Extraction, Purification, and Biological Activities of Flavonoids from Branches and Leaves of *Taxus cuspidata* S. *et* Z.

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The optimal operational and process parameters were determined for the enzymatic hydrolysis and ultrasound-assisted extraction (EHUE) method of flavonoids extracted from *Taxus cuspidata* branches and leaves (TCBL), and the biological activity of obtained flavonoids was evaluated. According to single factor and central composite design experiments, the optimum key experimental parameters for EHUE were that pectinase enzyme concentration was 0.10 mg·mL^{-1}, enzymatic hydrolysis temperature was 48 °C, and enzymolysis time was 39 min. The yield of flavonoids from TCBL under the optimized conditions was 5.23% ± 0.18%. Four purified flavonoid compounds from TCBL extract were identified as 1) (*E*)-1-methoxy-2-O-(p-coumaroyl)-myo-inositol, 2) catechin, 3) epicatechin, and 4) quercetin-3-O-glucoside. Among the 4 compounds, compounds 2 and 3 showed higher antioxidant capacities, α-amylase, and α-glucosidase inhibitory activities. The statistical analysis showed that epicatechin and catechin were potent antioxidants and active agents for inhibiting type II diabetes. In addition, all 4 compounds exerted clear antitumor activity against MCF-7, Hela, and HepG2 cells. Especially, compound 4 had highest antitumor capacity against MCF-7 and Hela, while compound 1 was best at suppressing the proliferation of HepG2 cells.

Keywords: Taxus cuspidata, Extraction; Purification; Flavonoids; Biological activity

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INTRODUCTION

The genus *Taxus* consists of 24 species and 55 varieties (Spjut 2007), which provides a source of biological and pharmacological active compounds such as taxanes. Paclitaxel is one of the most broadly used anticancer drugs, and there are many other taxane derivatives (Van Rozendaal *et al*. 2000; Hao *et al.* 2011). *Taxus cuspidata* Sieb *et* Zucc. (Japanese yew or spreading yew), which is native to Japan, Russia, Korea, and northeast China, has been used to treat inflammation, diabetes, renal disorders, and cancer as traditional medicine (Bajpai *et al*. 2014). More than 100 taxane compounds have been identified from this yew, including various skeletons (Kobayashi and Shigemori 1999, 2002; Shigemori and Kobayashi 2004), but there are still new taxane compounds being isolated (Li *et al.* 2013). Moreover, *Taxus* spp. contain numerous other compounds with pharmacological and biological activities other than taxane diterpenoids. A variety of fatty acids obtained from *T. chinensis* leaves have been identified using gas chromatography-

mass spectrometry (GC-MS) (Tang *et al.* 2013). Yew alkaloid toxoids, which are commercially available, were quantified and discerned by liquid chromatography-mass spectrometry/ mass chromatography (LC-MS/MS) (Grobosch *et al.* 2013). Lignins and flavonoids have been isolated from *Taxus* (Tezuka *et al.* 2011). A new kind of heteropolysaccharide has been separated and purified from *T. yunnanensis* leaves using anion-exchange chromatography and GPC (Yan *et al.* 2013).

The extracts from *Taxus* have been reported to possess potent antidiabetic, antitumor, and immunity enhancing activities (Wu and Wu 2009; Zhang *et al.* 2012; Zheng *et al.* 2014). Phenolic compounds from *T. cuspidata* exert clear anticancer activity against Jurkat, MCF-7, T24, HT-29, and Hela cells (Elansary *et al.* 2019). Flavonoid extracts of *T. yunnanensis* could be underlying candidates for development of paclitaxel oral formulation, as they inhibit CYP3A4 and P-gp activity concurrently (Li *et al.* 2013). Aqueous extract from *T. baccata* has potent antitumor activities due to its inhibitory activity against adenosine deaminase (Durak *et al.* 2014). The alcohol extract of *T. baccata* has anti-inflammation activity, such as decreasing bronchial hyperreactivity and bronchodilating activity (Dutta *et al.* 2010; Patel *et al.* 2011).

The main purpose of this study was to develop an effective way to extract flavonoids from *T. cuspidata.* Ultrasound-assisted extraction intensifies cell disruption, improves mass transfer, and accelerates the solvent osmotic effect (Cai *et al.* 2014). In a previous study (Jiang *et al.* 2017a), the optimum process conditions of ultrasound-assisted extraction were an ethanol concentration of 60%, extraction temperature of 60 °C, extraction time of 60 min, liquid to material of 19:1 (v/w), and ultrasonic power of 168 W. In this study, enzymatic hydrolysis was added before ultrasound-assisted extraction, and an enzymatic hydrolysis and ultrasound-assisted extraction (EHUE) method was developed to increase the yield of flavonoids from *T. cuspidata.*

In this study, experimental design was used to determine the optimum values for the process variables, including enzymatic hydrolysis temperature, enzymolysis time, and enzyme concentration. Furthermore, the crude flavonoids were separated and purified with macroporous adsorption resin and Sephadex LH-20 column chromatography. The antioxidant activities of flavonoids from *Taxus cuspidata* branches and leaves (TCBL) were evaluated by radical scavenging of DPPH, and the *in vitro* inhibition of α-amylase and α-glucosidase activities were examined for antidiabetic properties. The anticancer properties of purified flavonoids were evaluated by inhibitory activities against MCF-7, Hela, and HepG2 cells *in vitro*.

EXPERIMENTAL

Material

Taxus cuspidata branches and leaves (moisture content: 6.33%) were harvested in April 2017 in Rongcheng, China. TCBL materials were crushed and air-dried in the shade then finely ground to particles (approximately 425 μm in diameter) using a multi-function pulverizer. Pectinase enzyme was purchased from Congdien Biological Co., Ltd in Shandong, China.

Extractions

The yield of flavonoids extracted by traditional solvent extraction method, ultrasound-assisted extraction method, and enzymatic hydrolysis and ultrasound-assisted

extraction method were compared. Each extraction method was as follows:

1. Traditional solvent extraction: 3 g TCBL was weighed into a 100 mL conical flask, to which 42 mL of aqueous solution was added. The TCBL solution was treated with water bath at 50 °C for 40 min and filtrated. Then the filtrate was transfer to a 100 mL volumetric flask. The residue was extracted under the same conditions with 60% ethanol solution and then filtrated. The filtrates were combined, and the total volume was made constant to 100 mL with 60% ethanol.

2. Ultrasound-assisted extraction: 3 g TCBL was weighed into a 100 mL conical flask, to which 42 mL of 60% ethanol solution was added, with sonication for 40 min at an ultrasonic power of 144 W. The solution was filtrated and transferred into a 100 mL volumetric flask. The residue was extracted in water bath with 60% ethanol solution under the same conditions and then filtrated. The filtrates were combined, and total volume was constant to 100 mL with extract solution.

3. Enzymatic hydrolysis and ultrasound-assisted extraction: 3 g TCBL was weighed into a 100 mL conical flask, to which 42 mL 0.1 mg/mL pectin enzyme solution was added (Jiang *et al*. 2019). The TCBL solution was treated with water bath at 50 °C for 40 min and filtrated. The filtrate was transferred into a 100 mL volumetric flask, and residue was extracted in water bath with 60% ethanol solution and sonicated for 40 min at an ultrasonic power of 144 W and then filtrated. The filtrates were combined, and total volume was made constant to 100 mL.

Single Factor Experiment

Three grams of TCBL powder was dissolved in 42 mL pectinase enzyme solution with water bath and then extracted with ultrasound. The influence factors of different enzymolysis time, enzymatic hydrolysis temperature, and enzyme concentration were studied to develop the EHUE method.

Response Surface Optimization

Based on the result of single factor experiment, enzymolysis time, enzymatic hydrolysis temperature, and enzyme concentration were selected to design response surface experiments with 3 factors and 3 levels.

Determination and Separation of Flavonoids

The 60% ethanol extract of TCBL was diluted 10 times, and the absorbance of diluent was measured by the $NaNO_2$-Al $(NO_3)_3$ colorimetric method (Han *et al.* 2015).

Desiccative and pulverized TCBL were extracted 3 times with 60% ethanol, and the extract solution was concentrated under reduced pressure to evaporate the ethanol. The extract was separated with different polarity solvents: petroleum ether, dichloromethane, ethyl acetate, n-butanol, and water for 5 times, respectively. The ethyl acetate extract was further isolated due to its highest antioxidant activity. Crude flavonoids from ethyl acetate extract were separated into several fractions by the macroporous adsorption resin method to remove pigments and other substances. Afterwards, components with higher flavonoid content were chromatographed over a Sephadex LH-20 column (Shanghai, China) eluted with water/methanol for purification.

Chromatographic analysis of flavonoids was performed on HPLC (Agilent 1200 Series, California, USA) using a C18 Column (100 mm × 4.6 mm × 3.5 μm Aglient Eclipse Plus C18) maintained at 25 °C. The mobile phases were 0.1% deionized acetic acid (A) and methanol (B) at a flow rate of 0.5 mL·min^{-1} with the following gradient programs: 10%

to 30% B (0 to 15min), 30% to 60% B (15 to 25 min), and 60% to 10% B (25 to 30 min). The eluent was detected at a wavelength of 280 nm. The injection volume was 5 μL for every sample (Jiang *et al.* 2017b).

Antioxidant Activity of Flavonoids from TCBL

The antioxidant ability of sample solution was measured by calculating the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging rate (Rosa *et al.* 2011), and the absorbance value was determined at 517 nm. Vitamin C (Energy Chemical, Shanghai, China) was used as the control group.

Antidiabetic Activity of Flavonoids from TCBL

The antidiabetic activity of purified flavonoids was tested by α-amylase and α-glucosidase inhibitory activity (Jiang *et al.* 2017b). Acarbose (Bayer Healthcare Company Ltd, Beijing, China) was selected as the control group.

Anticancer Activity of Flavonoids from TCBL

The antitumor activity of purified flavonoids was examined in different cancer cell lines (MCF-7, Hela, and HepG-2) by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Jiang *et al*. 2019). The antitumor drug etoposide (Beyotime Biotechnology, Nanjing, China) was selected as a control.

Statistical Analyses

Statistical analyses were conducted using Origin 8 software (OriginLab, Northampton, USA). Data were expressed as mean ± SD. The response surface optimization experimental design was performed by Box-Behnken model of Design-Expert 8.0.6.1 software (Statease, Minneapolis, USA). All determinations were repeated 3 times. When p-value <0.05 was considered significant different.

RESULTS AND DISCUSSION

Comparison of Different Enzymatic Hydrolysis and Ultrasound-Assisted Extraction (EHUE) Methods

Table 1 shows that the highest total flavonoids yield (5.16%) was using pectinase aqueous solution after water bath and then ultrasonic extraction with 60% ethanol solution.

Table 1. Comparative Results of Different Enzymatic Hydrolysis and Ultrasound-assisted Extraction (EHUE) Methods

Trial Number	Methods	Total Flavonoids Yield (w/w dry material %)
1	Aqueous solution water bath treatment + 60% ethanol solution water bath extraction	4.08±0.07
2	60% ethanol solution ultrasonic treatment	4.86±0.02
3	Enzyme aqueous solution water bath treatment +60% ethanol solution ultrasonic extraction	5.16±0.05

The yield was 26.47% higher than that of the traditional solvent extraction method (water bath treatment + 60% ethanol solution water bath extraction) and 6.17% higher than that of ultrasound-assisted extraction (60% ethanol solution ultrasonic treatment + 60% ethanol solution ultrasonic extraction). Due to the high content of pectin and viscose resin in the TCBL, it was very difficult to filter the solution by enzyme treatment and ultrasound at the same time, and the yield was decreased by 3.61% (data not shown). Therefore, ultrasonic extraction of pectinase from TCBL by water bath and then 60% ethanol solution with ultrasound-assisted coupling was the best method for the extraction of flavonoids, pectinase. The extraction process of this method was studied.

Results of Single Factor Experiments of Flavonoids Extraction Process

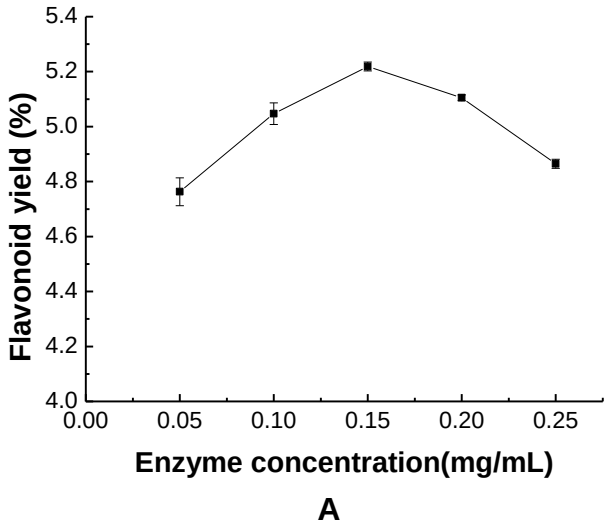
Effects of enzyme concentration on total flavonoids yield

Enzymatic hydrolysis was first carried out at 50 °C and 40 min at different enzyme concentrations (0, 0.05, 0.1, 0.15, 0.2, and 0.25 $mg \cdot mL^{-1}$). The second extraction was sonicated with 60% ethanol at 144 W for 40 min. The effects of different enzyme concentration on total flavonoids yield extracted from TCBL by EHUE are shown in Fig. 1(A). When the enzyme concentration was between 0.05 and 0.15 $mg \cdot mL^{-1}$, the yield of total flavonoids increased gradually as the enzyme concentration increased. When the enzyme concentration reached 0.15 $mg \cdot mL^{-1}$, the total flavonoids yield was the maximum of 5.20%; after the concentration exceeded 0.15 $mg \cdot mL^{-1}$, the yield began to diminish. The possible reason is that with the increasing of pectinase dosage, pectinase decomposes pectin into small molecular substances in the TCBL, making cell wall loose, reducing the diffusion resistance, making flavonoids fully dissolved, and thus increasing the yield of total flavonoids. However, as the concentration of pectinase continued to increase, large doses of pectinase hydrolyzed pectin in the cell wall of TCBL. The hydrolysate produced by pectinase had strong adsorption on flavonoids, but the yield of total flavonoids decreased (Wang and Liu 2004). Therefore, the concentration of pectinase at 0.15 $mg \cdot mL^{-1}$ (2.24 mg/g of the dry weight of the raw material) was initially determined as the optimal enzymatic concentration of the coupling of enzymatic and ultrasound-assisted extraction method for the total TCBL flavonoids.

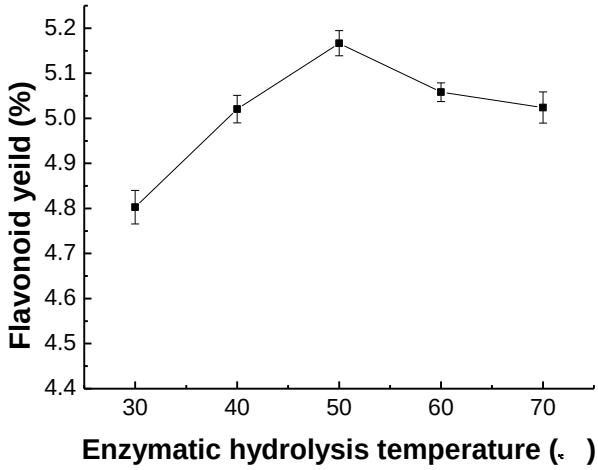
Effects of Enzymatic Hydrolysis Temperature on Total Flavonoids Yield

The pectinase concentration of 0.15 $mg \cdot mL^{-1}$ (2.24 mg/g of absolute dry weight of raw materials) was selected, and the enzymatic hydrolysis time was 40 min, under different enzymatic hydrolysis temperatures. The second extraction was then sonicated with 60% ethanol at 144 W for 40 min. The effects of enzymatic hydrolysis temperatures at 30, 40, 50, 60 and 70 °C, respectively, on total flavonoids yield from TCBL were investigated. The results are shown in Fig. 1(B).

From Fig. 1(B), as enzymatic hydrolysis temperature increased in the range of 30 to 50 °C, the yield of total flavonoids gradually increased. The yield reached the maximum of 5.17% when the enzymatic hydrolysis temperature was 50 °C. When the temperature went beyond 50 °C, the total flavonoids yield started to decrease gradually. This might be due to the fact that the optimum temperature of pectinase was 40 to 50 °C in general and the activity of pectinase was the best in this temperature range. When the temperature of enzymatic hydrolysis was from 30 °C to 50 °C, the activity of pectinase gradually increased with temperature rising.



A



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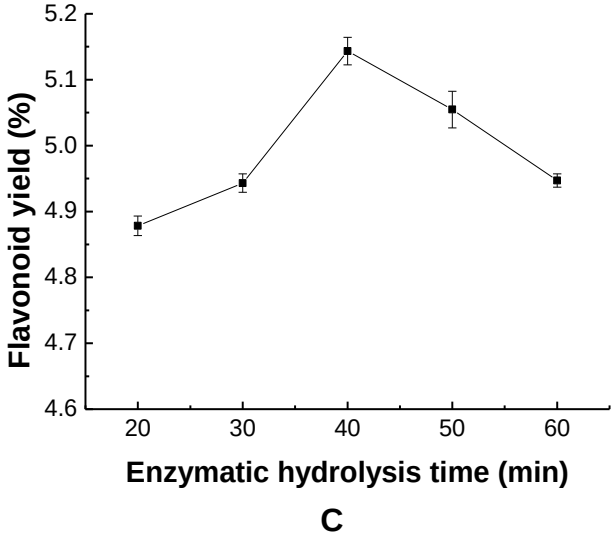


C

Fig. 1. Effect of different conditions on total flavonoids yield

The extraction rate gradually increased as a result; but when the temperature of enzymatic hydrolysis was over 50 °C, with the increase in temperature, the yield of total flavonoids in TCBL began to decrease, which might be due to the heat denaturation of enzyme proteins and the decrease of pectinase activity caused by high temperature, thus resulting in the decrease of the extraction rate of total flavonoids in TCBL. Therefore, 50 °C was judged to be the optimal temperature to extract total flavonoids from TCBL by EHUE.

Effects of Enzymolysis Time on Total Flavonoids Yield

The pectinase concentration of 0.15 mg·mL^{-1} (2.24 mg/g of absolute dry weight of raw materials) was selected. The enzymatic hydrolysis was carried out at 50 °C under different enzymatic hydrolysis times. The second extraction was then sonicated with 60% ethanol at 144 W for 40 min. Finally, the two portions of extract were combined. The effects of different enzymatic hydrolysis time (20, 30, 40, 50, and 60 min) on yield of total flavonoids from TCBL by EHUE method were explored. The results are shown in Fig. 1(C). As the enzymatic hydrolysis time reached 40 min, the total flavonoids yield reached to the maximum value 5.14%. Within 20 min to 40 min, the yield of total flavonoids gradually increased with enzymatic hydrolysis time increasing, which might due to the full utilization of the catalytic ability of the enzyme. When time was more than 40 min, the yield of total flavonoids began to decrease, which may due to the long enzymatic hydrolysis time leading to the inactivation of part of pectinase, thus reducing the yield of total flavonoids. Therefore, 40 min was adopted as the optimal enzymatic hydrolysis time.

Table 2. Results of Response Surface Analysis

Run.	X_1 (Enzyme concentration, mg·mL^{-1})	X_2 (Enzymatic hydrolysis temperature, °C)	X_3 (Enzymolysis time, min)	Total flavonoids yield(%)
1	0.15	60	50	4.93
2	0.20	50	30	4.74
3	0.10	60	40	5.04
4	0.15	40	50	4.81
5	0.10	50	50	4.98
6	0.15	50	40	5.06
7	0.15	40	30	4.88
8	0.20	40	40	4.72
9	0.20	50	50	4.78
10	0.20	60	40	4.92
11	0.15	50	40	5.05
12	0.15	60	30	4.92
13	0.15	50	40	5.08
14	0.10	50	30	5.03
15	0.15	50	40	5.05
16	0.15	50	40	5.07
17	0.10	40	40	5.08

Analysis of Optimal Extraction Process by Response Surface Methodology

Experimental design was carried out according to Box-Behnken model (Hou *et al*. 2013), based on results of single-factor experiment and results were displayed in Table 2.

The quadratic polynomial regression equation is shown in Eq. 1.

$$Y=5.06-0.12X_1+0.04X_2-8.75*10^{-3}X_3+0.060X_1X_2+0.022X_1X_3+0.020X_2X_3-0.062X_1^2-0.060X_2^2-0.12X_3^2 \quad (1)$$

To test the accuracy of the regression equation, further statistical analysis was conducted on the test results in Table 2, and the total flavonoids yield response surface variance analysis results were obtained as shown in Table 3.

Table 3. Variance Analysis for Extraction Yield of Total Flavonoids

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Value	p-Value	Significant
model	0.25	9	0.027	191.38	<0.0001	**
X_1	0.12	1	0.12	819.19	<0.0001	**
X_2	0.013	1	0.013	89.15	<0.0001	**
X_3	6.125×10^{-4}	1	6.125×10^{-4}	4.27	0.0777	
X_1X_2	0.014	1	0.014	100.30	<0.0001	**
X_1X_3	2.025×10^{-3}	1	2.025×10^{-3}	14.10	0.0071	**
X_2X_3	1.6×10^{-3}	1	1.6×10^{-3}	11.14	0.0124	*
X_1^2	0.016	1	0.016	113.64	<0.0001	**
X_2^2	0.015	1	0.015	104.70	<0.0001	**
X_3^2	0.058	1	0.058	403.18	<0.0001	**
residual	1.005×10^{-3}	7	1.436×10^{-4}			
lack of fit	3.250×10^{-4}	3	1.083×10^{-4}	0.64	0.6295	
pure error	6.800×10^{-4}	4	1.700×10^{-4}			
correlation total	0.25	16				

X_1: enzyme concentration, X_2: enzymatic hydrolysis temperature, X_3: enzymolysis time
* Significant difference ($0.01 < p < 0.05$); ** Significant difference ($p < 0.01$).

As shown in Table 3, the lack of fit p-value ($0.6295 > 0.05$) was non-significant, and $p < 0.0001$ indicated that the regression equation was significant. The determination coefficient $R^2 > 0.9$ demonstrated that the model was well-fitted (Zou *et al.* 2012). Thus, the regression equation was reliable to determine the optimal flavonoids extracting process.

In the regression model, the p-value of enzyme concentration (X_1) and enzymatic hydrolysis temperature (X_2) was less than 0.0001, which indicated the effect on the extraction rate of total flavonoids from TCBL was significant. The main factors influencing total flavonoids yield extracted from TCBL by enzymatic hydrolysis and ultrasound-assisted extraction were: enzyme mass concentration (X_1) > enzymatic hydrolysis temperature (X_2) > enzymolysis time (X_3). The interaction terms X_1X_2 and X_1X_3 had an extremely significant effect, while the interaction term X_2X_3 had a significant effect. The quadratic terms X_1^2, X_2^2, and X_3^2 had an extremely significant effect, indicating that the factors had a significant interaction effect rather than a simple linear relationship on the total flavonoids yield ($p < 0.0001$). The results of response surface methodology are shown in Fig. 2.

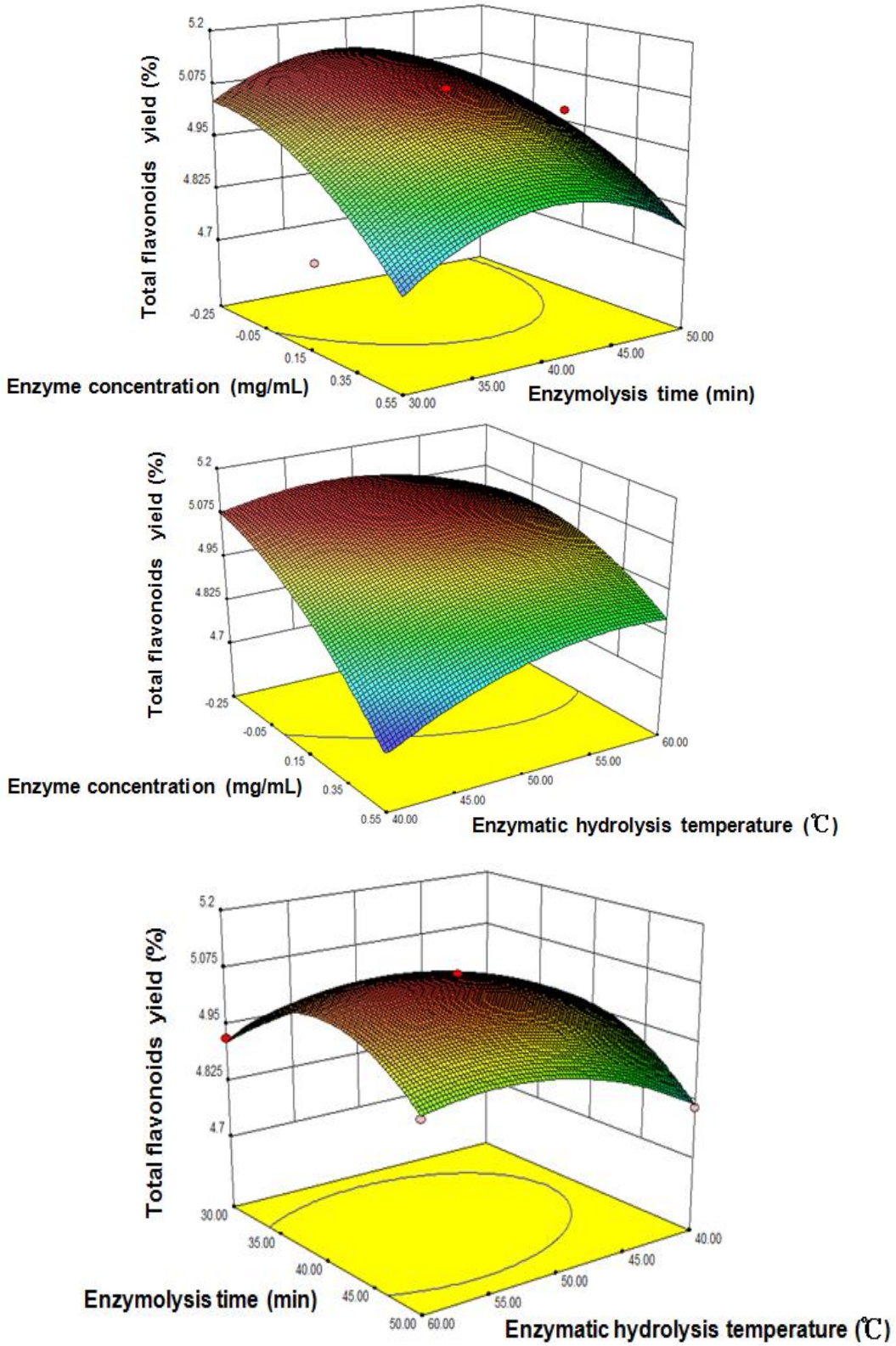


Fig. 2. Results of response surface experiments

In the response surface diagram, the steeper the surface was, the greater the effect this factor had on total flavonoids yield (Jiang *et al.* 2017a). As shown in Fig. 2, the surface of enzyme mass concentration and enzymatic hydrolysis temperature was steep, indicating

that these two factors had a significant influence on the yield of total flavonoids, while the surface of enzymatic hydrolysis time was relatively smooth, indicating that the influence was weak. Through analysis of response surface software, optimal extracting conditions were obtained: enzyme mass concentration of 0.1 mg·mL^{-1} (enzyme accounted for 1.49 mg g^{-1} of the absolute dry weight of the raw material), enzymatic hydrolysis temperature of 48 °C, enzymatic hydrolysis time of 39 min. Under these conditions, the predicted value of total flavonoids in TCBL was 5.13%. In order to test whether the experimental results were consistent with the real situation, 6 confirmatory tests were conducted on the optimal extraction technology conditions, and the actual yield of total flavonoids extracted from TCBL was 5.28%, 5.34%, 5.19%, 5.24%, 5.10%, and 5.25%, respectively. The average actual value was 5.23%, which had a relatively small error compared with the predicted data. Moreover, the measurement results were stable and had good repeatability, indicating that the process conditions obtained by the response surface optimization were reliable. Therefore, the optimal technological conditions for enzymatic hydrolysis and ultrasound-assisted extraction were 0.1 mg·mL^{-1} mass concentration enzyme solution (the absolute dry weight of the enzyme in the raw material was 1.49 mg g^{-1}), the enzymatic hydrolysis temperature was 48 °C, and the enzymatic hydrolysis time was 39 min.

Purified flavonoids from TCBL

The 5 g ethyl acetate extract was measured, and after gradient elution by AB-8 macroporous adsorption resin, 16 separated components Fr1-Fr16 were obtained with a total recovery rate of 85.07%.

Through HPLC analysis, the chemical components of Fr5 and Fr7 were relatively small, and there were substances with large peak areas, which are relatively pure. Therefore, Fr5 and Fr7 were selected for further separation and purification.

Compound 1 and compound 2 were obtained from Fr7 by water elution and 40% methanol elution of LH-20 glucan gel chromatographic column, while compound 3 was obtained from Fr5 by 60% methanol elution of LH-20 glucan gel chromatographic column. At the same time, a relatively pure compound 4 was also isolated from Fr5. Then HPLC-MS, UV, IR, and NMR were performed regarding the structural characterization of chemicals.

Structural Characterization of Compound 1

Compound 1 is yellow powder with a melting point of 120 to 122 °C. The UV absorption spectrum in Fig. 3 shows that the compound had a maximum absorption wavelength at 235 and 311 nm, and the liquid phase shows a single peak at the retention time t_R=13.033 min (Fig. S1). The purity of compound 1 was calculated to be more than 95% by area normalization. According to mass spectrogram 8, the mass charge ratio of the excimer ion peak $[M-H]^-$ of the compound was 339, so the molecular weight of the compound was 340. As shown in the infrared spectrum, 1629 cm^{-1} may be the absorption peak of the compound C=C, the wide peak of 3403 cm^{-1} may be the stretching vibration peak of -OH, 1701 cm^{-1} may be the stretching vibration peak of the ester carbonyl group, 2927 cm^{-1} may be the antisymmetric stretching vibration peak of CH in -O-CH_3, and 1604 cm^{-1}, 1517 cm^{-1}, and 1439 cm^{-1} may be the absorption peaks of C=C on the benzene ring. The molecular formula of this compound is $C_{16}H_{20}O_8$, which is a new compound, named (E) -1-methoxy-2-o-(p-coumaroyl)-myo-inositol (Wu *et al.* 2010). Its structure is shown in Fig. 6.

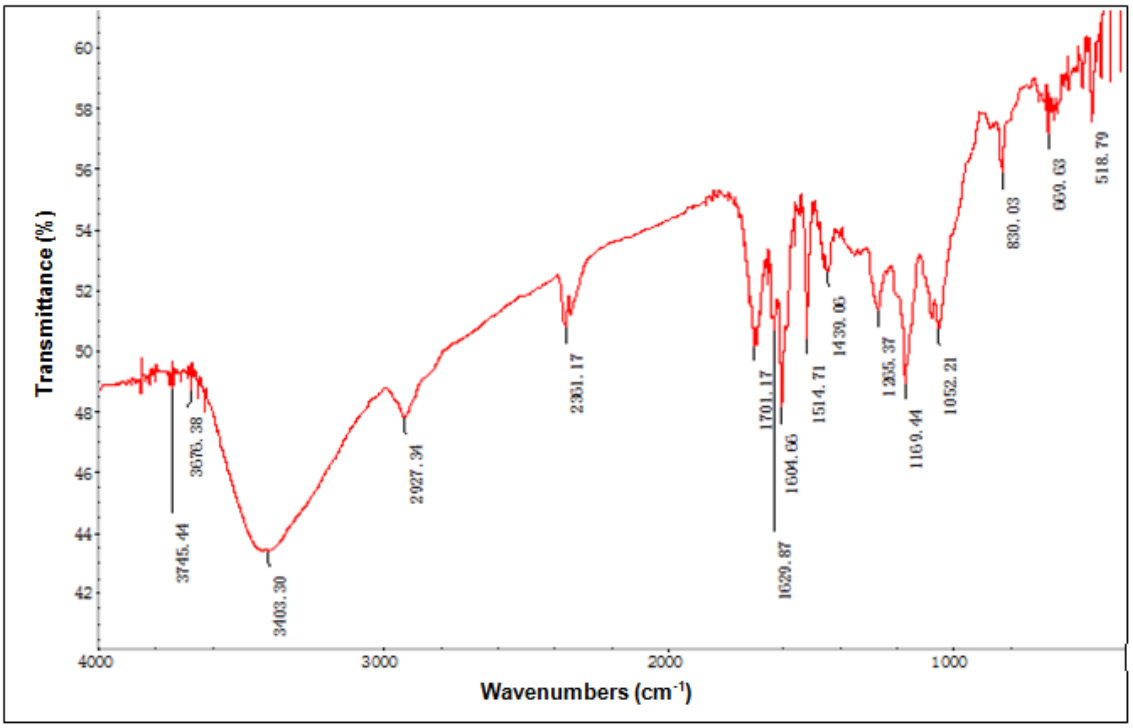


Fig. 3. FTIR spectrum of compound 1

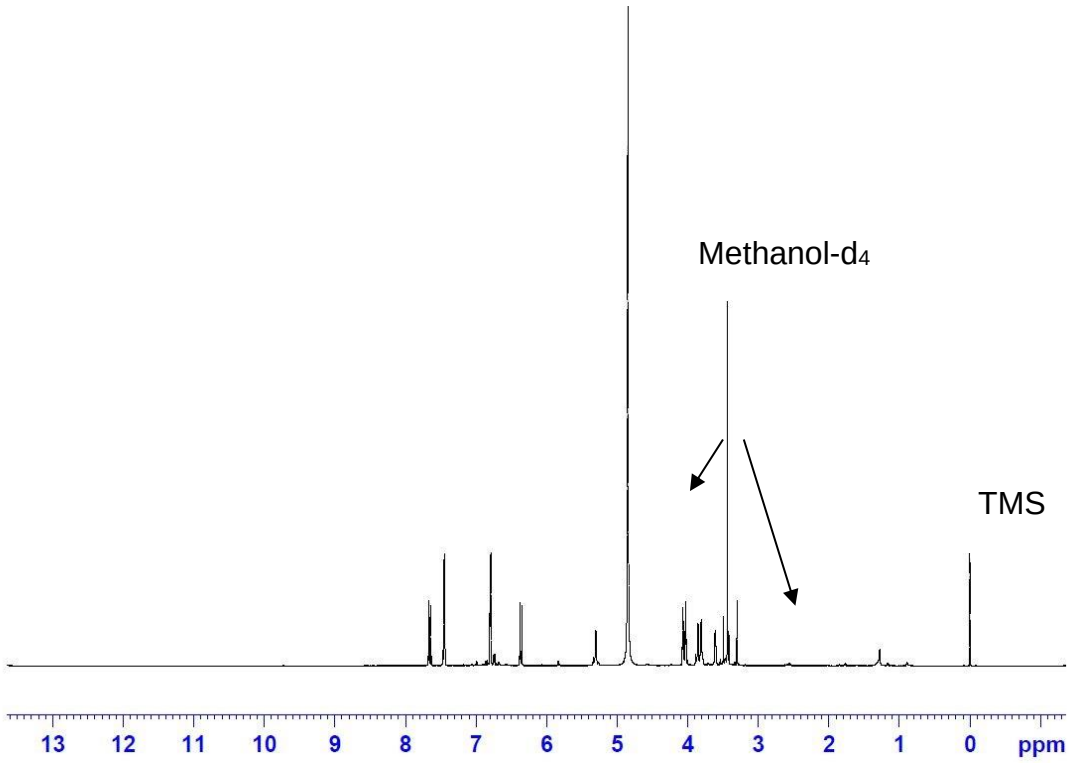


Fig. 4. ^{1}H NMR spectrum of compound1

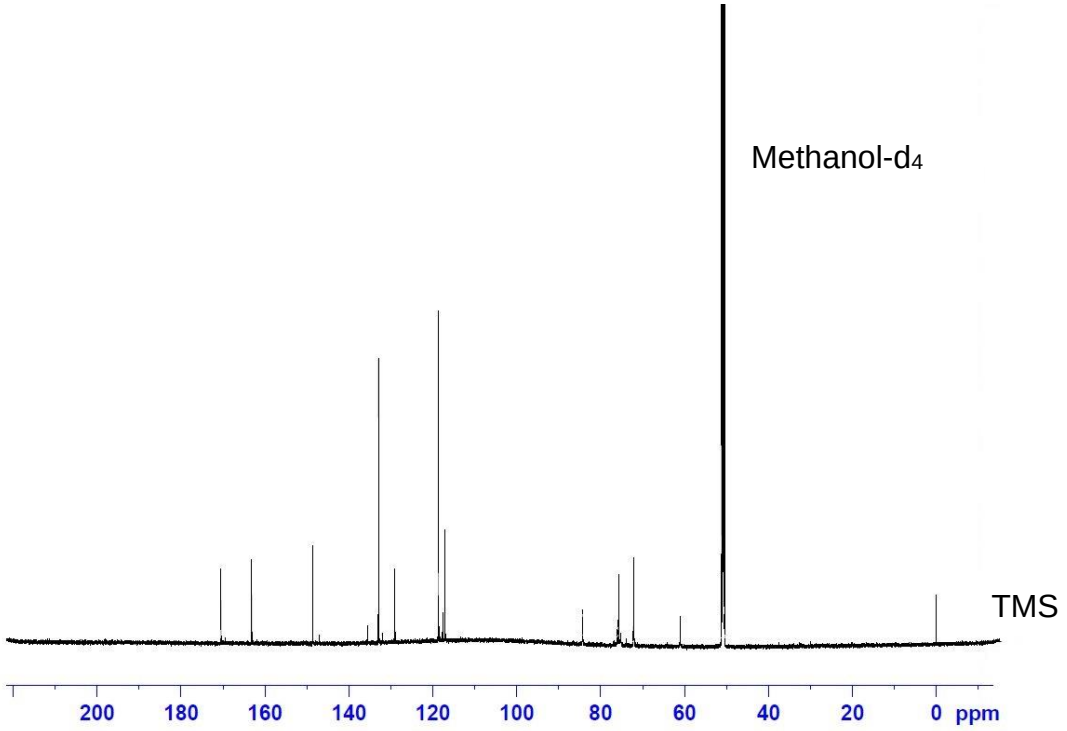


Fig. 5. ^{13}C NMR spectrogram of compound1

1H-NMR spectrum information: 1H NMR (700 MHz, methanol-d_4):Delta TMS [3.53 H-1, dd, J=3.2, 6.5 Hz), 5.33 (H-2, dd, J=3.2, 6.7 Hz), 4.00 (H-3, dd, J=6.7, 11.6 Hz), 3.85 (H-4, dd, J=6.6, 11.6 Hz), 3.70 (H-5, dd, J=6.6, 13.3 Hz), 4.07 (H-6, DDD, J=3.2, 8.1, 13.3 Hz].7.45 [H-2', d, J=9.8 Hz), 6.88 (H-3', d, J=9.8 Hz), 6.88 (H-5', d, J=9.8 Hz], the delta 7.45 [H-6 ', d, J=9.8 Hz), 7.62 (H-7', d, J=18.1 Hz), 6.66 (H-8', d, J=18.1 Hz), 3.49 (1-OCH_3, s)

13C-NMR spectrum information: 13C NMR (175 MHz, methane-d_4): δ_{TMS} 84.2 (C-1), 75.2 (C-2), 72.1 (C-3), 75.6 (C-4), 75.2 (C-5), 72.0 (C-6), 128.9 (C-1'), 132.9 (C-2'), 118.4 (C-3'), 163.1 (C-4'), 118.4 (C-5'), 132.9 (C-6'), 146.9 (C-7'), 117.8 (C-8'), 170.4 (C-9'), 61.0 (1-OCH_3). The data above basically agreed with the literature (Wu *et al*. 2010).

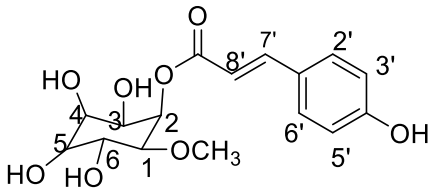


Fig. 6. Structure of compound 1

Structural Characterization of Compound 2

Compound 2 is dark brown powder with a melting point of 236 to 237 °C. Figure S4 shows that there is a maximum absorption peak at 234 and 278 nm, and Fig. S5 shows that the liquid phase diagram has a single peak at the retention time t_R=14.367 min. According to the mass spectrogram in Fig. S6, the mass charge ratio of the excimer ion peak $[M-H]^-$ of the compound was *m*/*z*=289, so the molecular weight of the compound was 290.

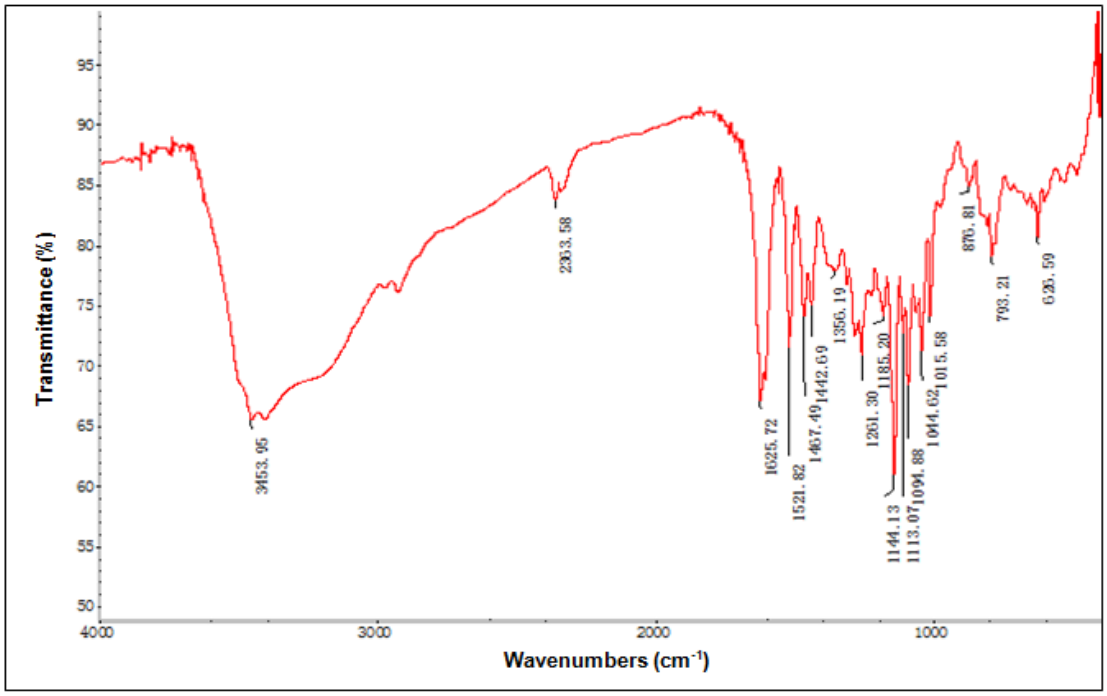


Fig. 7. FTIR spectrum of compound 2

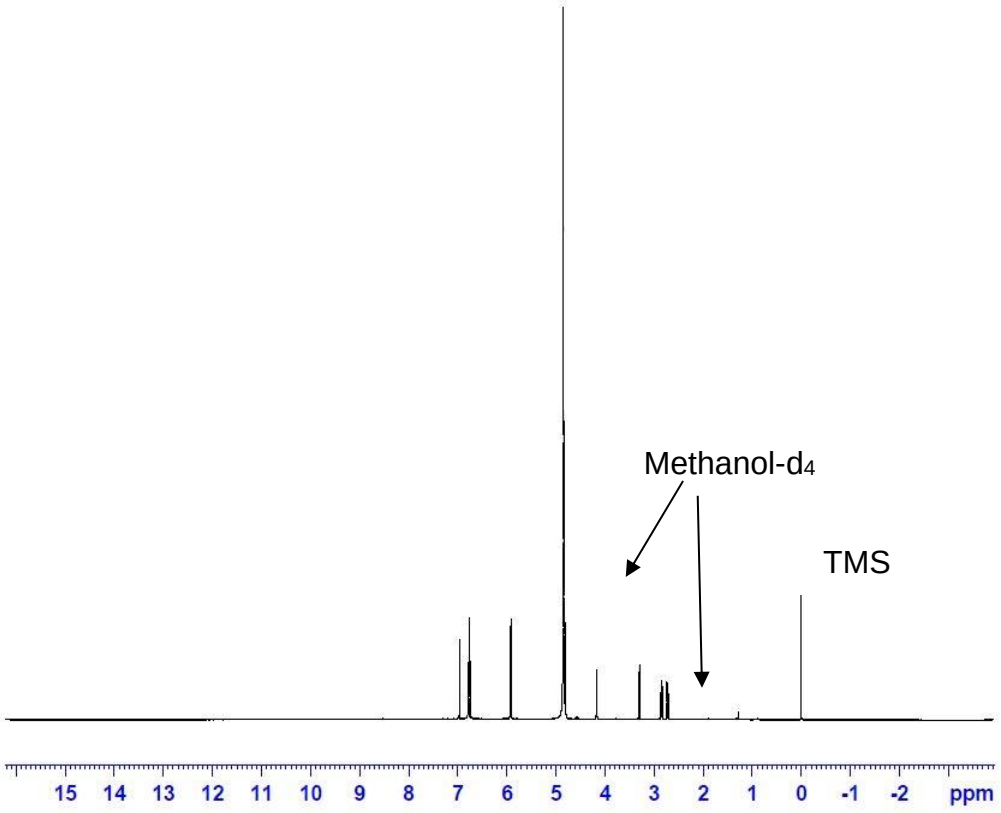


Fig. 8. ^{1}H spectrum of compound 2

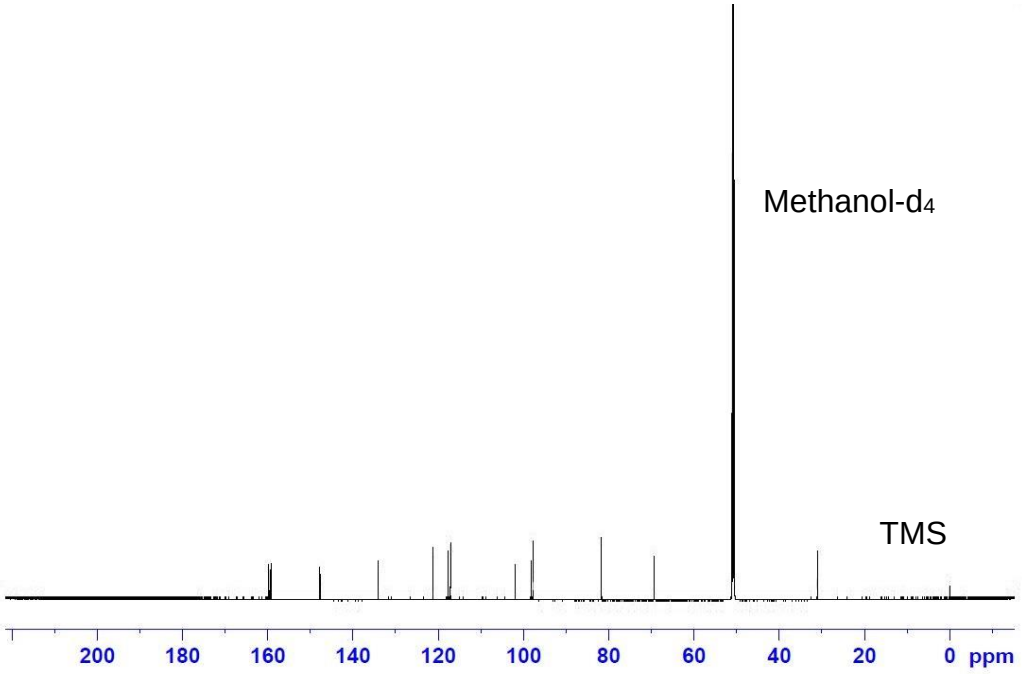


Fig. 9. ^{13}C NMR spectrogram of compound 2

According to the infrared spectrum in Fig. 7, the wide peak of 3453 cm^{-1} may be the stretching vibration peak of -OH, 1356 cm^{-1} may be the in-plane deformation vibration of phenolic hydroxyl, 1144 cm^{-1} may be the stretching vibration of C-O bond of phenolic hydroxyl, and 1625 cm^{-1}, 1521 cm^{-1}, 1469 cm^{-1}, and 1442 cm^{-1} may be the absorption peaks of carbon-carbon double bond on the benzene ring. The molecular formula of this compound is $C_{15}H_{14}O_6$, which is epicatechin. The structure is shown in Fig.10.

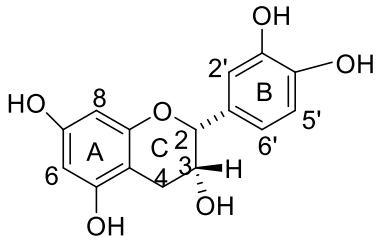


Fig. 10. Structure of compound 2

1H-NMR spectrum information: 1H NMR (700 MHz, methanol-d_4): δ_{TMS} 4.55 [H-2, m], 4.21 [H-3,ddd,J=3.2,4.6,1.6Hz],2.48[H-4a,ddd,J=4.6,16.1, 0.8Hz], 2.82 [H-4b, ddd, J=3.2,16.1, 0.9Hz], 5.85 [H-6, d, J=2.2Hz], 5.92 [H-8, d, J=2.2Hz], 7.03 [H-2′, d, J=1.8Hz], 6.75 [H-5′, d, J=8.1Hz], 6.70 [H-6′, ddd, J=1.8 , 8.1 ,0.7Hz]

13C-NMR spectrum information: 13C NMR (175 MHz, methanol-d_4): δ_{TMS} 81.6 (C-2), 69.2 (C-3), 31.0 (C-4), 101.8 (C-4a), 159.4 (C-5), 97.6 (C-6), 159.8 (C-7), 98.1 (C-8), 159.1 (C-8a), 134.0 (C-1'), 117.0 (C-2'), 147.7 (C-3'), 147.5 (C-4'), 117.6 (C-5'), 121.1 (C-6'). The data above basically agreed with the literature (Cren-Olivé *et al.* 2002; Davis *et al.* 2015).

Structural Characterization of Compound 3

Compound 3 was a white powder with a melting point of 160 to 162 °C. According to Fig. S7, compound 3 had the maximum absorption peaks at 235 and 278 nm. As shown in the liquid phase (Fig. S8), there was a single peak at the retention time t_R=6.567 min, and the purity of compound 3 was calculated to be more than 95% by area normalization. According to mass spectrometry in Fig. S9, the compound presented *m*/*z* 289 $[M-H]^-$ as precursor ion in negative ionization mode, showing molecular weight 290 of the compound.

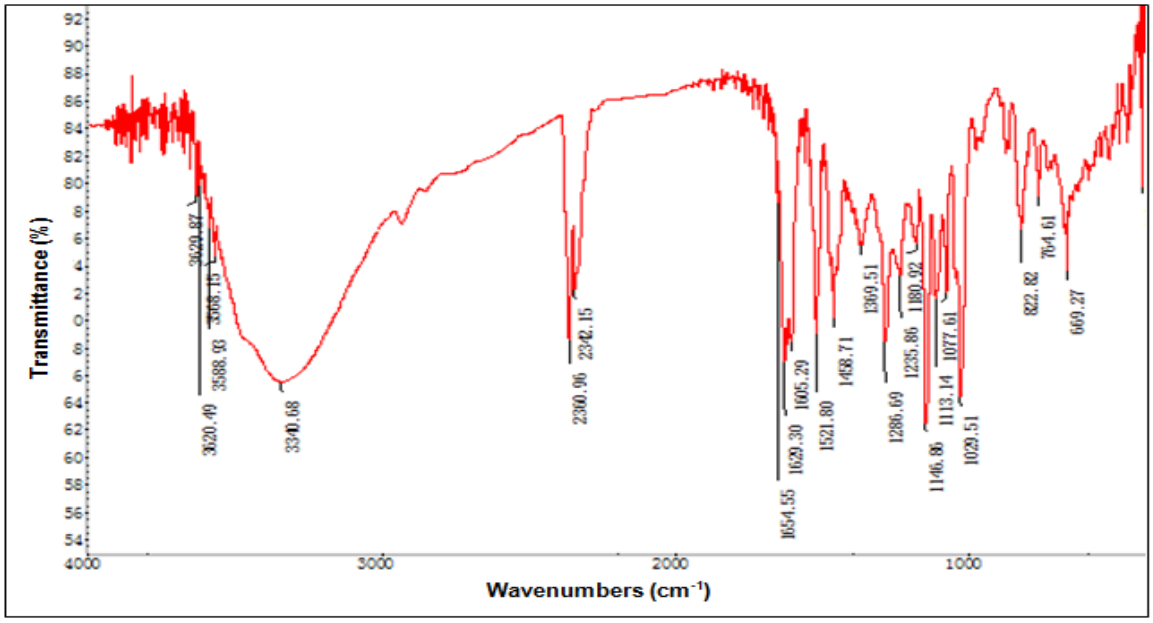


Fig. 11. FTIR spectrum of compound 3

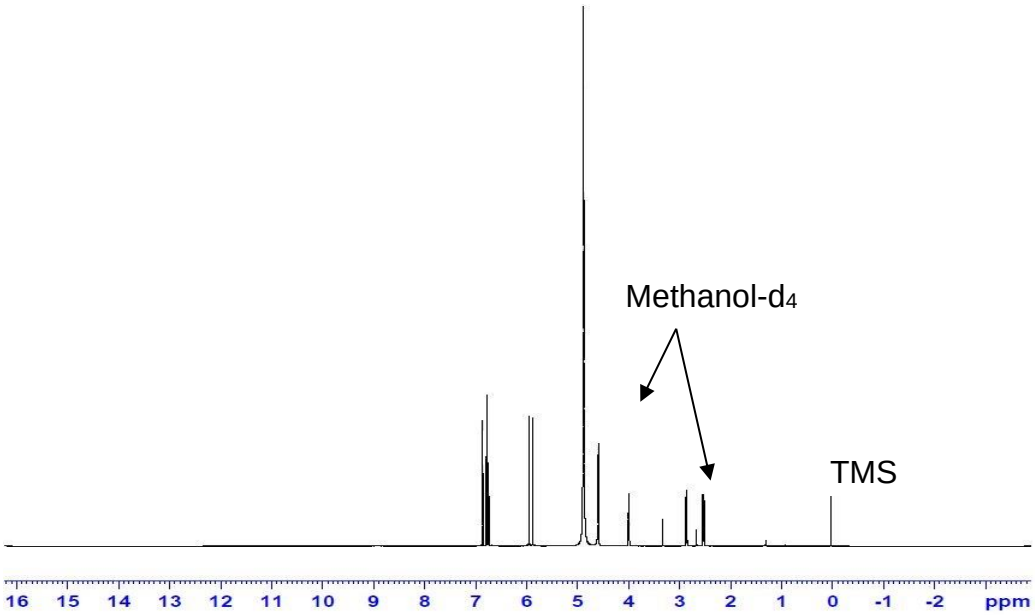


Fig. 12. ^{1}H spectrum of compound 3

As shown in the infrared spectrum in Fig. 11, the wide peak of 3340 cm^{-1} may be the stretching vibration peak of -OH, 1369 cm^{-1} may be the in-plane deformation vibration of phenolic hydroxyl, 1146 cm^{-1} may be the stretching vibration of C-O bond of phenolic hydroxyl, and 1629 cm^{-1}, 1605 cm^{-1}, 1521 cm^{-1}, and 1458 cm^{-1} may be the characteristic absorption peaks of C=C on the benzene ring. The molecular formula of this compound is $C_{15}H_{14}O_6$, which is catechin, and its structure is shown in Fig.14. Compound 3 was also analyzed by TLC thin plate chromatography in Fig. S10. Chloroform: methanol 8:2 was used as the developing agent to draw fluorescent spots under ultraviolet light. Iodine/silica gel color developer was used for color development.

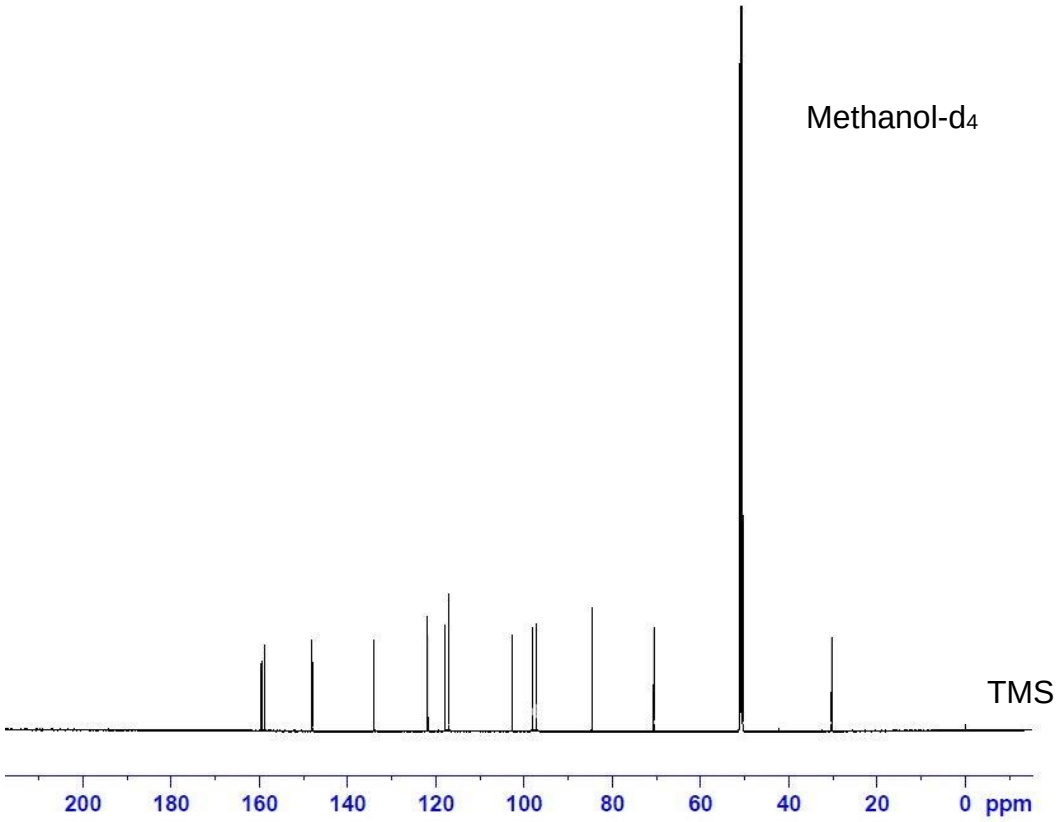


Fig. 13. ^{13}C NMR spectrogram of compound 3

1H-NMR spectrum information: 1H NMR (700 MHz, methanol-d_4): δTMS 4.55 [H-2, d, J=7.8 Hz], 3.96 [H-3, ddd, J=5.6,8.1,7.8Hz], 2.48 [H-4a, dd, J=8.1,16.1Hz], 2.82 [H-4b, dd, J=5.6, 16.1Hz], 5.85 [H-6, d, J=2.2 Hz], 5.92 [H-8, d, J=2.2 Hz], 6.83 [H-2′,d, J=1.8 Hz], 6.75 [H-5′,d, J=8.1 Hz], 6.70 [H-6′,dd, J=1.8, 8.1 Hz]

13C-NMR spectrum information: 13C NMR (175 MHz, methanol-d_4): δTMS 84.6 (C-2), 70.6 (C-3), 30.3 (C-4), 102.6 (C-4a), 159.3 (C-5), 97.2 (C-6), 159.6 (C-7), 98.0 (C-8), 158.7 (C-8a), 134.0 (C-1'), 117.0 (C-2'), 148.0 (C-3'), 148.0 (C-4'), 117.8 (C-5'), 121.8 (C-6'). The data above basically agreed with the data in literature (Hye *et al.* 2009; Davis *et al.* 2015).

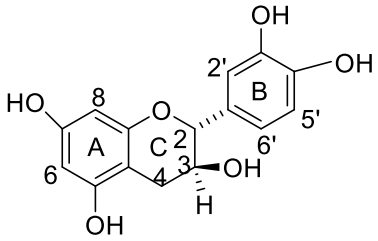


Fig. 14. Structure of compound 3

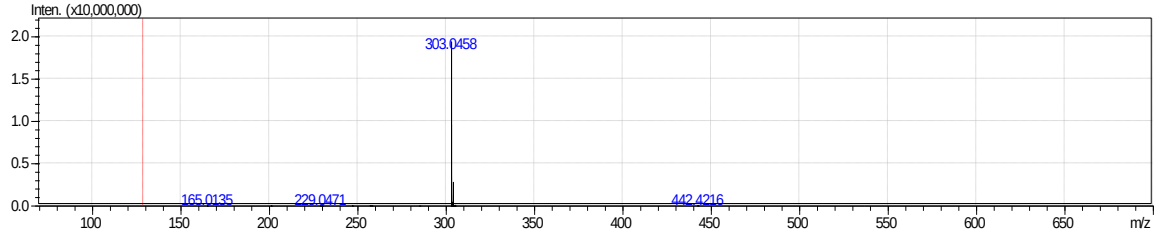


Fig. 15. Ms/Ms spectrum of compound 4

Compound 4 was isolated from Fr5 with the largest wavelength ultraviolet absorption at 255 and 368 nm. The quasi molecular ion peaks of $[M+1]^+$ *m/z* 465 indicates that the molecular weight was 464. The MS^2 spectrometry showed a fragmentation ion at $[M+1]^+$ *m/z* 303, so the fragment was tentatively identified as quercetin. Compared with the compound precursor ion $[M+1]^+$ *m/z* 465, the molecular weight decreased by 162 which probably meant that one molecule of the glucose group had fallen off. The liquid retention time of the compound was t_R=32.1 min, which was compared with that of the reference substance quercetin-3-O-glucoside. Their retention time was the same. Therefore, the molecular formula of the compound was identified as $C_{21}H_{20}O_{12}$, and the compound 4 was identified as quercetin-3-O-glucoside. Because the purity of this compound was relatively low and the yield was not considerable, it was not been studied for antioxidant and antidiabetic activity.

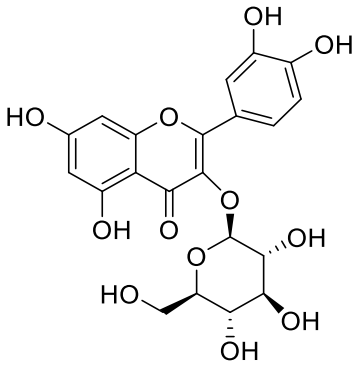


Fig. 16. Structure of compound 4

Antioxidant Activity

Three purified flavonoids were assayed at a range of concentrations (10 to 60 μg·mL^{-1}). Vitamin C (Vc) was used as a positive control. As shown in Fig.17, the DPPH• radical clearance rate of compounds 2 and 3 increased with the mass concentration rising. By comparing the IC_{50} value, V_C (14.48 μg·mL^{-1}) > compound 2 (16.88 μg·mL^{-1}) > compound 3 (20.20 μg·mL^{-1}) were listed in the order from large to small. This result

indicated that compounds 2 and 3 had significant antioxidant activity, among which the IC_{50} of compound 2 was only slightly less than that of V_C, indicating that compound 2 had very strong antioxidant activity. With the increase of the concentration of compound 1, DPPH• radical scavenging rate remained almost unchanged and low, indicating that compound 1 had almost no antioxidant activity. This may be because compounds 2 (epicatechin) and 3 (catechin) have polyhydroxyl structures in the benzene ring, and they are effective at scavenging free radicals.

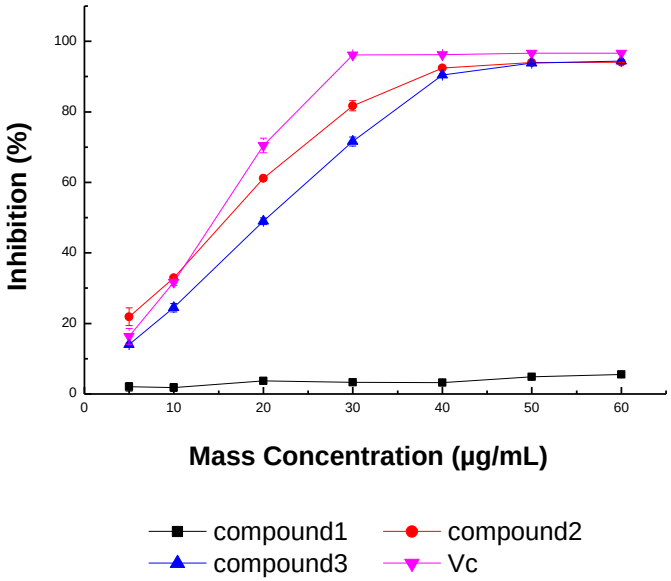


Fig. 17. Relationship between 3 purified flavonoids and DPPH scavenging rate

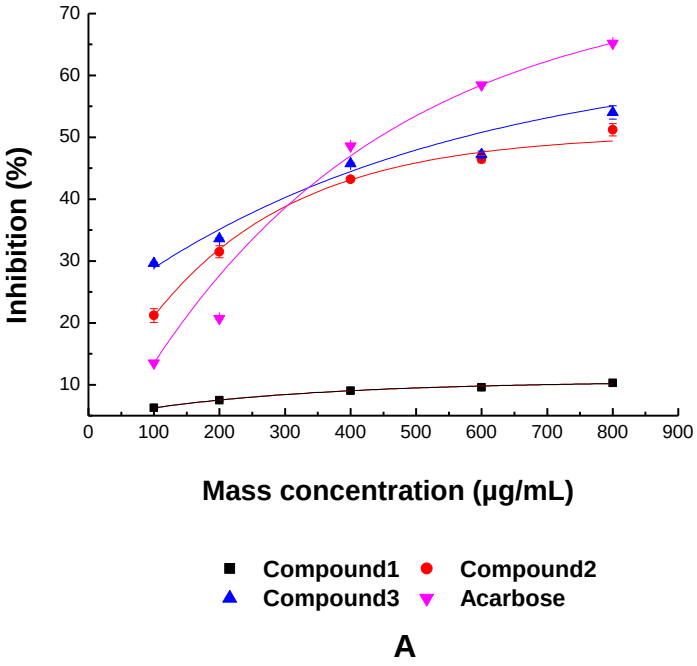
Antidiabetic Activities

Figure 18 (A) shows that the IC_{50} values of compound 2 and compound 3 were 0.752 mg·mL^{-1} and 0.655 mg·mL^{-1}, while compound 1 had no semi-inhibition rate. The IC_{50} value of the positive control acarbose was 0.456 mg·mL^{-1}. Therefore, the order of inhibitory activity of α-amylase was as follows: compound 3 (catechin) > compound 2 (epicatechin) > compound 1 ((E) -1-methoxy-2-o -(p-coumaroyl)-myo- inositol). This may be because that the 3-OH in the structure of catechins and epicatechins, and the hydroxyl substitution on its B ring would increase their ability to inhibit the activity of α-amylase (Liu *et al.* 2011).

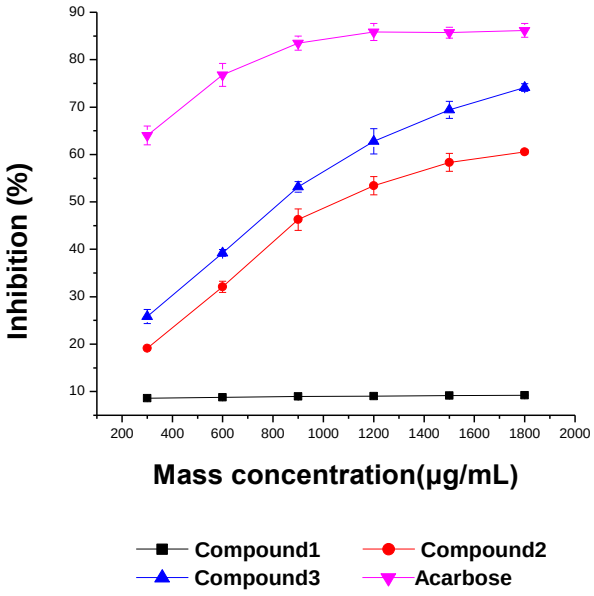
α-Glucosidase inhibitors delay and alleviate the time and progression of a diabetic patient's postprandial blood glucose elevation effectively; thus α-glucosidase inhibitors may make great contribution in treating diabetes and the occurrence of complications (Jiang *et al.* 2017b; Chen *et al.* 2019).

Figure 18 (B) shows the relationship between the α-glucosidase inhibitory activity of acarbose and 3 purified flavonoids. The inhibitory activity of α-glucosidase increased gradually with increasing concentration of the samples. However, the α-glucosidase inhibition capacity of compound 1 was very weak, with no significant change when the concentration increased from 300 to 1800 g·mL^{-1}. The order of α-glucosidase inhibition of the three compounds was compound 3 (catechin) > compound 2 (epicatechin) > compound

1 ((E)-1-methoxy-2-o-(p-coumaroyl)-myo-inositol). *α*-Glucosidase inhibitory activities means that the glucose absorption progress and postprandial hyperglycemia have been suppressed, which affected the serum glucose concentration, which is related to type II diabetes (Kellogg *et al.* 2014). Compared with the ability of α-amylase inhibitory activity of three pure compounds, catechin and epicatechin were good inhibitors for type II diabetes.



A



B

Fig. 18. Results of antidiabetic activity of 3 purified flavonoids against α-amylase (A) and α-glucosidase (B)

Antitumor Activity

The antitumor activity against MCF-7, Hela, and Hepg-2 cells were evaluated using *in vitro* essays (Fig. 19 and Table 4).

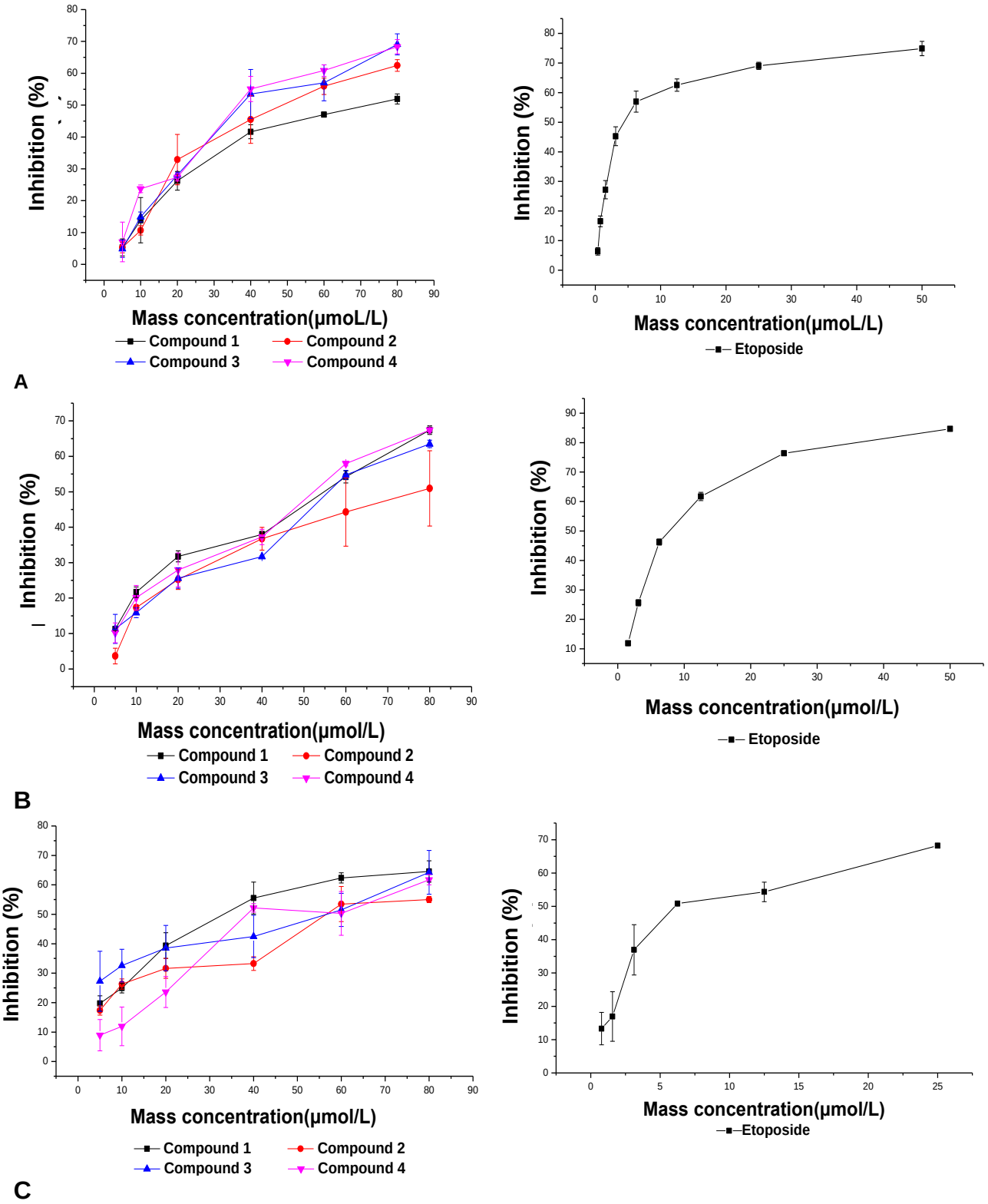


Fig. 19. Antitumor activities of 4 purified flavonoids against MCF7 (A), Hela (B), and HepG2 (C)

Compound 4 had the greater capacity inhibiting MCF7 and Hela activity with IC_{50} values of 36.4 μmol·L^{-1} and 52.5 μmol·L^{-1}, respectively. Compound 1 demonstrated a good inhibitory capacity against HepG2 with an IC_{50} value of 33.8 μmol·L^{-1}, while the IC_{50} value of etoposide, an antitumor drug, was 5.9 μmol·L^{-1}. Therefore, it was concluded that the flavonoids from TCBL can serve as potential candidates for natural bioactive antitumor drugs.

Table 4. IC_{50} Value of 4 Purified Flavonoids Against MCF7, Hela and HepG2

4 Purified Flavonoids	IC_{50} μmol·L^{-1}		
	MCF7	Hela	HepG2
Compound 1	75.7±0.05	54.7±0.04	33.8±0.05
Compound 2	49.2±0.02	77.8±0.07	56.7±0.02
Compound 3	37.3±0.02	56.1±0.02	57.1±0.02
Compound 4	36.4±0.05	52.5±0.03	38.3±0.01
Etoposide	4.5±0.03	7.8±0.01	5.9±0.04

CONCLUSIONS

1. The highest flavonoid yields from *Taxus cuspidata* branches and leaves (TCBL) were obtained by the enzymatic hydrolysis and ultrasound-assisted extraction (EHUE) method. The optimal conditions were: an enzymatic hydrolysis temperature of 48 °C, an enzymolysis time of 39 min, and an enzyme concentration of 0.10 mg·mL^{-1}. The flavonoids yield obtained under the optimized conditions was 5.23% ± 0.18%.
2. Compared to the authors' previous study of TCBL flavonoids using ultrasound-assisted extraction methods alone (yield up to 4.48%), the current study for enzymatic hydrolysis and ultrasound-assisted extraction (EHUE) method is novel and more beneficial (6% higher yield). The method is a combination of enzyme treatment and ultrasound-assisted extraction methods. The raw materials of TCBL after being treated with pectinase enzyme has a higher extraction rate of total flavonoids than the ultrasound-assisted extraction alone and traditional extraction methods. Enzymatic hydrolysis extraction can loosen and break up the plant cell wall, thus reducing the mass transfer resistance, enhancing the permeability of plant cell wall, and improving the extraction rate of effective ingredients. The extraction conditions are mild, which greatly improve the extraction efficiency. Therefore, this method can be widely used in the extraction of flavonoids of TCBL.
3. Four compounds (*E*)-1-methoxy-2-*O*-(*p*-coumaroyl)-*myo*-inositol, catechin acid, epicatechin, and quercetin-3-O-glucoside were purified from TCBL ethyl acetate extract. These were mainly composed of flavonoids. Epicatechin showed the greatest antioxidant capacity (IC_{50} value: 16.88 μmol·L^{-1}), while catechin also revealed a potent antioxidant activity (IC_{50} value: 20.20 μmol·L^{-1}). They both have strong inhibitory activities on α-amylase and α-glucosidase as well, which demonstrated the TCBL extract could be potential inhibitors for type II diabetes. Moreover, the extracts exerted clear anticancer activities against MCF-7, Hela, and HepG2 cells. The strongest anticancer activities against MCF7 and Hela were exerted by compound 4 with IC_{50} values of 36.4μmol·L^{-1} and 52.5 μmol·L^{-1}, respectively and compound 4 possessed a good inhibitory capacity against HepG2 cells with an IC_{50} value of 33.8 μmol·L^{-1}.

TCBL also contained various health-benefited polysaccharides. Therefore, flavonoids and other effective components in TCBL presented good bioactivities and make it a source of potential inhibitors of type II diabetes and human cervical cancer activity, and may contribute to the future biomedical industry. Further research may be performed on the bioavailability, bioaccessibility, and *in vivo* bioactivities to confirm the benefit.

ACKNOWLEDGEMENTS

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APPENDIX

Supplementary Figures

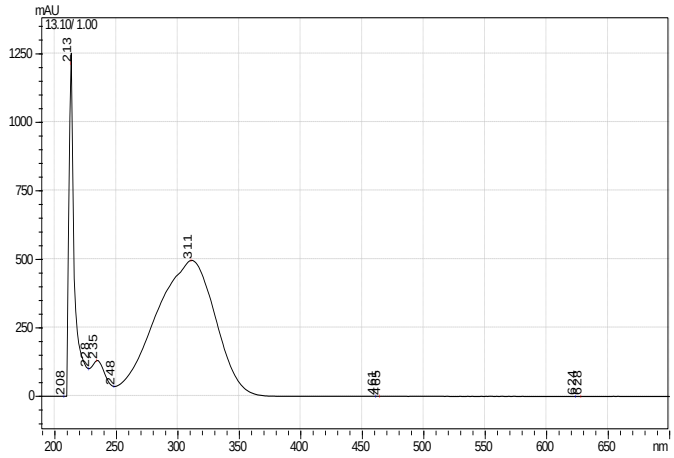

Fig. S1. UV absorption spectrum of Compound1

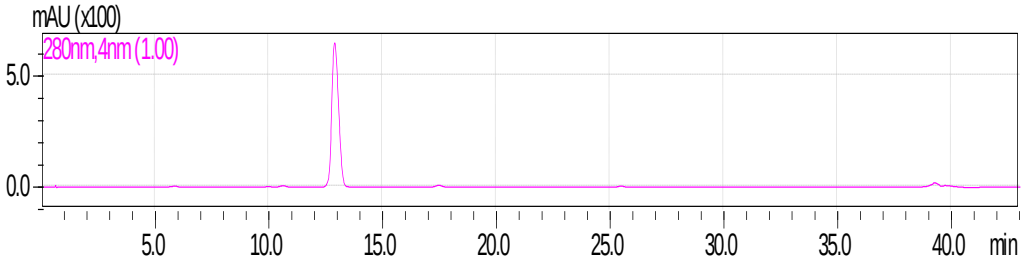

Fig. S2. High performance liquid chromatogram of Compound1

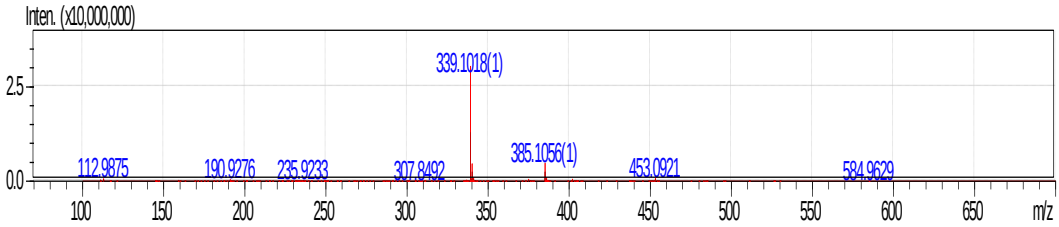

Fig. S3. ESI-MS^{-} spectrum of compound1

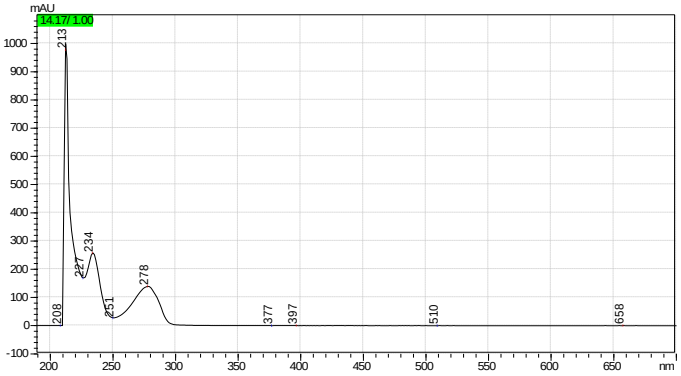

Fig. S4. UV absorption spectrum of compound 2

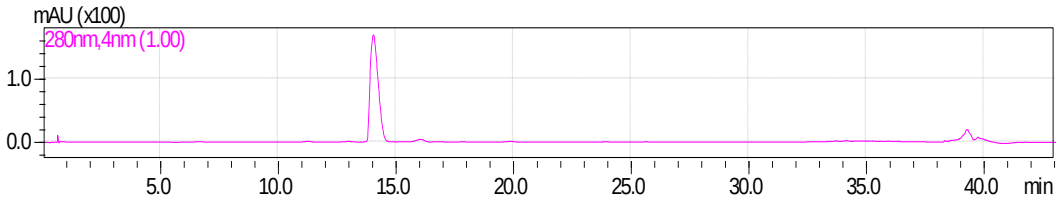

Fig. S5. High performance liquid chromatogram of compound 2

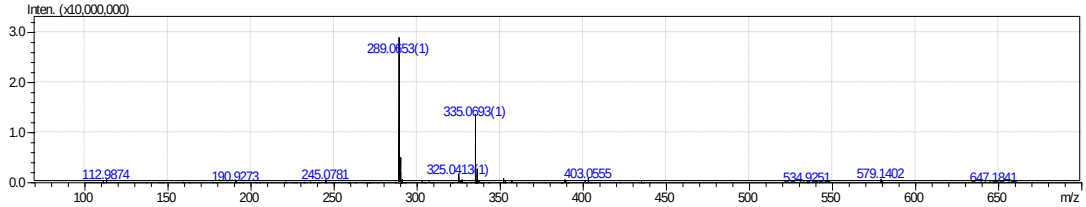

Fig. S6. ESI-MS$^-$ spectrum of compound 2

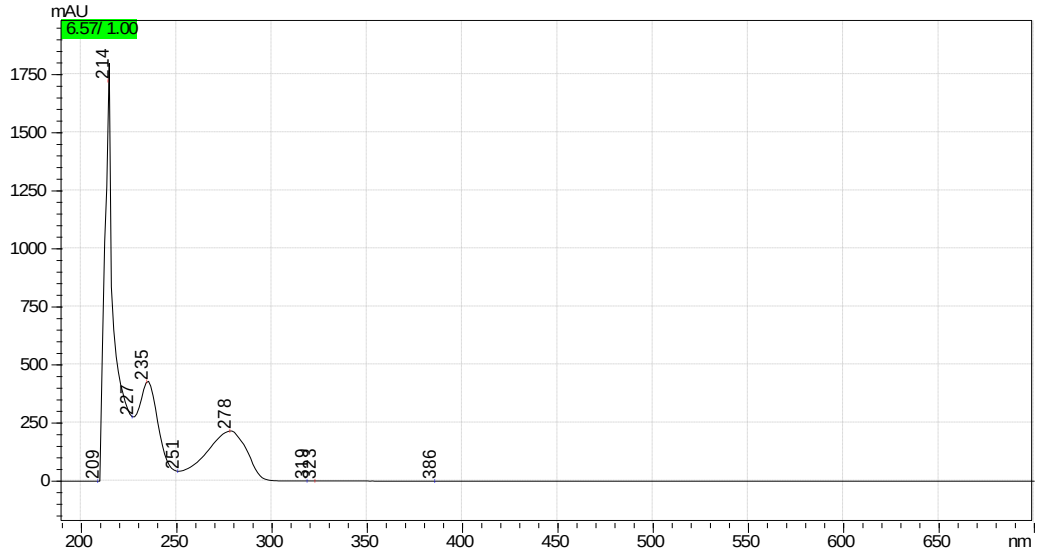

Fig. S7. UV absorption spectrum of compound 3

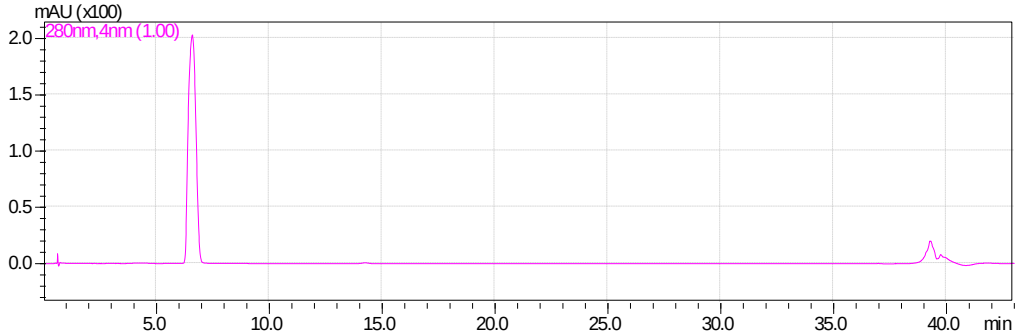

Fig. S8. High performance liquid chromatogram of compound 3

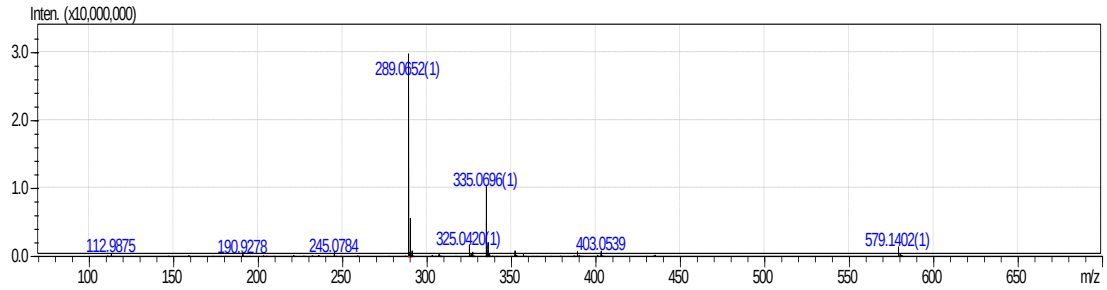

Fig. S9. ESI-MS$^-$ spectrum of compound 3

Fig. S10. The thin-layer chromatography of compound 3

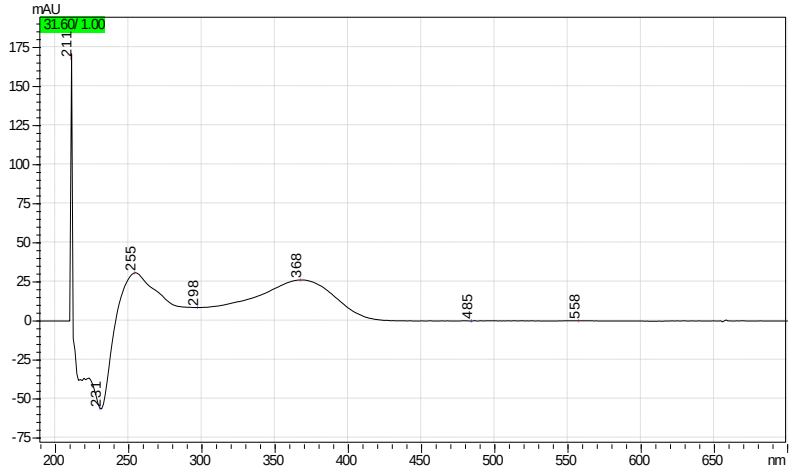

Fig. S11. UV absorption spectrum of compound 4

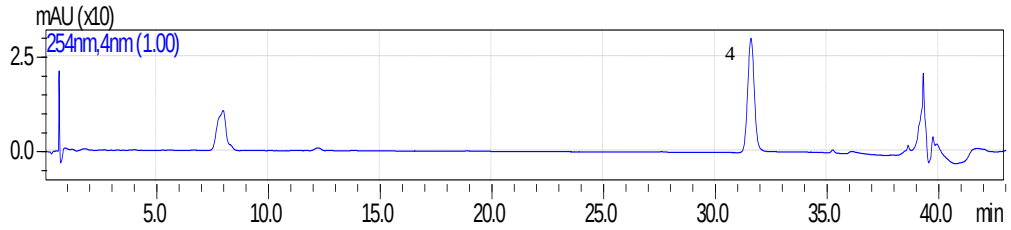

Fig. S12. High performance liquid chromatogram of compound 4

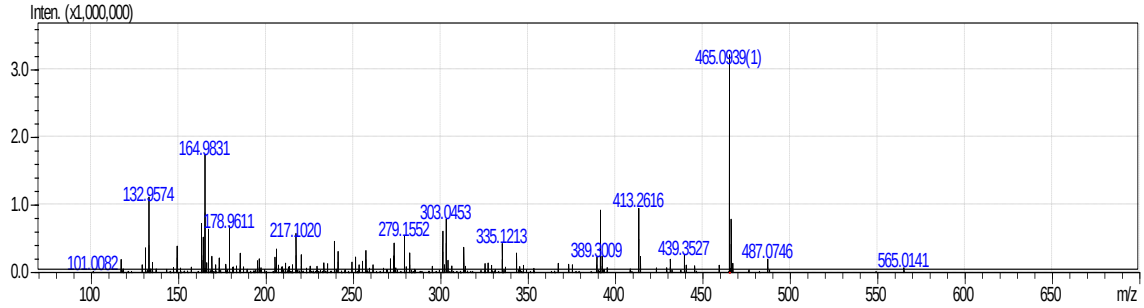

Fig. S13. ESI-MS$^+$ spectrum of compound 4