successfully screened and identified as chlorogenic acid, echinacoside, and isorhamnetin-rutinoside with UV and MS data. The fluorescent quenching analysis showed that three inhibitors quenched the fluorescence intensities of enzyme with different binding capacities. For further exploring the bioactivity of three inhibitors, the protective effects on hydrogen peroxide-induced oxidative stress was investigated using human normal liver cell (LO2), human gastric mucosal epithelial cell (GES-1), and human umbilical vein endothelial cell (HUVEC). As a result, they exhibited protective effects on three injured cells in dose-dependent manners without cytotoxicity. To evaluate the difference among different jute species obtained in our laboratories, the amounts of three compounds in ten samples were assessed and analyzed. The results showed that it could be divided into three groups. The jute leaves showed nutrient and medical potentials and deserved further research on pharmaceutical and biochemical utilization in future.

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1 Introduction

Jute (*Corchorus capsularis* L.) is an annual herb of the bast fiber plant that belongs to the family of Tiliaceae. It is a short-day crop suitable for growing in warm and humid climates. Jute fiber is often used to make ropes and produce paper because of its good toughness, low cost, easy availability, and renewable resource [1]. In recent years, secondary metabolites from plants attracted widespread attention because of their abundant activities and benefits for human health. Currently, among these secondary metabolites, phenolic compounds were well explored and studied. Due to their antioxidant properties, they are often used as natural antioxidants instead of synthetic antioxidants in foods [2]. Natural antioxidants showed protective activities against cardiovascular diseases and cancers by inhibiting and scavenging free radicals [3]. Many studies have shown that the tender leaves of jute were rich in carotenoids, trace elements, vitamins, and dietary fiber. As one of the plants that have long history in China, jute has a great potential in food and medicinal applications because of its diuretic, antipyretic, analgesic, antibacterial activity, and their beneficial antitumor ingredients [4]. Nowadays, it has become a type of dish in China. Therefore, finding active compounds in it and further bioactivity analyses were necessary.

Xanthine oxidase is an enzyme that catalyzes xanthine to uric acid through oxidation reactions. The overexpression of xanthine oxidase would generate many types of diseases such as hyperuricemia and gout [5]. Hyperuricemia occurs when there is high uric acid level in human blood and is an induction factor for gout [6]. Gout is a typical chronic disease due to the increasing concentration of uric acid and results in the deposition of urate crystals. Therefore, the control of uric acid by inhibiting the activity of xanthine oxidase is required in curing gout and related diseases [7]. However, several commercial xanthine oxidase

inhibitors including allopurinol and thiopurinol have many undesirable side effects, which limit their use [8]. Also, these inhibitors show more more side effects [9–11]. On the basis of these results, novel xanthine oxidase inhibitors with fewer side effects should be explored in natural products for better effects [12,13].

As natural extracts contain numerous bioactive compounds, it is difficult to separate ingredients, and many high-throughput screening analysis methods were introduced [14,15]. Ultrafiltration combined with liquid chromatography-mass spectrometry (UF-LC-MS) was a widely used technique in separating active compounds [16]. UF-LC-MS showed many advantages such as simple procedures, speed analysis, and low cost for the whole experiment, and it could accelerate the efficiency of active compounds discovery [17]. Due to these advantages, UF-LC-MS was widely applied in screening of many types of enzyme inhibitors [18–21].

In this study, xanthine oxidase inhibitors in jute leaves extract were screened and identified by UF-LC-MS. After identification of screened active compounds, inhibition of three screened active compounds was verified and the kinetic parameters were studied. The interactions between three inhibitors and xanthine oxidase were observed. Besides, the *in vitro* assays about enzyme inhibitions, antioxidant capacities, and the protective effects on hydrogen peroxide-induced oxidative stress of three inhibitors were studied. Finally, the amounts of three inhibitors in ten jute species were determined and compared. This research could give more evidence on the application of jute leaves in food and nutrients preventing some chronic diseases.

2 Materials and methods

2.1 Chemicals and materials

Freeze-dried powder of xanthine oxidase, chlorogenic acid, and superoxide dismutase kit were purchased from Yuanye Biotechnology Co. (Shanghai, China). Echinacoside and isorhamnetin-rutinoside were purchased from Tauto Biotech Co., Ltd (Shanghai, China). Acetic acid and acetonitrile in HPLC grade were bought from Merck KGaA (Darmstadt, Germany). Polyphenol oxidase, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), β -carotene, linoleic acid, tween 60, and chloroform were bought from Alfa Aesar (Thermo Fisher Scientific, MA, USA). Ultrapure water

(18.2 M Ω cm resistivity) was obtained from an ELGA water purification system (ELGA Berkefeld, Veolia, Germany). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and were of analytical grade, unless otherwise stated.

2.2 Preparation of jute leaves extract

A total of 1.50 g of jute leaves powder were mixed in 250 mL of ethanol solution (v:v, 50:50). The mixture was reflux extracted for 1 h at 90°C. After first extraction, the solvent was poured out and 100 mL of fresh ethanol solution (v/v, 50:50) was added for the second extraction. The third extraction was carried out as described earlier, and three parts of solvents were combined. Finally, the solvent of extract was evaporated by a rotary evaporator, and the residues were stored at 4°C.

2.3 Screening of xanthine oxidase inhibitors in jute leaves extract

Ultrafiltration was performed in screening of xanthine oxidase with slightly modifications [21]. One milliliter of jute leaves extract (43.90 mg/mL in water) and xanthine oxidase solution (1.0 μ mol/L) were mixed together and shaken at 180 rpm and 27°C for 30 min. After incubation, 500 μ L of the mixture was transferred to the ultrafiltration centrifuge tube (YM-30, 10 kDa, Microcon, Merck Millipore, Germany), and the tube was centrifuged at 12,000 rpm for 20 min under 4°C (Beckman Coulter Allegra 64R, Brea, CA, USA). The filtrate was collected, and the mixture of enzyme and the extract remaining in the filter was washed with 500 μ L of water under the same conditions as mentioned earlier. Then, the filtrate was discarded, and 500 μ L of 80% methanol solution was added to the filter, and the tube was centrifuged under the same conditions. The control experiment was carried out using denatured enzyme as substitution.

2.4 Identification of screened xanthine oxidase inhibitors

The qualitative analyses of jute leaves extract and target peaks were achieved by the LC-MS analysis on an Agilent

1260 HPLC combined Agilent 6460 Triple Quadrupole LC-MS system (Agilent Technologies Inc., Santa Clara, CA, USA) [22]. A C₁₈ reverse phase column (Waters Xbridge™, 250 mm × 4.6 mm i.d., 5 µm, Milford, MA, USA) was used for HPLC separation under a gradient elution program: 0–5 min, 5% of acetonitrile, and 95% of water; 5–15 min, 5–25% of acetonitrile, and 95–75% of water; 15–25 min, 25–30% of acetonitrile, and 75–70% of water. A total of 0.8 mL/min of flow rate and 25°C of column temperature were set as parameters. For mass spectrometry, negative ionization mode was used in the electron spray ionization source with the full-scan mode from 100 to 1,000 *m/z*.

2.5 Enzyme inhibition assays of screened inhibitors

2.5.1 Xanthine oxidase

The inhibition assay of xanthine oxidase was carried out by UV-Vis spectrophotometer (UV2700, Shimadzu, Kyoto, Japan) [22,23]. First, 20 µL of xanthine oxidase solution and 980 µL of sample were mixed in a quartz cuvette (1.0 cm). Then, 1 mL of xanthine solution (1 mg/mL) was added to start the reaction, and the absorbance increase in 60 s was measured at 295 nm. All experiments were repeated for three times. The inhibition rate of xanthine oxidase was calculated by the following formula:

$$\text{Inhibition \%} = (1 - \Delta A_s / \Delta A_0) \times 100\%, \quad (1)$$

where ΔA_s and ΔA_0 are the changes of absorbance for sample and blank, respectively. The inhibition of sample was defined as the concentration of sample needed to inhibit 50% of the enzymatic activity (IC₅₀).

2.5.2 Superoxide dismutase (SOD)

The riboflavin–nitroblue tetrazolium (NBT) assay was used according to the previous study, with slight modification [24]. Hundred microliters of enzyme solution containing samples were mixed with 1,900 µL of phosphate buffer (20 mmol/L, pH 7.4) containing 200 µL of ethylenediaminetetraacetic acid (0.1 mol/L) and 200 µL of NBT (1.5 mmol/L). After incubation at 37°C for 10 min, 50 µL of riboflavin (1.2 mmol/L) was added, and the mixture was illuminated with a 15 W light tube for 15 min in a sealed box. The UV-Vis spectrophotometer was used to measure the absorbance at 560 nm. The same amount of water was used as a blank instead of the sample. The inhibition rate of SOD was calculated by the following formula:

$$\text{Inhibition \%} = (1 - A_s / A_0) \times 100\%, \quad (2)$$

where A_s and A_0 are the absorbance for a sample and a blank, respectively.

2.5.3 Polyphenol oxidase (PPO)

Twenty microliters of PPO solution (1 µmol/L in a buffer solution) and 80 µL of different concentration samples were mixed. Then, 1,900 µL of L-dopa (500 µg/mL) was added to start the reaction, and an increase in absorbance at 475 nm in 200 s was measured. Equal amount of water was used as a blank control instead of a sample. The inhibition rate of PPO was calculated by formula (1).

2.6 Antioxidation assays of screened inhibitors

2.6.1 DPPH assay

The DPPH free radical scavenging test was measured according to the method reported in the previous study, with minor adjustment [22,25]. A total of 1.0 mL of samples were thoroughly mixed with 2 mL of DPPH working solution (0.05 mg/mL in ethanol). After kept in dark for 5 min, the absorbance of solution was measured at 519 nm. The same amount of water was used as a blank control. The scavenging activity of DPPH was calculated by formula (2) and expressed as the concentration of sample needed to scavenge 50% of DPPH (EC₅₀).

2.6.2 ABTS assay

The ABTS assay was measured using a previously described procedure [26]. ABTS stock solution (7 mmol/L of ABTS solution mixed in 2.45 mmol/L of potassium persulfate solution with equal volume) was incubated for 12 h and diluted for use. A total of 1.0 mL of sample was mixed thoroughly with 2 mL of ABTS working solution and kept in dark for 6 min. Then, the absorbance of solution was measured at 734 nm. The same amount of water was used as a control. The ABTS-radical scavenging activity of the sample was calculated using formula (2).

2.6.3 β-Carotene bleaching assay

The β-carotene bleaching test was slightly modified according to the method suggested by Koleva et al. [27].

One milligram of β -carotene, 20 mg of linoleic acid, and 140 mg of tween-60 were dissolved in 10 mL of chloroform, dried on a rotary evaporator at 50°C, and then thoroughly shaken by adding 50 mL of water to get an emulsion for further usage. Then, 2 mL of sample (0.2 mg/mL) was mixed with 1 mL of the emulsion and incubated at 50°C for 2 h. After reaction, the absorbance of mixture was measured at 470 nm. The control test was performed under the same conditions but using water instead of sample. The β -carotene bleaching activity of sample was evaluated using the following formula:

$$\beta\text{-carotene bleaching \%} = [1 - (A_0 - A_t)/(C_0 - C_t)] \times 100\%, \quad (3)$$

where A_0 and A_t are the absorbance of sample before and after reaction, respectively; C_0 and C_t are the absorbance of control before and after reaction, respectively.

2.7 Determination of the kinetic parameters

Lineweaver–Burk plot and Dixon plot analyses were performed to determine the inhibition mode, the inhibition mechanism, and kinetic parameters of screened inhibitors according to our previous study [22]. The kinetics were measured using different concentrations of xanthine as a substrate and various concentration of inhibitors with fixed xanthine oxidase concentration. The maximum reaction rate ($V_{\max}$) and the Michaelis–Menten constant (K_m) were calculated by the double-reciprocal plot using the following formula:

$$\frac{1}{V} = \frac{K_m + [C]}{V_{\max}[C]} = \frac{K_m}{V_{\max}} \frac{1}{[C]} + \frac{1}{V_{\max}}, \quad (4)$$

where V is the reaction rate and $[C]$ is the substrate concentration. The inhibition constant K_i was calculated from the secondary plot constructed using slopes or y -intercepts of Lineweaver–Burk plots. K_i is the equilibrium constant for the binding of inhibitors to enzyme.

2.8 Fluorescence spectra analysis

The quenching of fluorescence spectra in xanthine oxidase was performed according to the procedures reported in the previous study [23]. Fluorescence spectra were recorded on a fluorometer (Hitachi F-7000, Tokyo, Japan). Different volumes of inhibitors (0–200 μ L, 1.0 mmol/L in methanol) were mixed with 2.0 mL of xanthine oxidase solution (1.0 μ mol/L in phosphate buffer). After incubation at 25°C for 5 min, the fluorescence emission spectra

of mixture were recorded from 300 to 450 nm under an excitation wavelength at 280 nm.

2.9 Effects of screened inhibitors on hydrogen peroxide-induced oxidative stress

2.9.1 Cell culture

Three normal cell lines were selected for research including human normal liver cell (LO2), human gastric mucosal epithelial cell (GES-1), and human umbilical vein endothelial cell (HUVEC). The LO2, GES-1, and HUVEC cell lines were purchased from Yuxi Biotechnology Co. Ltd (Jiangyin, China) and incubated in Dulbecco's modified eagle medium containing 10% fetal bovine serum (Gibco, Australia), 1% penicillin, and 1% streptomycin (Yuxi Biotechnology, Jiangyin, China) under a humidified 5% CO₂ atmosphere at 37°C in a cell incubator.

2.9.2 Cytotoxicity assay

Cell counting kit-8 (CCK-8) assay was used to evaluate the cytotoxicity of hydrogen peroxide-induced cells. Consider the HUVEC cell as an example, the HUVEC cell suspension ($2\text{--}3 \times 10^5$ cells/well) was incubated in 96-well plates overnight. Then, 250 μ mol/L of hydrogen peroxide was added into cells and incubated for 72 h. Then, 10 μ L of CCK-8 reaction solution was added into each well, and the plate was incubated for another 2 h. Finally, the absorbance at 450 nm of each well was observed using a microplate reader (Epoch, BioTek Instruments Inc., Winooski, VT, USA). For the evaluation of protective effects, the cells were exposed to hydrogen peroxide containing 10, 50, and 100 μ mol/L of chlorogenic acid, echinacoside, and isorhamnetin-3-O-rutinoside. Under the same procedures, the absorbance at 450 nm was compared. The HUVEC cells without exposure of hydrogen peroxide were set as the control group.

2.9.3 Intracellular reactive oxygen species (ROS) assay

The intracellular ROS was measured by a dihydroethidium probe (DHE; Beyotime Institute of Biotechnology, Nantong, China) according to the previous study [28]. The investigated cells were exposed to 250 μ mol/L of hydrogen peroxide for 72 h in the presence or absence of chlorogenic acid, echinacoside, and isorhamnetin-3-O-

rutinoside (10, 50 and 100 $\mu\text{mol/L}$). For the determination of ROS, cells were incubated in 5 $\mu\text{mol/L}$ of DHE for 30 min after exposure. Finally, the fluorescence of cells was observed at 600 nm using a microplate reader under the excitation light at 530 nm (Epoch, BioTek, USA).

2.9.4 Intracellular SOD assay

The activity of intracellular SOD was determined by a SOD assay kit (Beyotime Institute of Biotechnology, China) according to manufacturer's instructions [29,30]. The investigated cells were exposed to 250 $\mu\text{mol/L}$ of hydrogen peroxide for 72 h in the presence or absence of chlorogenic acid, echinacoside, and isorhamnetin-3-O-rutinoside (10, 50, and 100 $\mu\text{mol/L}$). After exposure, the cells were washed, homogenated, and centrifuged with buffer solution at 4°C. The supernatant was used as a test sample and mixed with 160 μL of WST-8/enzyme working solution and 20 μL of reaction triggering working solution. After incubation for 30 min at 37°C, the absorbance at 450 nm was recorded by a microplate reader (Epoch, BioTek, USA).

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Screening of xanthine oxidase inhibitors in extracts of jute leaves

After the optimization of chromatographic conditions, the HPLC analysis could be completed in 25 min. The chromatogram of jute leaves extract is shown in Figure 1.

The inhibition tests showed that the jute leaves extract has an inhibition on xanthine oxidase with IC_{50} value at 7.48 $\mu\text{g/mL}$. Accordingly, ultrafiltration screening of jute leaves extract was conducted to find active xanthine oxidase inhibitors. After ultrafiltration, the filtrate and methanol elute were subjected to LC-MS analysis, and the chromatograms of them are shown in Figure 1.

In the chromatogram of filtrate, three main peaks marked with numbers showed reduction of peak areas, and these three peaks appeared in the corresponding chromatogram of the eluent. According to the principle of ultrafiltration, the active binders were bound to enzyme and trapped by membrane through ultrafiltration. Therefore, fewer active binders were shown in the chromatogram of filtrate (fewer peak areas). Then, the active binders were eluted by organic solution and shown in the chromatogram of eluent. Based on these findings, three peaks could be confirmed as potential inhibitors (binders) to xanthine oxidase. Further identification and activity confirmation of these compounds are needed.

3.2 Optimization of screening conditions

3.2.1 Concentration of enzyme

To investigate the effects of screening conditions and optimize them, the calculation of binding degree was done to show the screening efficiency of compound using the following formula.

$$\text{Binding degree \%} = A_e/A_0 \times 100\%, \quad (5)$$

where A_e and A_0 are the peak areas of a compound in chromatograms of eluent and extract, respectively. The higher binding degree means higher screening efficiency, which showed that more compounds were bound to enzyme and then eluted by organic solutions.

Figure 1: The chromatograms of (a) jute leaves extract, (b) filtrate, and (c) eluent after ultrafiltration.

Certain amount of enzyme was needed for the screening of active compounds. However, excess usage of enzyme was a kind of waste and increased the cost of experiments. Hence, to study the effect of enzyme concentrations on binding degree of compounds, various concentrations of xanthine oxidase (0.1, 0.25, 0.5, and 1.0 $\mu\text{mol/L}$) were incubated with jute leaves extract. Figure 2a displays the changes of binding degrees for three compounds under the increasing concentrations of xanthine oxidase. When the concentration increased from 0.1 $\mu\text{mol/L}$ to 0.25 $\mu\text{mol/L}$, the binding degrees of them increased and kept stable when the concentration was higher than 0.25 $\mu\text{mol/L}$. As a result, satisfied screening could be achieved when the concentration of xanthine oxidase was 0.25 $\mu\text{mol/L}$.

3.2.2 Incubation time

Different incubation times from 7.5 min to 60 min were investigated to find an optimal incubation time for screening. As shown in Figure 2b, the binding degrees reached the highest value at 30 min of incubation. When the time was prolonged, gradual decrease of binding degrees could be observed for three compounds. On the basis of this result, 30 min of incubation time was set for the following experiments.

3.2.3 Temperature

As high temperature could influence the status of enzyme and deactivate it and as the enzyme was commonly sensitive to temperature of environment, the effect of temperatures on binding was investigated from 20 to 60°C (Figure 2c). As illustrated in figure, all of the binding degrees of three compounds reached the highest values at 30°C. This level of temperature could protect the

activity and stability of xanthine oxidase [31]. Therefore, to gain the highest screening efficiencies for three compounds, the temperature for screening was determined at 30°C.

3.3 Identification of screened xanthine oxidase inhibitors

The jute leaves extract was subjected to LC-MS analysis, and the chromatographic and MS data of three screened compounds are shown in Table 1. Compound 1 was identified as chlorogenic acid by analyzing the parent ion fragmentation pattern and retention time in comparison with the authentic standard. Compound 2 exhibited a precursor ion and a fragment ion by the loss of a caffeic acid moiety at 785 and 623 m/z , respectively. On the basis of the previously reported literature, this compound was identified as echinacoside in comparison with the authentic standard. Compound 3 appeared at 17.7 min with precursor ion at 623 m/z . With further collision resulted in fragment ion at 315 m/z , it was designated as isorhamnetin-3-O-rutinoside. The chromatograms of jute leaves extract and three authentic references were compared (Figure 3). The retention time of three authentic studies and those in extract were the same. The comparison indicated the identifications of them were credible.

3.4 Fluorescent quenching of xanthine oxidase

As many studies reported, fluorescent quenching of xanthine oxidase by three screened inhibitors was conducted to investigate the interaction between inhibitors and enzyme (Figure 4). Typically, xanthine oxidase

Figure 2: Effects of (a) concentration of enzyme, (b) incubation time, and (c) temperature on the screening efficiencies of three compounds.

Table 1: The identifications of screened compounds by LC-MS

No.	Identification	R_t (min)	Formula	Precursor ion (m/z)	Fragments (m/z)
1	Chlorogenic acid	14.5	$C_{16}H_{18}O_9$	353	191, 179
2	Echinacoside	14.6	$C_{35}H_{46}O_{20}$	785	623
3	Isorhamnetin-3-O-rutinoside	17.7	$C_{28}H_{32}O_{16}$	623	315

Figure 3: The chromatograms of (a) jute leaves extract, (b) chlorogenic acid, (c) echinacoside, and (d) isorhamnetin-rutinoside.

Figure 4: The quenching effects of (a) chlorogenic acid, (b) echinacoside, and (c) isorhamnetin-rutinoside on xanthine oxidase fluorescence spectra. $\lambda_{ex} = 280\text{ nm}$; xanthine oxidase, $1.0\text{ }\mu\text{mol/L}$; the addition of inhibitors was from 10 to $100\text{ }\mu\text{mol/L}$. (d) The Stern–Volmer plots for fluorescence quenching by three inhibitors. (e) The plots of $\lg[(F_0 - F)/F]$ versus $\lg[Q]$ for three inhibitors.

showed a fluorescence emission spectrum with the emission peak at 335 nm . When the inhibitor was respectively added in enzyme, the fluorescence intensities reduced gradually. The emission peak appeared in blue shift in

the addition of chlorogenic acid, red shift in the addition of isorhamnetin-rutinoside, and nearly no shift for echinacoside. These phenomena meant that the molecular conformation of xanthine oxidase was changed when

the presence of inhibitor, which was in agreement with previous studies [32,33]. It showed the structures of enzymes would be changed in binding with polyphenols [34,35]. The Stern–Volmer plots of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside were then calculated by the following Stern–Volmer formula and is also plotted in Figure 4d [36]:

$$F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q], \quad (6)$$

where F_0 and F are the peak values of fluorescence spectra in the absence and presence of inhibitor, respectively, $[Q]$ is the concentration of sample, K_q is the quenching rate constant, τ_0 is the average lifetime (6.2 ns), and K_{sv} is the Stern–Volmer quenching constant. The Stern–Volmer plots were linear with fitting degrees higher than 0.970, indicating that related calculations were acceptable and a single way of quenching playing the key role in binding [37]. Commonly, the quenching mode would be supposed as static quenching when K_q is apparently greater than 2.0×10^{10} L/mol/s [33]. In this study, all K_q values were higher than 2.0×10^{10} L/mol/s (Table 2), as the quenching mode of them could be accordingly considered as static quenching.

For further research of static quenching, the binding constants (K_a) and number of binding sites per protein molecule (n) were calculated by the double-logarithm curve as follows:

$$\lg(F_0 - F)/F = \lg K_a + n \lg [Q]. \quad (7)$$

The plots and related values of K_a , n , and fitting degrees are shown in Figure 4e and Table 2. The high fitting degrees of linear curves (higher than 0.976) showed that the calculation was acceptable [38]. Accordingly, the rank of binding capacity of these inhibitors from high to low was isorhamnetin-rutinoside, chlorogenic acid, and echinacoside.

3.5 Kinetics studies for xanthine oxidase

The inhibition details of chlorogenic acid, echinacoside and isorhamnetin-rutinoside on xanthine oxidase were

tested *in vitro*. As a result, they showed inhibitions on xanthine oxidase with IC_{50} values of 65.6, 1.63, and 1.52 mmol/L, respectively. Chlorogenic acid showed the highest inhibitory activity, followed by isorhamnetin-rutinoside and echinacoside. In comparison, allopurinol showed the IC_{50} value at 16.2 μ mol/L. This result could confirm that the screening for xanthine oxidase using ultrafiltration was effective. The inhibition of chlorogenic acid on xanthine oxidase was reported by another study [39]. The activities of xanthine oxidase were tested with various concentrations of substrates and inhibitors for inhibition parameters. Figure 5 illustrates the Lineweaver–Burk plots for chlorogenic acid, echinacoside, and isorhamnetin-rutinoside and some related parameters. It could be found that the inhibition mode of echinacoside was competitive, and the inhibition modes of chlorogenic acid and isorhamnetin-rutinoside were uncompetitive type. When the Michaelis–Menten constant (K_m) was increasing with the increasing concentrations of inhibitor, and the maximum reaction velocity (V_{max}) was stable, it could be considered as a competitive inhibition. The competitive inhibition indicated that the occupation of inhibitor prevented substrate from binding with the active site [22]. For uncompetitive inhibition, the decreases could be observed in both K_m and V_{max} during increasing concentrations of inhibitor, which indicated the inhibitor blocked the formation of enzyme–substrate complex reversibly with weak interactions [40].

3.6 Antioxidant activities and enzyme inhibitions

The enzyme inhibitions and antioxidant activities of jute leaves extract and three inhibitors were determined. The enzyme inhibitions results including xanthine oxidase, PPO, and SOD and antioxidant activities results including DPPH, ABTS, and β -carotene were presented as EC_{50} values in Table 3. Allopurinol, ursolic acid, and ascorbic acid were applied as the positive control for enzyme inhibition and antioxidant activities. It could be found that

Table 2: The affinity constants of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside for xanthine oxidase

No.	Compounds	K_q (10^{12})	K_{sv} (10^4)	R^2	$\lg K_a$	n	R^2
1	Chlorogenic acid	0.124	0.766	0.970	1.96	1.53	0.976
2	Echinacoside	0.125	0.776	0.992	1.41	1.27	0.988
3	Isorhamnetin-3-O-rutinoside	0.067	0.414	0.979	2.04	1.41	0.997

Figure 5: Lineweaver–Burk plots for inhibition of xanthine oxidase by (a) chlorogenic acid, (c) echinacoside, and (e) isorhamnetin-rutinoside. Secondary plots of slopes against concentrations of (b) chlorogenic acid, (d) echinacoside, and (f) isorhamnetin-rutinoside to calculate K_i .

three active compounds showed activities for all of these assays with different degrees. Chlorogenic acid showed

the highest activity in xanthine oxidase and ABTS assays. Echinacoside exhibited the highest activities in both

Table 3: IC₅₀ values for enzyme inhibitions and EC₅₀ values for antioxidant activities of jute leaves extract and three inhibitors

Compounds	Xanthine oxidase	PPO	SOD	DPPH	ABTS	β-Carotene
Jute leaves extract (μg/mL)	7.48 ± 0.15	36.5 ± 1.57	12.7 ± 0.8	21.2 ± 0.2	5.48 ± 0.2	18.9 ± 0.6
Chlorogenic acid (μmol/L)	65.6 ± 0.3	547 ± 5	419 ± 7	110 ± 2	0.511 ± 0.02	1,194 ± 35
Echinacoside (μmol/L)	86.2 ± 1.5	129 ± 3	69.5 ± 2.7	52.3 ± 0.9	1.83 ± 0.03	36.5 ± 2.5
Isorhamnetin-rutinoside (μmol/L)	75.9 ± 0.8	111 ± 4	0.954 ± 0.02	214 ± 2	17.8 ± 0.2	896 ± 5
Allopurinol (μmol/L)	16.2 ± 0.2	—	—	—	—	—
Ursolic acid (μmol/L)	—	50.2 ± 0.15	—	—	—	—
Ascorbic acid (μmol/L)	—	—	1.25 ± 0.05	20.3 ± 0.2	0.86 ± 0.03	22.1 ± 0.1

DPPH and β-carotene assays, and isorhamnetin-rutinoside showed the highest activity in PPO and SOD inhibitions. These results showed that these three inhibitors have many types of antioxidant activities and enzyme inhibitions. Further research could be conducted based on these findings.

3.7 Protective effects on hydrogen peroxide induced oxidative stress

3.7.1 Cytotoxicity

The protective effects of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside on hydrogen peroxide-induced cells (LO2, GES-1 and HUVEC) were employed (Figure 6). The cell viability reduced about 25% after the exposure of hydrogen peroxide (the red column). When some amounts of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside were respectively added into cells during the exposure of hydrogen peroxide, the cell viabilities increased a lot. The protective effect improved with the increasing concentrations of inhibitors. Control tests showed that there was no cytotoxicity of these compounds (data not shown). These results showed that 100 μmol/L of chlorogenic acid, echinacoside, and isorhamnetin-

rutinoside could ameliorate oxidative stress of LO2, GES-1, and HUVEC cells without additional cytotoxicity.

3.7.2 Intracellular ROS

The effects of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside on intracellular ROS level of hydrogen peroxide-induced cells were investigated (Figure 7). The assay results showed that ROS activities of cells were obviously improved in the presence of hydrogen peroxide (nearly 4.7 times). However, the addition of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside apparently decreased the ROS level. The ROS level almost restored to normal when 100 μmol/L of inhibitors were added into the systems. These results showed that chlorogenic acid, echinacoside, and isorhamnetin-rutinoside could relieve the ROS levels of LO2, GES-1, and HUVEC cells induced by hydrogen peroxide.

3.7.3 Intracellular SOD

As an important factor in modulating the ROS level, the activity of SOD in cells should be evaluated [41]. It could be found that hydrogen peroxide treatment obviously impeded the activities of SOD in LO2, GES-1, and HUVEC

Figure 6: Effects of chlorogenic acid (CA), echinacoside (EC), and isorhamnetin-rutinoside (IR) on cell viability of hydrogen peroxide induced (a) LO2, (b) GES-1, and (c) HUVEC cells.

Figure 7: Effects of chlorogenic acid (CA), echinacoside (EC), and isorhamnetin-rutinoside (IR) on intracellular ROS levels of hydrogen peroxide-induced (a) LO2, (b) GES-1, and (c) HUVEC cells.

Figure 8: Effects of chlorogenic acid (CA), echinacoside (EC), and isorhamnetin-rutinoside (IR) on intracellular SOD levels of hydrogen peroxide-induced (a) LO2, (b) GES-1, and (c) HUVEC cells.

cells compared to those in the control group. It was clear that the addition of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside restored the SOD activity in a dose-dependent manner (Figure 8). It suggested that chlorogenic acid, echinacoside, and isorhamnetin-rutinoside could protect the SOD activities in cells.

3.8 Contents of three inhibitors jute samples

For a better understanding of the jute species in the aspect of xanthine oxidase inhibitors, the contents of three screened inhibitors in ten jute samples obtained from our research labs are collected and analyzed. The contents of three inhibitors, chlorogenic acid, echinacoside, and isorhamnetin-rutinoside were evaluated by HPLC and presented in Table 4. For the specific compound, species 319 (7) possessed the highest content of chlorogenic acid. Species 315 (5) possessed the highest content of echinacoside. Moreover, species 447 (10) showed the highest content of isorhamnetin-rutinoside.

The results of the cluster analysis for ten samples using SPSS are shown in Figure 9. It was found that the five species including 313 (3), 317 (6), 319 (7), 445 (8), and

446 (9) could be place in one group due to relatively small distances. The content of echinacoside was not detected in these five species as well, which might become one of the reasons for the classification. Another four species including 306 (1), 314 (4), 315 (5), and 447 (10) could be divided into another group. In these four species, the contents of chlorogenic acid and isorhamnetin-rutinoside were relative higher than that of others. Finally, the species 312 (2) was placed in a separate group

Table 4: The contents of three inhibitors in ten jute samples

No.	Species	Chlorogenic acid (mg/g)	Echinacoside (mg/g)	Isorhamnetin-rutinoside (mg/g)
1	306	4.58	1.08	6.47
2	312	1.40	1.02	2.24
3	313	5.67	0	6.69
4	314	7.04	1.86	6.98
5	315	6.74	2.42	7.29
6	317	5.94	0	4.51
7	319	8.61	0	2.28
8	445	6.35	0	4.38
9	446	7.37	0	4.66
10	447	5.32	1.38	8.85

Figure 9: The cluster analysis of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside in ten samples.

because the contents of three inhibitors were the lowest among ten samples.

The principal component analysis (PCA) was also applied, and the score plot generated between PC1 and PC2 is shown in Figure 10. It could be found in the score plot that ten samples were successfully segregated in three distinct groups: Group one including 317 (6), 319 (7), 445 (8), and 446 (9); Group two 306 (1), 313 (3), 314 (4), 315 (5), and 447 (10); and Group three with 312 (2). These results were much similar with that in the cluster analysis. The only difference was the position of species 313 (3), which was assigned to different groups. It might be due to the rank of three active compounds contents in species 313 (3) was medium. In conclusion, ten jute species showed relatively apparent differences in the

Figure 10: PCA score plots of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside in ten samples.

contents of three inhibitors. The content analysis of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside could become a kind of classification and estimation for jute species.

4 Conclusions

In this study, three compounds were screened out as xanthine oxidase inhibitors from jute leaves extract using UF-LC-MS. The factors affecting screening efficiency were optimized such as concentration of enzyme, incubation time, and temperature. Under the optimum conditions, three inhibitors were identified as chlorogenic acid, echinacoside, and isorhamnetin-rutinoside with UV and MS data. The fluorescent quenching analysis showed three inhibitors could quench the fluorescence intensities of xanthine oxidase. The rank of binding capacity from high to low was isorhamnetin-rutinoside, chlorogenic acid, and echinacoside. The analyses of inhibition kinetics constants of them showed that the inhibition mode of xanthine oxidase of echinacoside was competitive type and that of chlorogenic acid and isorhamnetin-rutinoside was uncompetitive type. These inhibitors also showed good antioxidant activities and enzyme inhibitions with various degrees. The protective effects of inhibitors on hydrogen peroxide-induced LO2, GES-1, and HUVEC cells were conducted in aspects of cell viability, SOD activity, and ROS activity. The content analysis of chlorogenic acid, echinacoside, and isorhamnetin-rutinoside in ten jute species showed that it could become a kind of classification and estimation for jute species. These results could explore more xanthine oxidase inhibitors in jute leaves and its protective effects on oxidative stress. Further research could be launched in future to explore the potentials in food and medical fields.

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