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复方枳葛橘提取物体外抗氧化活性评价

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摘 要:复方枳葛橘水提液依次用氯仿、乙酸乙酯和正丁醇萃取,与水层得到4个不同极性部位萃取物,以清除DPPH·、ABTS+·,以及络合Fe^{2+}能力,综合评价其抗氧化作用;采用芦丁显色法检测各部位总黄酮含量,并分析黄酮类物质含量与抗氧化活性的相关性;采用HPLC法分析其抗氧化能力较强部位可能的化学成分。结果显示4个不同极性部位均具有不同程度的抗氧化能力,其中乙酸乙酯部位抗氧化能力最强,其次是正丁醇、水层,而氯仿层较弱;其抗氧化能力与总黄酮含量呈正相关,且与黄酮类化合物结构类型相关;HPLC分析结果显示乙酸乙酯合并正丁醇部位主要成分为柚皮苷、野漆树苷和槲皮素等黄酮类化合物。复方枳葛橘提取物可以作为天然抗氧化剂用于药物研究和相关疾病的防治。

关键词:复方枳葛橘;提取物;自由基;总黄酮;抗氧化活性

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Antioxidant Activity of the Extracts of Formulation *Zhigeju*

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Abstract:The formulation *Zhigeju* was fractionated by using 3 different organic solvents and water to provide chloroform extract (CE),ethyl acetate extract (EAE),n-butanol extract (BE) and water extract (WE). Different antioxidant assays (DPPH,ABTS scavenging activity and metal chelating activity) were employed to evaluate the antioxidant activities of the four extracts. The contents of total flavonoids in different extracts were measured through UV spectroscopy using rutin as control by color reaction. The correlation between the concentration of total flavonoids and their antioxidant activities was then investigated. HPLC was used to analysis the components of the extract with the highest antioxidant activity. The results showed that the EAE had the highest antioxidant activity while the CE had the lowest antioxidant activity. All of the four extracts showed concentration dependent antioxidant activity,however the IC_{50} values of the four extracts on antioxidant activity were different. The results of HPLC analysis showed that the main components of EAE and BE were flavonoids,including naringin,rhoifolin and quercitin. The extracts of formulation *Zhigeju* had the potential to be used as natural antioxidant for the drug research and related disease prevention.

Key words:formulation *Zhigeju*;extracts;flavonoids;free radicals;antioxidant activity

Introduction

Alcoholism is the world's first public nuisance,and its toxicity effects all the organs,especially the liver. The patients with alcoholic liver injury have gradually increased in our country in recent years. However,so far, there are no satisfactory drugs for it. Hence,it is important and urgent to look for an effective drug. The formulation *Zhigeju*, constituted of Flos Puerariae,Semem Hoveniae and exocarpium Citrus Grandis,was traditionally used to treat alcoholic liver injury. The Flos Puerariae and Semem Hoveniae's effects of antialcoholism are recorded in Qian Jin Yao Fang and Compendium of Materia Medica,modern pharmacological research has shown that those herbs have obvious inhibitory activity on alcoholic liver damage[1-3]. Exocarpium Citrus Grandis is listed as a tribute in previous dynasties,as a folk medicine used to help breaking down alcohol in the

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genuine region HuaZhou. The supplement to Compendium of Materia Medica also records its antialcoholism function. The previous researches of our group showed that the flavonoids of exocarpium Citrus Grandis significantly ameliorated the liver damage on rats and mouse alcoholic models [4,5].

Since, the related medical research indicated that lipid peroxidation plays a very important role on formation and development in alcoholism liver damage [6]. The objectives of this study are to evaluate the antioxidant properties of different extracts from formulation *Zhigeju*, to investigate the relationship between concentration of crude drug and its antioxidant properties, and finally to explore the features of Chinese medicine and LingNan medicine on prevention and treatment of alcoholic liver diseases.

Materials and Methods

Chemicals

Trichloroacetic acid (TCA), 2-deoxy-D-ribose, linoleic acid, gallic acid, tween-40, 3-(2-pyridyl)-5,6 bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), phenazine methosulphate (PMS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ehtylenediamine trtra acetic acid (EDTA), ascorbic acid, potassium ferricyanide, thiobarbituric acids (TBA), nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced (β-NADH) and ammonium thiocyanate were obtained from Sigma Aldrich Chemical Co. (USA). All other chemicals and solvents were of analytical or HPLC grade.

Crude drugs

Flos Puerariae and Semem Hoveniae were purchased from ZhiXin medicine health CO., LTD (Guangzhou, China). Exocarpium Citrus Grandis was picked in GAP production base HuaZhou. All of the three crude drugs were identified by Prof. LIN Li, a specialist on Chinese medicine resources researching. The crude drugs were dried at 60 ℃ and then grounded into a fine powder with a grinder. The voucher specimens (Flos Puerariae No. 20121120-GH, Semem Hoveniae No. 20121120-ZJZ, Exocarpium Citrus Grandis No. 20120513-HJH) were deposited in botanic specimen center of college of Chinese Materia Medica, Guangzhou University of Chinese Medicine.

Sample preparation

Five gram of the dried powder of formulation *Zhigeju* (Flos Puerariae: Semem Hoveniae: Exocarpium Citrus Grandis, 3:3:2) were extracted with 3 × 400 mL water at 100 ℃ for 1.5 h each time. The water solutions were combined, and then evaporated to dryness under reduced pressure. The residue was re-dissolved in 200 mL 30% aqueous ethanol (v/v). The resulting solution was kept at 4 ℃ overnight, and then centrifuged at 5000 rpm for 10 min to eliminate chlorophyll and polysaccharose. The supernatant solution was collected and evaporated to dryness. The residue was added 100 mL distilled water, and shaken vigorously to form a suspension, followed by liquid-liquid extraction using different solvents in order of: chloroform, ethyl acetate and n-butanol. Three extracts and the water phase were evaporated to dryness under reduced pressure by rotary evaporator to provide chloroform extract (CE), ethyl acetate extract (EAE), n-butanol extract (BE) and water extract (WE). All of these extracts were stored at 4 ℃ before used.

The stock solution of these extracts (0.4 mg/mL) were prepared weekly with 50% aqueous methanol (v/v) and stored at 4 ℃. The working solutions of these extracts were daily fresh prepared by diluting the stock solutions with 50% aqueous methanol (v/v).

DPPH radical scavenging assay

The free radical scavenging activity was measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) assay. 3.2 mg of DPPH(were dissolved in 100 mL of 82% aqueous methanol (v/v). 2.8 mL of the DPPH(solution was added to glass vial followed by the addition of 0.2 mL of tested sample solution dissolved in 50% aqueous methanol (v/v), leading to the final concentration of extracts between 0.05-4.0 mg/mL. Mixture of DPPH and the four different extracts were kept in the dark at controlled room temperature for 1 h. After incubation, the mixtures were measured at 517 nm. Mixture of 2.8 mL of 82% aqueous methanol (v/v) and 0.2 mL of 50% aqueous methanol (v/v) was used as blank, while 0.2 mL of 50% aqueous methanol (v/v) and 2.8 mL of DPPH(solution was taken as control. Each

extract was tested in triplicate. The IC_{50} value was calculated by graph pad prism software. The radical-scavenging capacity (RSC) was calculated using the equation:

$$RSC = [1-(A_1-A_0)/A_2] \times 100\%$$

Where A_1 is the absorbance of probe, at a given sample concentration level (average of three probes); A_0 is the correction or the absorbance of the extract alone (without reagents), and A_2 is the absorbance of the DPPH (radical reagent without extracts.

ABTS radical cation scavenging assay

ABTS (2, 2′-azinobis-3-ethylbenzthiazoline-6-sulphonate, 7.4 mM) was treated with potassium persulfate (2.45 mM) to produce free radicals. The initial absorbance of the reagents in the glass cuvette was recorded at 414 nm. 100 μL of sample solution with different concentrations of extracts between 0.05-4.0 mg/mL were transferred into the cuvette and examined after 90 min using a UV-vis spectrophotometer. Antioxidant capacity of the ascorbic acid was also determined. All tests were run in triplicate and averaged. The IC_{50} value was calculated by graph pad prism software. The capability to scavenging the ABTS radical cation was calculated using the following quation:

$$\text{ABTS radical cation scavenging ability} = (A_1-A_2/A_1) \times 100\%$$

Where A_1 is the absorbance of the control (ABTS solution without tested sample), and A_2 is the absorbance in the presence of the tested sample.

Fe^{2+} chelating activity assay

1 mL of different concentrations of sample solution (0.05-4.0 mg/mL), 3.7 mL of 50% aqueous methanol (v/v) and 0.1 mL of 2 mmol/L $FeSO_4$($7H_2O$ solution were mixed and reacted for 30 s. The reaction was initiated by adding 0.1 mL of 5 mmol/L ferrous triazine solution. Absorbance A_1 at the wavelength of 562 nm was measured after 10 min of incubation at room temperature. The sample solvent was used as blank A_0. Percentage chelating was measured according to following formula and IC_{50} value was calculated by graph pad prism software.

$$\text{Chelating ability} = (A_1-A_0) / A_0 \times 100\%$$

Determination of total flavonoids content

0.25 mL of the extracts (supernatants after centrifuge, mentioned in above section, 1 mL diluted to 2.5 mL) or rutin standard solution (64-320 μg/mL) was mixed with 1 mL of distilled water in a testing tube, followed by addition of 250 μL of a 5% (w/v) sodium nitrite aqueous solution. After 6 min, 250 μL of a 10% (w/v) aluminum nitrate aqueous solution was added and the resulting mixture was allowed to stand for a further 5 min before 0.5 mL of 1 mol/L NaOH solution was added. The mixture was topped up to 2.5 mL by adding distilled water and mixed well. The absorbance at 510 nm was measured immediately. The results of triplicate analyses were expressed as mg of rutin equivalents of extractable compounds.

HPLC analysis

The sample solution was analyzed by HPLC (Dionex Ultimate 3000, DAD detector) under the following conditions: Dionex Acclaim C_{18} column (250 mm × 4.6 mm, 5 μm); Mobile phase: 0.1% phosphoric acid water solution (A)-methanol (B), gradient elution program: 0-15 min, B 80-60%; 15-45 min, B 60-55%; 45-50 min, B 55-0%; Wavelength scanning range: 190-400 nm.

Statistical analysis

All assays were carried out in triplicates, and results are expressed as. ANOVA test was used to analyze the differences among IC_{50} of various samples for different antioxidant assays, with least significance difference (LSD) $P < 0.01$ as a level of significance. Experimental results were further analyzed for Pearson's correlation coefficient of the content of flavonoids with different antioxidant assays and tested for significance at $P < 0.05$. The IC_{50} values were calculated using graph pad prism software and all the results was analyzed by SPSS16.0.

Results and Discussion

Antioxidant activity assay

Fig. 1 (A) showed the dose-response curves of DPPH (radical scavenging activities of the extracts from *Zhigeju*. The scavenging effects of EAE, BE and WE performed similar on DPPH (radical scavenging activity, while CE was a considerably less effective DPPH radicals scavenger. All the extracts were capable of scavenging DPPH radicals in a concentration-depend-

ent manner.

The results of Duncan's multiple Range Tests showed that there were no significant differences ($P>0.05$) among IC_{50} (concentration of crude drug, similarly hereinafter) values of WE (1.077 ± 0.014) mg/mL and BE (0.920 ± 0.013) mg/mL, while IC_{50} of EAE (0.301 ± 0.012) mg/mL and CE (54.808 ± 0.214) mg/mL were significantly different ($P<0.01$) from the others. The abilities of scavenging DPPH (radicals were in descending order: EAE > BE > WE > CE.

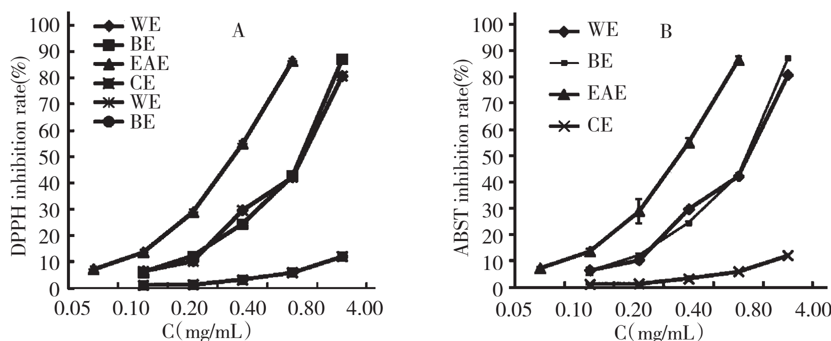

Fig. 1 Scavenging activities of different extracts on DPPH (A) and ABTS + (B) ($\bar{x} \pm s, n=3$)

Fig. 1 (B) showed the dose-response curves of ABTS radical scavenging activities of the extracts from *Zhigeju*. Again, the scavenging effects of EAE, BE and WE performed similar on ABTS radical scavenge activity, while CE was a considerably less effective ABTS radicals scavenger. All the extracts were capable of scavenging ABTS radicals in a concentration-dependent manner. The results of Duncan's multiple Range Tests showed that there were no significant differences ($P>0.05$) among IC_{50} values of WE (0.345 ± 0.013) mg/mL and BE (0.192 ± 0.009) mg/mL, while IC_{50} of EAE (0.006 ± 0.001) mg/mL and CE (13.306 ± 0.312) mg/mL were significantly different ($P<0.01$) from the others. The abilities of scavenging ABTS radicals were in descending order: EAE > BE > WE > CE.

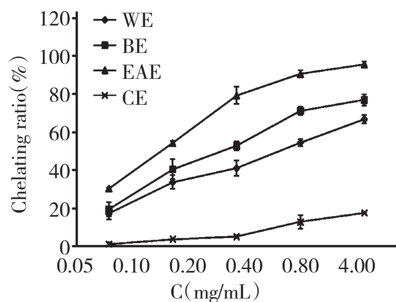

Fig. 2 Effect of different extracts on chelating Fe^{2+} ($\bar{x} \pm s, n=3$)

CE, EAE, BE and WE were assessed for their ability to compete with ferrozine for Fe^{2+} in free solution. All the extracts demonstrated an ability to chelate Fe^{2+} in a dose-dependent manner (Fig. 2). IC_{50} values of WE (0.864 ± 0.011) mg/mL, BE (0.427 ± 0.032) mg/mL, EAE (0.156 ± 0.021) mg/mL and CE (20.320 ± 0.418) mg/mL were significantly different ($P<0.01$).

Chemical composition analysis

The content of flavonoids in extracts was calculated according to regression equation of calibration curve ($Y = 0.1074X-0.0051, r = 0.9998$) and expressed in rutin equivalents. The results showed that CE, EAE, BE and WE contained (1.82 ± 0.25), (263.54 ± 3.95), (226.54 ± 3.49) and (55.08 ± 4.32) mg/g (by crude drug) flavonoids, respectively. Significant differences ($P<0.05$) existed between each two extracts in content of flavonoids.

In order to further identify the effective components existing in formulation *Zhigeju* extract, high-performance liquid chromatography (HPLC) analysis was conducted. The HPLC chromatogram of EAE and BE was similar and the content of total flavonoids was with no significant differences ($P>0.05$), hence the two extracts were combined to do the HPLC analysis, and 19 major compounds were detected (Fig. 3), among which, compound 14 (relative peak area was 45.01%) was identified as 4′, 5, 7-trihydroxyflavanone-7-rhamno-glucoside ($C_{27}H_{32}O_{14}$, naringin), compound 15 (relative peak area was 1.45%) was identified as apigenin-7-O-

neohe-speridoside ($C_{27}H_{30}O_{14}$, rhoifolin) and compound 19 (relative peak area was 3.96%) was identified as 4H-1-benzo-pyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-flavones ($C_{15}H_{10}O_7$, quercetin) by comparing their retention times and UV spectra with the reference substances obtained from Sigma Aldrich Chemical Co. (USA). The relative peak areas of compound 4, 11 and 12 were 4.58%, 23.05% and 12.56%, respectively. Further study needs to be carried out on the identification of these three compounds.

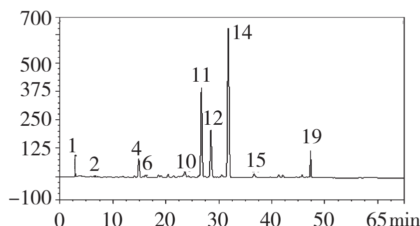

Fig. 3 HPLC chromatogram of EAE of formulation *Zhigeju* (detection wavelength 280 nm)

Based on the previous reports that the strong antioxidant activity of flavonoids were caused by their phenolics hydroxyl groups[7-9], the presence of three polyphenolic flavonoids (naringin, rhoifolin, quercetin) in EAE might play an important role on its antioxidant activity.

Correlation between flavonoids and antioxidant activity

According to the Pearson correlation analysis in each assay that mentioned above, it was known that the whole experiment showed a high correlation between concentration of flavonoids and antioxidant activity. Table 1 showed the Duncan's Multiple Range tests between modified IC_{50} values of different extracts, in which the concentration of crude drug was replaced by concentration of flavonoids. There were significant differences ($P < 0.05$) between different extracts in each assay, except EAE and WE in Fe^{2+} chelating activity assay. The Tukey tests showed the consistent results with Duncan's tests. This result illustrated that flavonoids with different structures had different antioxidant activity. For DPPH radical scavenging activity, polar flavonoids occurring in WE was highest, and the medium-polar flavonoids in EAE was the next. For ABTS radical scavenging activity, medium-polar flavonoids occurring in EAE was highest. For metal chelating activity, non-polar flavonoids in CE were highest.

Table 1 IC_{50} (concentration of crude drug replaced by concentration of flavonoids, mg/mL) of different extracts from formulation Zhigeju

Extracts	DPPH radical scavenging	ABTS radical cation assay	Fe^{2+} chelating activity
WE	$0.0593 \pm 0.0061^{a*}$	$0.0190 \pm 0.0056^{b*}$	$0.0476 \pm 0.0048^{f*}$
BE	$0.2084 \pm 0.0045^{d*}$	$0.0435 \pm 0.0031^{d*}$	$0.0967 \pm 0.0112^{b*}$
EAE	$0.0793 \pm 0.0047^{b*}$	$0.0016 \pm 0.0004^{a*}$	$0.0411 \pm 0.0083^{e*}$
CE	$0.0998 \pm 0.0054^{c*}$	$0.0242 \pm 0.0078^{c*}$	$0.0370 \pm 0.0105^{a*}$

a*, b*, c*, d*, e*, f* values with different letters in the same column were significantly ($P < 0.05$) different.

Conclusion

Based on the results obtained in the present study, it was concluded that formulation *Zhigeju* was rich in flavonoids. Different extracts of the formulation *Zhigeju* possessed different antioxidant activities. The antioxidant activity of each extract was related to the polarity and concentration of flavonoids contained in each extract. The extracts from formulation *Zhigeju* could be used as an antioxidant for adjuvant therapy.

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