

Preparation and activity evaluation of chrysin- β -D-galactopyranoside

Zhen-Yuan Zhu¹ · Ling Chen¹ · Fei Liu¹ · Li-Jing Chen² · Meng Meng¹ · Hui-Qing Sun¹ · Yong-Min Zhang³

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Abstract Chrysin- β -D-galactopyranoside was efficiently synthesized, evaluated for its inhibitory activities against H22 cell lines compared with chrysin, the scavenging of hydroxyl radical, DPPH radical and superoxide anion, inhibitory effect against bacteria and fungi. The structures of all compounds were fully characterized by spectroscopic data (NMR, MS). The anti-tumor, antioxidant and antimicrobial activities of chrysin- β -D-galactopyranoside were proved to be enhanced significantly compared with chrysin.

Keywords Chrysin · Glycosylation · Derivative · Antitumor activity · Antioxidant activity · Antimicrobial activity

Introduction

Despite the large number of anti-tumor drugs available for medical use, the treatment of cancer remains a challenging therapeutic problem (Zhu et al. 2014), and the emergence of antimicrobial strains constitutes a substantial need (Zheng et al. 2011). Thus, the search for novel anti-tumor

and antimicrobial agents has turned to natural sources in particular plants used in medicines now.

Chrysin namely 5,7-dihydroxyflavone, a form of flavonoids is widely found in honey and plants. The anti-inflammatory, anti-bacterial, anti-oxidation, anti-anxiety, anti-strain, anti-diabetic, anti-hypertensive, anti-viral, anti-tumor and a variety of pharmacological effects such as vasodilation of chrysin have been reported in some studies. These studies also proved that it have strong chemical activity and little toxicity. Its structural can be easily modified (Mentzer et al. 1953).

Although many common chrysin have effective physiological function and little side effects, some research and clinical application of chrysin were limited by its low water solubility and low bio-availability. Therefore, it is of great importance to modify the structure of dialogue salicin chemically to enhance its water solubility and biological activity. Thus, to improve the targeting in vivo effect of genistein (Zheng et al. 2011; Mentzer et al. 1953).

Results and discussion

Chemistry

In the previous study, the chrysin-7-O-glucoside had been effectively synthesized by a reaction of chrysin and Acetyl bromgalactoside in 1999 (Alluis and Dangles 1999). In this experiment, benzoyl group was chosen as a protecting group of sugar ring hydroxyl group to form the compound a under the reaction of pyridine and benzoyl chloride (Park et al. 2005; Lönn 1985), and then hydrogen bromide was used as a hydroxyl activating group to obtain compound b a kind of glycosyl donor with high stereoselectivity. The yield of benzoyl- β -D-galactopyranoside and 1-Br-benzoyl-

✉ Zhen-Yuan Zhu
zhyuanzhu@tust.edu.cn

¹ Key Laboratory of Food Nutrition and Safety, Ministry of Education, College of Food Science and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, People's Republic of China

² Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 200090, People's Republic of China

³ Université Pierre et Marie Curie-Paris 6, Institut Parisien de Chimie Moléculaire UMR CNRS 8232, 4 place Jussieu, 75005 Paris, France

β -D-galactopyranoside could both reach above 85 %. 1-Br-benzoyl- β -D-galactopyranoside and chrysin were then dissolved in the K_2CO_3 and acetone solution to conduct the following reaction. The yield of compound c obtained by the glycosylation reaction is about 70.6 %. Followed by the synthesis of compound d which final yield was about 52.5 % by reaction with sodium methoxide to remove the protection group of the compound c in a solution consisted of anhydrous dichloromethane and methanol (v:v = 1:1). The stereochemistry of the newly introduced linkage was determined to be β on the basis of the Gal H-1, H-2 coupling constant ($J_{1,2} = 7.6$ Hz) (Bregant et al. 1999). The specific reaction process was shown in Fig. 1. All the synthesized compounds were characterized by IR, 1H -NMR, ^{13}C -NMR, and elemental analyses.

The ability of the new derivative such as anti-tumor, antioxidant and antimicrobial were evaluated by the method mentioned in MTT assay, Fenton-salicylic acid method and bacteriostatic circle measurement method respectively.

Anti-tumor activity

The anti-tumor activity, inhibition of H22 tumor cells of chrysin-galactopyranoside and chrysin was evaluated by

the MTT assay. As it could be clearly seen from the Fig. 2a, the anti-tumor effect towards H22 tumor cell decreased with the decreasing of concentration. The tumor inhibition rate of chrysin- β -D-galactopyranoside at the concentration of 2 μ mol/mL remained the highest through out the period. To be specific, this figure reached 44.95, 69.21 and 79.34 % at the 24th, 48th and 72th h respectively. This result was in consistence with or even much more superior than the some past researches in regarding to the anti-tumor activity (Tran et al. 2012). Surprisingly, beneficial effect toward the growth of H22 cells was observed after 24 h of cultivation when the concentration of chrysin- β -D-galactopyranoside was below 0.25 μ mol/mL. The reason for this phenomenon was yet to be determined. In comparing to chrysin- β -D-galactopyranoside, chrysin has exhibited with a stronger anti-tumor ability under the concentration of 2 μ mol/mL during throughout the period.

However, the ability is weaker than chrysin- β -D-galactopyranoside at other concentrations. This fact was the most obvious under the concentration of 2 μ mol/mL, at which point, its inhibition rate was almost 20 % less than that of chrysin- β -D-galactopyranoside. This phenomenon might be a result of the coexisted function group galactopyranoside depended its anti-tumor effect heavily

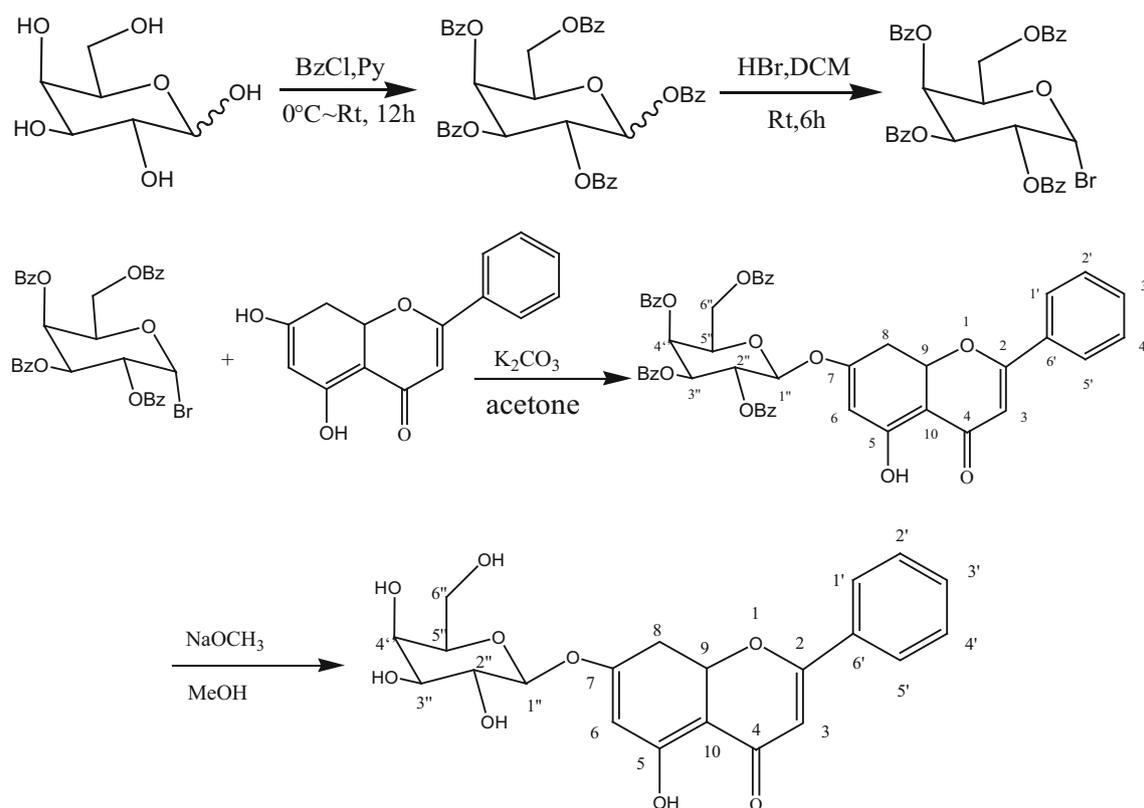


Fig. 1 Synthetic route of Chrysin- β -D-galactopyranoside

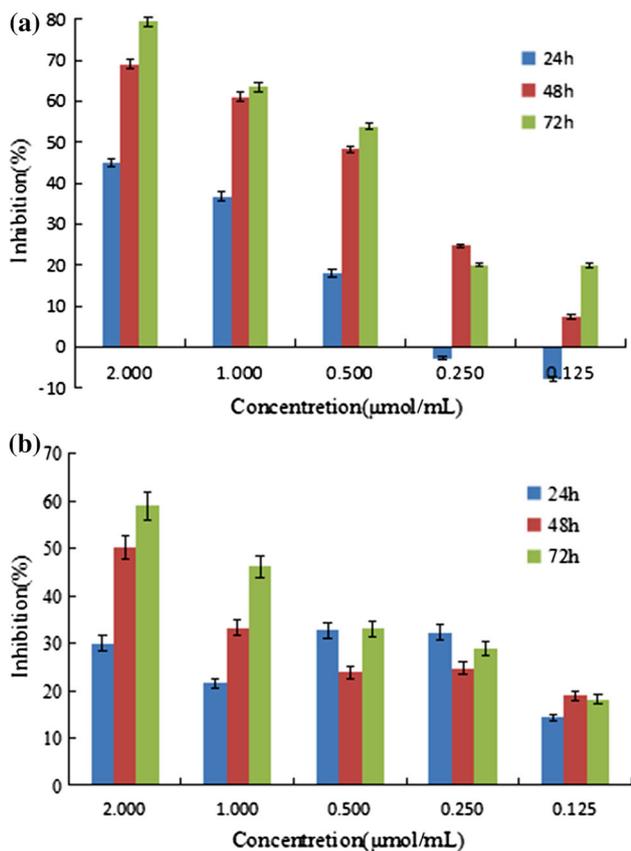


Fig. 2 Inhibition of H22 cells incubated with for 24, 48, 72 h **a** Chrysin- β -D-galactopyranoside **b** Chrysin

on the concentration. In conclusion, the result showed that the inhibition effect is strongly dosage dependent, and that, this effect generally increased with the passing of time. Since H22 cells belongs to mouse, further research is required to confirm its effect toward human cells.

Antioxidant activity

The $\text{O}_2^{\cdot-}$ scavenge effect of chrysin and chrysin- β -D-galactopyranoside were shown in Fig. 3. From which it could be seen that oxygen free radical scavenging ability of chrysin- β -D-galactopyranoside obtained from glycosylation was superior to that of chrysin. Without the radiation of the UV light, the superoxide anion scavenging rate of chrysin- β -D-galactopyranoside was 32.79 %, about 15 % superior to that of chrysin. After 1 h UV irradiation, growth was shown in the scavenging rate of glucoside. The figure for glucoside reached almost as high as 60 % almost the twice as the figure for chrysin. After this point, decline in oxygen free radical scavenging rate were observed both in chrysin and chrysin- β -D-galactopyranoside with the increasing period of exposure to UV light.

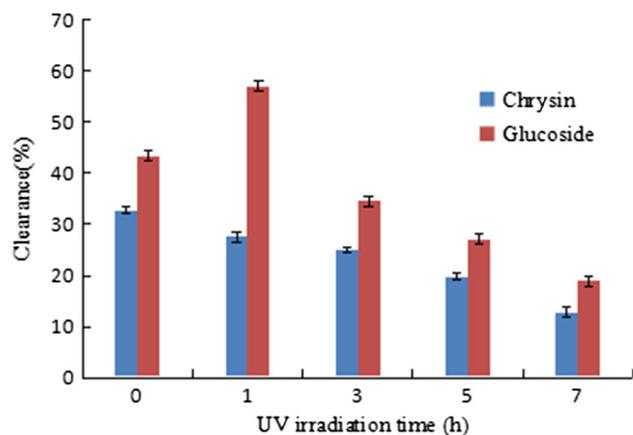


Fig. 3 Scavenging ability of compounds on $\text{O}_2^{\cdot-}$ radical, chrysin and chrysin-galactopyranoside

The DPPH \cdot scavenging rate of chrysin and chrysin-galactosidase were illustrated in Fig. 4. As can be seen from Fig. 4, the DPPH \cdot scavenging rate of chrysin and glucoside was similar without the treatment of UV irradiation at a little below 30 %. This figure for chrysin was significantly declined with prolonged UV exposure time to only around 10 % after 7 h UV irradiation. Unlike chrysin, the figure for glucoside remained relatively stable in the first 5 h. It was at the fifth hour that the greatest difference was identified in the DPPH \cdot scavenging rate. At this moment, the figure for glucose was around 23 %, only 7 % less than without UV irradiation treatment almost twice as that of chrysin. Surprisingly, at the 7th h, a significant decline was observed in the figure for glucoside, to around 14 %, while the figure for glucoside stabled at 10 % still about 5 %, less than that of glucoside.

Similarly, the hydroxyl radical scavenging rate of chrysin and chrysin-galactosidase were shown in Fig. 5. As it could be seen from Fig. 5 the hydroxyl radical

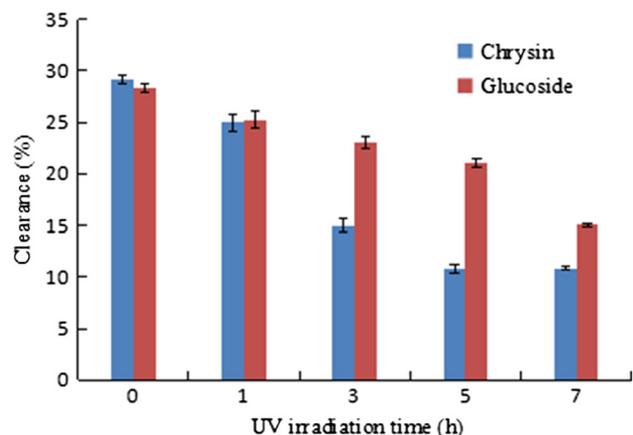


Fig. 4 Scavenging ability of compounds on DPPH \cdot radical, chrysin and chrysin-galactopyranoside

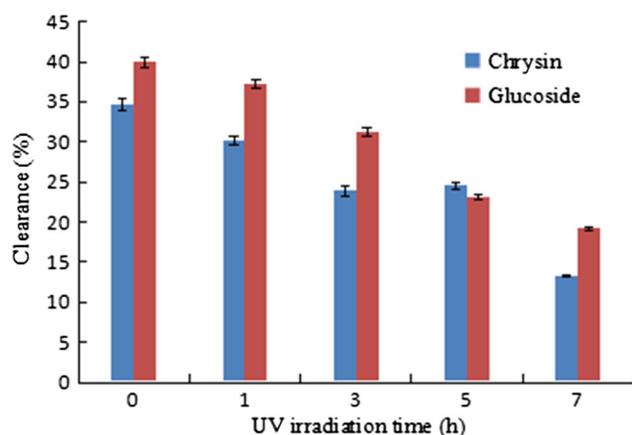


Fig. 5 Scavenging ability of compounds on OH· radical, chrysin and chrysin- β -D-galactopyranosid

scavenging ability of both chrysin and glucoside were both strong without UV radiation, which clearance rate was around 35 and 40 %, respectively. The figure for the two both gradually declined with prolonged UV exposure in the first 3 h. During this period, the figure for glucoside remained about 5–8 percent superior than that of chrysin. Surprisingly, at the 5th h, with the dramatic decline of this figure for glucoside and a slightly increase of this figure for chrysin, the figure for the latter slightly surpassed the former at around 24 %. Afterward, the figure for glucoside retrieved its dominant position at round 20 % with the dramatic decline of the figure for chrysin to around 13 % at the end of this test. It was obvious that during the whole process, the hydroxyl radical scavenging ability of glucoside was generally superior to that of chrysin.

Antimicrobial activity

In-vitro anti-microbial activity was evaluated using the minimum inhibitory concentration (MIC) with different strains. Table 1 showed that chrysin and chrysin- β -D-galactopyranoside with MIC value >64 mg/mL had a little

Table 1 The antimicrobial effect of the two chemicals based on the diameter of antimicrobial zone (mm)

Tested microorganism	Chrysin- β -D-galactopyranoside	Chrysin
<i>Escherichia coli</i>	6.18	6.05
<i>Bacillus subtilis</i>	6.12	6.02
<i>Staphylococcus aureus</i>	6.09	6
<i>Salmonella Typhimurium</i>	6.11	6.03
<i>Mucor</i>	9.75	6.37
<i>Rhizopus</i>	6	6
<i>Penicillium</i>	6	6
<i>Aspergillus</i>	6	6

inhibitory effect on food spoilage bacteria like *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella*, and the antibacterial effect of chrysin- β -D-galactopyranoside was weaker compared with chrysin. The diameter of antibacterial zone of chrysin was 6.18, 6.12, 6.09 and 6.11 mm, respectively. While the diameter of antibacterial zone of chrysin- β -D-galactopyranoside was 6.05, 6.02, 6 and 6.03 mm, respectively. In addition, the diameter of control group was 6 mm.

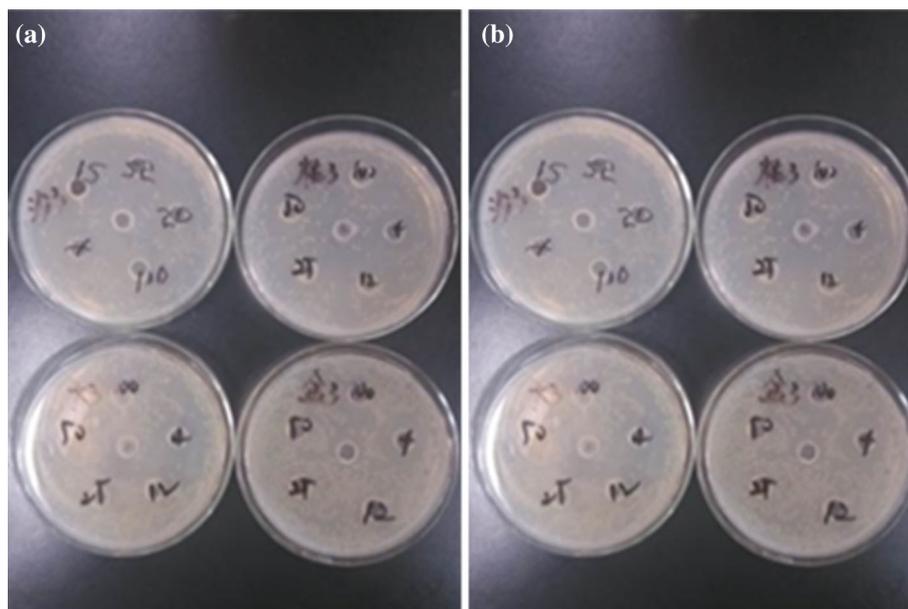
Similarly, the drugs with minimum inhibitory concentration (MIC) which is more than 64 mg/mL was used in the testing of their inhibitory effect against fungus, basically no inhibitory effects on *Rhizopus*, *Penicillium* and *Aspergillus* in the two samples, but chrysin- β -D-galactopyranoside had an obvious inhibitory effect on *mucor*, which chrysin did not have. As it could be seen from Table 1, the diameters of inhibition zone of chrysin and the complex were 9.75, 6.37 mm. However, the diameters of inhibition zone of these two samples on *Rhizopus*, *Penicillium* and *Aspergillus* were 6 mm, which indicated they didn't change at all.

In conclusion, both compounds were inactive against tested bacterial species except *mucor* that showed greatest inhibition with a inhibitory diameter of 9.75. It suggested that the introducing galactopyranoside to the chrysin could not increase the antimicrobial activity of most food spoilage bacteria and fungus, and that the two might exert their antimicrobial activity through multiple mechanisms or different pathways (Fig. 6).

Conclusion

One derivatives of chrysin named chrysin- β -D-galactopyranoside was synthesized and tested for several of its bio-activities such as antioxidant, anti-tumor and antimicrobial activity. The antioxidant ability decreased with the increasing of irradiation period, however. This trend was less obvious in the modified product which antioxidant effect was always superior to that of chrysin. It might be a result of the introduced group with certain ability to improve the antioxidant effect worked as a protection group against the UV treatment. In regarding to the anti-tumor effect, they both showed excellent anti-tumor ability against H22 cell at certain concentrations. The modified chemical showed a stronger dosage dependent effect in this activity. The difference in their anti-tumor was most obviously observed at the concentration of 2 mg/mL. Finally, although both the two chemicals showed little inhibition effect toward most food spoilage bacteria and fungus, chrysin- β -D-galactopyranoside showed excellent inhibition effect toward *mucor* which a inhibitory diameter of 9.75. This might due to the fact that the

Fig. 6 The antimicrobial effect of chrysin- β -D-galactopyranoside. **a** common spoilage bacteria **b** common fungi



introducing of galactopyranoside changed its mechanisms or different pathways relating to the antimicrobial activity. Further research is required to gain more knowledge about these switch of pathways and more compounds need to be designed and synthesized for further investigation using chrysin as the lead compound.

Materials and methods

Chemistry

Mass spectra were measured on a MS-700(GERMANY). IR spectra were recorded(in KBr) on a FTIR1730. $^1\text{H-NMR}$ spectra were measured on a Bruker DRX400 spectrometer with TMS as the internal standard. Optical rotation was measured on a Perkin Elmer 241 polarimeter type at a condition of room temperature (25 ± 2 °C), length 10 cm and volume 10 mL. CS Chemdraw ProTM computer software was used to calculate the theoretical value of the molecular weight, mass spectrometry and molecular structural formula diagram draw synthetic compounds. Reagents were at analytical grade and commercially available (individual reagents and solvents were treated before use). Reaction courses were monitored by TLC on silica gel-precoated F254 Merck plates.

Preparation of the benzoyl- β -D-galactopyranoside (a)

1.8 g β -D-Galactose was dissolved in pyridine 10 mL, then cooled at 0 °C for 20 min before 6.25 mL benzoyl chloride was added in slowly. Then, it was stirred in ice-water for 30 min followed by 12 h reaction at room temperature.

After the reaction was indicated completed by TLC, the reactant was diluted by CH_2Cl_2 . By which means, the organic phase was extracted. Afterward, it was washed with 1 mol/L HCl solution and saturated NaHCO_3 solution for several times before it was washed again by saturated NaCl solution. Then, the organic phase was dried by Na_2SO_4 and was evaporated under reduced pressure. Finally, the purified product purified by silica gel column chromatography (Petroleum ether: ethyl acetate = 3:1) to furnish the compound **a** (1.656 g, 90.5 %).

Preparation of the 1-Br-benzoyl- β -D-galactopyranoside (b)

1.656 g of benzoyl- β -D-galactopyranoside obtained from the previous step was dissolved in 10 mL dichloromethane dried with specific methods, then 10 mg 4A molecular sieves was added before it was stirred until the temperature was cooled to 0 °C. Afterward, 6 mL 47 % HBr-acetic acid was added to the solution slowly to react for 6 h at room temperature. The reaction was quenched by cold saturated NaHCO_3 solution when the result of TLC indicated the reaction was completed. The product was successively extracted by adding a certain amount of dried CH_2Cl_2 . Then, the organic phase was dried by anhydrous sodium sulfate. Eventually, it was concentrated and purified by silica gel column chromatography (Cyclohexane: ethyl acetate = 3:1) to furnish the compound **b** (1.43 g, 86.7 %). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.05-7.14 (m, 25H, H arom); 6.90 (d, 1H, $J = 3.9$ Hz, H-1); 6.14 (d, 1H, $J = 3.1$ Hz, $J < 1$ Hz, H-4); 6.08 (dd, 1H, $J = 10.3$ Hz, $J = 3.1$ Hz, H-3); 5.69 (dd, 1H, $J = 10.3$ Hz, $J = 4.0$ Hz, H-2); 4.94 (m, 1H, H-5); 4.66 (dd, 1H, $J = 11.5$ Hz,

$J = 6.8$ Hz, H-6); 4.48 (dd, 1H, $J = 11.5$ Hz, $J = 6.0$ Hz, H-6'). $^{13}\text{C-NMR}$ (100.6 MHz, CDCl_3): δ 165.86, 165.50, 165.30, 165.26 (C=O); 133.76–128.30 (C arom); 88.41 (C-1); 71.89 (C-5); 68.96 (C-3); 68.63 (C-2); 68.14 (C-4); 61.72 (C-6,6').

Preparation of the chrysin- β -D-2,3,4,6-4-O- benzoyl-galactopyranoside (c)

Chrysin (0.4 mmol), anhydrous potassium carbonate (4 mmol) and acetone (4 mL) were added in a 50 mL round bottom flask and stirred together. After it was fully dissolved, another 0.6 mmol 1-Br-benzoyl- β -D-galactopyranoside was dissolved in 2 mL acetone solution slowly. The mixture was then stirred again for 4 h at 40 °C. The organic layer was successively concentrated under reduced pressure to furnish a yellow syrup, which was purified by column chromatography (EtOAc-petroleum ether = 1:3) to furnish compound **c** (Yellow powder, 70.6 %). $[\alpha]_{\text{D}}^{+75}$ (c = 0.1, CH_2Cl_2); IR: ν_{max} cm^{-1} 3435(O–H), 1617 (C=O), 1658(C=C), 1266 (Ar–O–C), 1141–1083 (C=C–O–C=C); $^1\text{H-NMR}$ (400 MHz, CDCl_3 , ppm): 12.74 (s, 1H, –OH), 8.15–7.28 (m, 25H, ArH); 6.69 (s, 1H, H-8); 6.67 (d, $J = 2.2$ Hz); 6.62 (d, $J = 2.4$ Hz); 6.14 (d, $J = 7.2$ Hz, 1H, H-1''); 5.76 (dd, $J = 3.6$ Hz, 1H, H-6); 5.74 (dd, $J = 3.6$ Hz, 1H, H-6''); 5.58(dd, $J = 7.6$ Hz, 1H, H-2''); 4.67 (dd, $J = 2.8$ Hz, 1H, H-6''); 4.64 (s, 1H); 4.62(t, 1H, H-5''); $^{13}\text{C NMR}$ (400 MHz, CDCl_3 -d6, ppm): 182.1 (C-4), 166.1, 165.5, 165.4, 165.1 (C=O, Bz) 164.4 (C-7); 162.4 (C-2), 162.2 (C-5), 157.4 (C-9), 133.7–126.3 (CH, Ar), 107.1 (C-3); 106.0 (C-10); 100.1 (C-1''Glc); 98.8 (C-6); 95.5 (C-8); 72.2 (C-5''); 71.4 (C-4''), 69.2 (C-2''); 67.9 (C-3''); 62.4 (C-6''); HRMS calcd for $[\text{M}+\text{H}]^+$ $\text{C}_{49}\text{H}_{36}\text{O}_{13}\text{H}$: 835.2312, found 835.2328.

Preparation of the Chrysin- β -D-Galactopyranoside(d)

Compound **c** (0.6060 g, 0.7 mmol) was dissolved in MeOH- CH_2Cl_2 (V:V = 1:1, 10 mL), followed by adding in 21.5 mg CH_3ONa . After stirring at 25 °C for 5 h, the solution was neutralized by ion-exchange resin (H^+), and then filtered and concentrated. The yellow residue was then purified by column chromatography (CH_2Cl_2 –MeOH = 8:1) to furnish **d** (yellow solid, yield 74.4 %). $[\alpha]_{\text{D}}^{25}$ –15° (c = 0.1, CH_2Cl_2). IR: ν_{max} cm^{-1} 3401(O–H), 1616(C=O), 1712(C=C), 1266(Ar–O–C), 1141–1083(C=C–O–C=C); $^1\text{H-NMR}$ (400 MHz, DMSO-d6, ppm); 12.82(s, 1H, –OH), 8.11–7.58 (m, 5H, ArH), 7.05(s, 1H, H-8), 6.87 (s, 1H, H-3), 6.48(d, $J = 2.4$ Hz, H-6), 5.05(d, $J = 7.6$ Hz, 1H, H-1''), 3.73–3.33 (m, 5H, H-2'' ~ 6''). $^{13}\text{CNMR}$ (400 MHz, DMSO-d6, ppm): 182.6 (C-4), 164.1 (C-7), 163.8 (C-2),

161.6 (C-5), 157.6 (C-9), 132.6–126.9 (C-1' ~ C-6'), 106.0 (C-10), 105.9 (C-3), 100.9 (C-1''), 95.3 (C-6, C-8), 76.2 (C-5''), 73.6 (C-4''), 70.53 (C-2''), 68.5 (C-3''), 60.75 (C-6''); HRMS calcd for $[\text{M}+\text{H}]^+$ $\text{C}_{21}\text{H}_{20}\text{O}_9\text{H}$: 419.1264, found 419.1285.

Anti-tumor activity

Research on the inhibition toward H22 tumor cells chrysin glucosidase was conducted in this experiment by MTT method (Tran et al. 2012). H22 tumor cells was obtained from Tianjin University of Science and Technology Laboratory of functional foods. Firstly, chrysin- β -D-galactopyranoside was weighed accurately, then 0.25, 0.5, 1.0, 1.5, 2 $\mu\text{mol/mL}$ sample solution were diluted by 2 $\mu\text{mol/mL}$ of concentrated 1640 cell culture medium after filter the original sample through 0.2 μm membrane to remove microorganisms.

100 μL tumor cells of 1×10^5 cell/mL configuration at the logarithmic growth phase were added into every hole of the counting board, followed by 100 μL of sample solution at different concentrations, each concentration were repeated three times. Then cultured for 20, 44, 72 h before being centrifuged at 1000 r/min for 6 min, the supernatant was decanted. 100 μL of MTT standard fluid was then added in the remained precipitation and cultured for another 4 h. Afterward, the culture was terminated by centrifugation at 1000 r/min for 10 min. The suspension of each hole was carefully cleaned before, the intracellular crystals was dissolved by 150 μL dimethylsulfoxide (DMSO) under oscillation. Finally, the OD value was measured by using a Multi-Mode Microplate Reader at 570 nm (Sargent 2003). Control group was set by the cell group without sample solution, while the blank group was set by 150 μL of the DMSO solution. Samples inhibit tumor cell inhibition rate calculation formula (1):

$$\begin{aligned} & \text{Inhibition percent (\%)} \\ &= \frac{\text{Control group (cpm)} - \text{Experimental group (cpm)}}{\text{Control group (cpm)}} \\ & \times 100 \end{aligned} \quad (1)$$

Antioxidant activity

Pyrogallol autoxidation was used in this experiment, the specific steps were mentioned as follows: placed 50 mmol/L, pH = 8.2 in Tris–HCl buffer 450 μL in 2 mL centrifuge tube before it was bathed at 25 °C for 20 min. Then 100 μL of 1.0 mg/mL sample processed with different period of UV irradiation were added into the solution. 50 μL 2.5 mmol/L phthalate solution was successively added into it before it was water bathed at 25 °C for 5 min.

Finally, 0.2 mL 8 mol/L HCl solution was added into the solution to terminate the reaction. Result was carried out by measuring the absorbance value of at 300 μ L samples which were placed in a 96-well microtiter plates. The formula shown in Formula (2.1):

$$\text{OH}^\bullet \text{ radical scavenging activity} = 1 - [\text{As}/\text{Ac}] \times 100 \% \quad (2.1)$$

As: absorbance of the OH \cdot solution containing samples. Ac: absorbance of the control solution without sample but with OH \cdot . The percentages of OH \cdot reduced were plotted against the samples.

The antioxidant activity was performed using the method described by Amarowicz et. al (2000). Added 500 μ L 1.5×10^{-4} mol/L DPPH-ethanol solution and 500 μ L 1.0 mg/mL of sample solution irradiated by UV at different times in 2 mL centrifuge tube. After pipetting until uniformed, closed in the water bath 25 $^\circ$ C to react for 60 min, 300 μ L of the reaction solution were successively added to 96-well microtiter plates, the measured absorbance values at 517 nm wavelength, was calculated by the formula (2.2):

$$\text{DPPH}^\bullet \text{ radical scavenging activity (\%)} = 1 - [\text{As}/\text{Ac}] \times 100 \% \quad (2.2)$$

As: absorbance of the DPPH solution containing samples. Ac: absorbance of the control solution without sample but with DPPH. The percentages of DPPH reduced were plotted against the samples.

Hydroxyl radical scavenging activity was measured using Fentons reaction method with slight modification (Bekhit et al. 2001). The reaction mixture generating hydroxyl radicals contained 0.1 mL of H₂O₂ (60 mmol/L), 0.1 ml of FeSO₄ (9 mmol/L), 0.1 ml of salicylic acid ethanolic solution (9 mmol/L) and 2.7 ml of the polysaccharides of varying concentrations. Distilled water was used as the blank control. After being incubated at 37 $^\circ$ C for 30 min, scavenging rate (%) was calculated using the formula (2.3). 300 μ L of the reaction solution were successively added to 96-well microtiter plates, the measured absorbance values at 510 nm wavelength.

$$\text{Radical scavenging activity (\%)} = 1 - [\text{As}/\text{Ac}] \times 100 \% \quad (2.3)$$

As: absorbance of the sample containing solution containing samples. Ac: absorbance of the control solution without sample. The percentages of H₂O₂ reduced were plotted against the samples.

Antimicrobial activity

Prepared a 6 mm diameter metal hole punch by heat sterilization at 120 $^\circ$ C for 20 min, the 10 g solid medium was

poured into dry sterilized dishes and melted after being autoclaved. Followed by solidification (Cheng et al. 2006) under room temperature.

Bacteria experiment: added 100 μ L bacterial suspension to 10 mL beef extract peptone medium and uniformed by air blowing. Then poured it into a layer that has been covered with plates of beef extract peptone medium. Afterward, holes with depth of 2 mm was penetrated by a sterile metal after it was solidified. 100 μ L of each sample gradient concentration solution was added in the holes, a penicillin was added as a control group. The hole in the middle of was added 100 μ L of sterilized acetone controls, three parallels were set for each bacteria sample. The plates were then placed in a incubator at 37 $^\circ$ C. Diameter of the inhibition zone were measured after 24 h incubation. Finally different antimicrobial effects were compared by the diameter.

Mold experiment: 10 mL PDA medium was melted in a petri dish that has already been covered by a layer of PDA medium and uniformed by air blowing. After it was solidified in room temperature, 100 μ L of each sample gradient concentration solution was added in the holes, a penicillin was added as a positive control group. The hole in the middle of was added 100 μ L of sterilized acetone controls, three parallels were set for each fungi sample. The plates were then placed in a incubator at 28 $^\circ$ C. Diameter of the inhibition zone were measured after 24 h incubation. Finally different antimicrobial effects were compared by the diameter.

An experiment of detecting the minimum inhibitory concentration of the compounds against the susceptible micro-organisms in the preliminary test (Gram-positive bacteria and Gram-negative bacteria). A twofold serial dilution technique (Bekhit et al. 2001) was followed to determine the minimum inhibitory concentration (MIC) of the compounds against. Test compounds dissolved in water were added to culture media (Brain Heart Infusion for *S. Mutans* and *Müller-Hinton* agar for other bacteria) to obtain final concentrations of 64–0.5 mg/mL. The final bacterial amount was determined to be 10⁵ CFU/mL. MIC values were read after incubation at 37 $^\circ$ C for 20 h. The lowest concentration of the test substance that completely inhibited growth of the micro-organism was recorded as the MIC (expressed in mg/mL). It was follow the similar process that the minimum inhibitory concentration against fungus was detected. All the experiments were repeated for 3 times.

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