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# Antioxidant Activities and the *In Vitro* Effects on K562 Cell Viability of Flavonoids Obtained from Onion Skin by Different Extraction Methods

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**Abstract:** In order to effectively extract specific flavonoids with high biological activity from onionskin, $NaNO_2$-$Al(NO_3)_3$-NaOH coloration method, spectrophotometry and MTT method were adopted to study the yield rate and content of flavonoids obtained from alcohol water and alkali water extraction processes, the antioxidant activities and the *in vitro* effects on K562 cell viability. The results indicated that yield rates of flavonoids obtained by alcohol water and alkali water extraction methods were 3.76% and 2.37%, and solid flavonoids contents were 42.20% and 76.21% respectively; under the same concentration of total flavonoids, alkali water extract has weaker effect on removing superoxide anions and DPPH • radicals, whereas they have similar effect on removing nitrite and hydroxyl free radicals; in the test concentration range (40 ~ 200 μg / mL), they have similar effect on inhibiting the proliferation activity of K562 cells; the maximum inhibition rate of flavonoids obtained by alkali water extraction method is 77.71%, while maximum inhibition rate of flavonoid obtained by alcohol water extraction method is 70.64%. Both the flavonoids obtained from onionskins by alcohol water extraction method and alkali water extraction method have good biological activity. The alkali water extraction method is simple and without any alcohol consumption, and the product purity is high. Although the yield rate and certain biological activity of flavonoids obtained by alkali water extraction method are lower than those obtained by alcohol water extraction method, it still has certain significance in industrial production.

**Keywords:** Onionskin, flavonoids, antioxidant activity, cell viability.

## 1. INTRODUCTION

Flavonoids refer to the compounds that exist in nature and have 2-phenyl-chromone (flavone) structure. They have a variety of physiological functions, and have good effects in relieving cough and asthma expectorant, diminishing inflammation, relieving allergy and improving capillary permeability. Besides, the superior antioxidant activity and potential anti-tumor effect of flavonoids have drawn more attention from people.

There are many researches in this area, for instance: silymarin can effectively prevent prostate cancer [1]. Scutellaria, flavin, chrysin, galangin and kaempferol can inhibit the cell proliferation of human esophageal squamous carcinoma cell lines KYSE-510 and OE33 by regulating gene expression and induce cell differentiation [2]. Kuntzs *et al.* selected more than 30 flavonoids, which were used to treat human renal tubular cell LLC-PK1, colon cancer cell Caco-2 and HT-29, and human breast cancer cell MCF-7. The results indicate that all these flavonoids have different effects on inhibiting the proliferation of these cancer cells, and have no significant cytotoxicity [3]. However, there are more researches on the antioxidant activity of flavonoids.

Onionskin is the waste in onion cultivation and processing; it is rich in bioflavonoids. There are very few studies on the biological activity of flavonoids obtained from onionskin. Chen Fengxiu, *et al.* found that the flavonoids in onion can significantly inhibit the proliferation of colon cancer HCT116 cells *in vitro* and induce their apoptosis [4]. Chen Jia studied the antioxidant activity *in vitro* of flavonoids obtained from onionskin by enzyme-assisted extraction method [5]. We used the method of combining single factor with three-factor quadratic regression general revolving combination design to discuss the alcohol water and alkali water extraction processes for extracting total flavonoids from onion skin. The optimum conditions for these two methods: alcohol water extraction method: 50% ethanol soaking, extraction for two times, extraction temperature is 71.6~73.8 °C, extraction time is 2.0~2.2 h, the ratio of liquid to solid is 30.1~34.2: 1 [6]; alkali water extraction method: pH 11 NaOH solution soaking, extraction for two times, extraction temperature is 83.0 °C ~ 84.0°C, extraction time is 1.9~2.0 h, the ratio of liquid to solid is 34.1~38.1: 1. The paper studies the antioxidant activity of extracts derived by different methods and the *in vitro* inhibition on the proliferation viability of K562K562 leukemia cell, in order to provide basis for choosing an effective method to extract flavonoids with high biological activity.

## 2. MATERIALS AND METHODOLOGY

### 2.1. Materials and Reagents

Onionskins are red onion peel wastes which are collected from the Beihuan Vegetable Wholesale Market of Zheng-

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zhou city. Onionskins are cleaned, dried and crushed for use. K562 cells are provided by the Pathophysiology Laboratory of School of Medicine of Zhengzhou University.

3-(4,5-dimethyl-thiazol-2)-2,5-diphenyl tetrazolium bromide (MTT, American Merck), 1,1- diphenyl-2-trinitrobenzene hydrazine (DPPH, Japanese Wako), 2,6-Di-tert-butyl-4-methylphenol (BHT, German Bayer), RPMI 1640 culture medium (American Gibco), fetal bovine serum (Chinese Academy of Medical Sciences), quercetin (≥98 %, National Institute for the Control of Pharmaceutical and Biological Products) and other reagents were domestic analytical reagents.

### 2.2. Instrument and Equipment

$CO_2$ gas incubator is made by Shanghai Yiheng Scientific instruments Co., Ltd; superclean bench is produced by Suzhou Purification Equipment Factory; BIO-RAD680 microplate reader is produced by Bio-Rad Company; SP752 UV visible spectrophotometer is made by Shanghai Spectrum Instruments Ltd.; SECURA124-1CN Electronic balance is made by German company Sartorius; microsyringe is from German company Eppendorf; high-speed centrifuge is from Sigma, USA; culture plate is made by American company Costar.

### 2.3. Extraction and Determination of Total Flavonoids from Onionskin

#### *2.3.1. Alcohol Water Extraction Method*

Accurately take 100g onionskin powder and put them in a 10 L round-bottom flask, put 3.2 L 50% ethanol solution into the flask for extraction, keep the extraction temperature at 73 ℃, and extract for two times, 2 h each time; then combine the extracts, reduce pressure and evaporate them until they are dry, measure the quality of solid extracts and the content of total flavonoids.

#### *2.3.2. Alkali Water Extraction Method*

Accurately take 100g onionskin powder and put them in a 10 L round-bottom flask, put 3.6 L pH11 NaOH solution into the flask for extraction, keep the extraction temperature at 83 ℃, and extract for two times, 2 h each time; then combine the extracts, use hydrochloric acid to adjust the pH to 2, while adding hydrochloric acid and stirring, after full precipitation, collect the precipitates, which are then washed with distilled water, dry precipitates, and measure the quality of solid extracts and the content of total flavonoids.

#### *2.3.3. Determination of Flavones*

According to Liu *et al.* method [6, 7], NaNO2-Al(NO3)3-NaOH method is adopted, quercetin is taken as standard, and absorbance value at the 318 nm is measured. It is found that the standard curve equation of concentration of quercetin $C$ and absorbance value $A$ is

$$A = 114.23C + 0.0016$$

Where

$A$---the absorbance value of solution;

$C$---the concentration of quercetin(mg/mL).

The content of total flavonoids in test samples is calculated according to quercetin.

### 2.3. Comparison of Antioxidant Activity

26.2 mg extracts obtained by alkali water extraction method and 47.4 mg extracts obtained by alcohol water extraction method are accurately weighed, put into solution with constant volume of 100 mL respectively, and formulated into sample solution with total flavonoid concentration of 200μg / mL for spare use.

#### *2.3.1. DPPH • Radical Scavenging*

According to Liuqing Yang etc. method [8], it was slightly modified. 2 mL DPPH• solution with concentration of $2\times10^{-4}$ mol/L and certain volume of sample solution are put into a 10 mL colorimetric tube, then anhydrous ethanol is added into it to make up a total volume of 5 mL. After shaking it, it is put in dark place at room temperature for 30 min. Taking anhydrous ethanol as a reference, the absorbance value at wavelength of 517nm is measured, then the free radical scavenging rate can be calculated according to the following formula:

$$R = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Where

$R$--- DPPH• scavenging rate (%)

$A_0$---the blank absorbance value of DPPH • solution without adding sample;

$A_1$---the absorbance value of DPPH• solution after adding sample;

$A_2$---the absorbance value of sample solution itself.

The DPPH• radical scavenging rate of Vitamin E ($V_E$) and 2,6-Di-tert-butyl-4-methylphenol (BHT) is measured by the similar method.

#### *2.3.2. Nitrite Scavenging*

According to Gang Xu *et al*. method [9], 2 mL $NaNO_2$ with concentration of 5 mg/L and certain volume of sample solution are put into a colorimetric tube, then anhydrous ethanol is added into it to make up a total volume of 5 mL. Shake it evenly. After reacting for 10 min, 2 mL sulfanilic acid with mass fraction of 0.4% is added, shaking it evenly, and leaving to rest for 5minutes. Then 1mL hydrochloric acid naphthylethylenediamine with mass fraction of 0.2% is added and shaken evenly. Distilled water is used to get a constant volume of 10 mL, then it is stored at room temperature for 15 min. Taking anhydrous ethanol as a reference, the absorbance value at wavelength of 538nm is measured, then

the nitrite scavenging rate can be calculated according to the following formula:

$$R = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Where

*R*---Nitrite scavenging rate (%)

$A_0$---the blank absorbance value of solution without adding sample;

$A_1$---the absorbance value of solution after adding sample;

$A_2$---the absorbance value of sample solution itself without adding $NaNO_2$.

The nitrite scavenging rate of Vitamin C (Vc) is measured by the similar method.

#### *2.3.3. Superoxide Anion Scavenging*

According to Gao Zhou *et al.* method [10], it was slightly modified. After adding 4.5 mL 0.05 mol/L tris-HCl (pH = 8.2) buffer solution into a colorimetric tube and keeping it immersed in a water bath preheated to 25 °C for 20 min, 0.4 mL 25 mmol/L pyrogallol and a certain volume of sample solution are added. After exact reaction for 4 min in water of 25 °C water, 2 drops of 8 mol/L dense HCl are added to stop the reaction. Distilled water is used to keep a constant volume of 10 mL. As for 4.5 mL tris-HCl buffer solution, distilled water is used to keep a constant volume of 10 mL for reference. The absorbance value at wavelength of 325nm is measured, then the superoxide anion free radical scavenging rate can be calculated according to the following formula:

$$R = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Where

*R*--- Superoxide anion radical scavenging rate (%)

$A_0$---the absorbance value of solution without adding sample;

$A_1$---the absorbance value of solution after adding sample;

$A_2$---the absorbance value of solution itself without adding pyrogallic acid sample.

#### *2.3.4. Hydroxyl Radical Scavenging*

According to Lili Zou *et al*. method [11], it was slightly modified. 3 mL of 2 mmol/L $FeSO_4$, 3 mL of 6 mmol/L $H_2O_2$, 3 mL of 6 mmol/L salicylic acid, and certain volume of sample solution are added into a 10 mL colorimetric tube in order, and then distilled water is used to get a constant volume of 10ml. Shake it evenly, and put it in water bath at 37℃ for 15 min. Then take the tube out, cool it, and keep it centrifuged for 10 min at 2000 r/min. Distilled water is used as a reference, the absorbance value at wavelength of 510nm is measured, then the hydroxyl free radical scavenging rate can be calculated according to the following formula:

$$R = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Where

*R*---Hydroxyl Free Radical Scavenging Rate (%)

$A_0$---the absorbance value of solution without adding sample;

$A_1$---the absorbance value of solution after adding sample;

$A_2$---the absorbance value of solution itself without adding salicylic acid sample.

The hydroxyl free radical scavenging rate of Vc, VE and BHT can be measured with similar method.

### 2.4. The Effects on K562 Cell Viability *in Vitro*

According to Li Cao *et al.* method [12], it was slightly modified. K562 cells at logarithmic growth phase are selected, and seeded in a 96-hole by the density of $1 \times 10^5$ cells / mL; each hole is inoculated (100 μL). Alcohol water group and alkali water group were treated with final concentration of total flavonoids (40, 80, 10, 160, 200 μg / mL) respectively. In addition, a blank group (which has only culture medium) and control group (without drug) are set, and six parallels are set in each group. After 72 h, MTT is added, and after culturing for another 4 h, get it centrifuged, the supernatant is discarded, and 150 μL of DMSO is added to each hole to fully dissolve the crystals. A microplate reader (Bio-Rad 3350 microplate reader) is used to measure the absorbance value at a wavelength of 570 nm, 0 is set for the blank group, and the inhibition rate is calculated according to following formula:

$$R = \frac{1 - A_0}{A_1} \times 100\%$$

Where

*R*--- Inhibition Rate (%)

$A_0$---the absorbance value of solution without adding sample;

$A_1$---the absorbance value of solution after adding sample;

## 3. RESULTS AND DISUSSION

### 3.1. Comparison of the Contents of Flavonoids Extracts and Yield Rate of Total Flavonoids Obtained by Two Methods

The extraction of total flavonoids from onionskin is carried out according to the method of combining single factor with three-factor quadratic regression general revolving combination design. The solid content, flavonoid content and yield rate of total flavonoids obtained by the alkali water extraction method and the alcohol water extraction method are shown in Table **1**.

**Table 1. The flavonoids extracts obtained by the alkali water extraction method and the alcohol water extraction method (100 g dry onionskin).**

| Extraction Method | Alcohol Water | Alkali Water |
|---|---|---|
| Quantity of total flavonoids (g) | 3.76±0.04 | 2.37±0.02 |
| Yield rate of total flavonoids (%) | 3.76±0.04 | 2.37±0.02 |
| Solid content (g) | 8.91±0.16 | 3.11±0.09 |
| Yield rate of solid content | 8.91±0.16 | 3.11±0.09 |
| Flavonoid content in extract (%) | 42.20±0.36 | 76.24±1.46 |

Data are presented as the mean ± SD of each triplicate test.

Compared with alcohol water extraction method, alkali water extraction method has lower yield of flavonoids, perhaps this is because that alkali water extraction method only obtains flavonoids with strong acidity, but the product purity is much higher, and the processing is simple.

## 3.2. Comparison of Antioxidant Activity of Extracts Obtained by Two Methods

### *3.2.1. DPPH • Radical Scavenging*

The main principle of DPPH• method was that DPPH• generated a stable nitrogen free radical in the solution, and the solution showed the typical purple color. It had the stronger absorption spectrum in ultraviolet visible (UV-Vis) region. When the antioxidant was added to DPPH• solution, the purple of DPPH• faded due to its scavenging action on free radical, so as to make the absorption spectrum intensity weakened with the increase of antioxidants.

Under the same condition with same concentration of total flavonoids, the DPPH• free radical scavenging rates of the extracts obtained by alcohol water and alkali water methods, VE and BHT are shown in Fig. (**1**).

As can be seen from Fig. (**1**), under the same concentration of total flavonoids, the DPPH• radical scavenging rate of alkali extracts is close to that of BHT; alcohol water extracts have similar effects as with $V_E$; and alcohol water extracts are better than alkali water extracts. The differences between the DPPH• radical scavenging rates of the two products may be caused by the differences in the components of flavonoids.

### *3.2.2. Nitrite Scavenging*

Nitrite could diazotize with sulfanilic acid under acidic condition, then coupled with N-Naphthylethylenediamine Dihydrochloride to generate a red clathrate. Its absorbance could be used to detect with the spectrophotometric method.

Because $V_E$ and BHT are insoluble in water, Vc is chosen as contrast. The scavenging ability of different substances in removing nitrite is shown in Fig. (**2**).

As can be seen from Fig. (**2**), within the test concentration range, the alcohol water extracts, alkali water extracts and Vc have similar ability in removing nitrite.

### *3.2.3. Superoxide Anion Scavenging*

The autoxidation reaction of pyrogallic acid occurred under alkaline conditions to produce the superoxide anion with the stable concentration and intermediate. The intermediate reacted with the superoxide anion to obtain the colored intermediate product. This product was absorbed in the ultraviolet so as to cause the linear accumulation of the absorbance at a certain wave length, which could react to the scavenging capacity of the antioxidant.

Pyrogallol method is adopted to measure the effects of extracts obtained by alkali water and alcohol water methods in scavenging superoxide anion radicals, the results are in Fig. (**3**).

It can be seen from Fig. (**3**) that at low concentrations, the extracts obtained by alkali water and alcohol water methods and pyrogallol have almost the same effect in scavenging superoxide anion radicals; with higher concentrations,

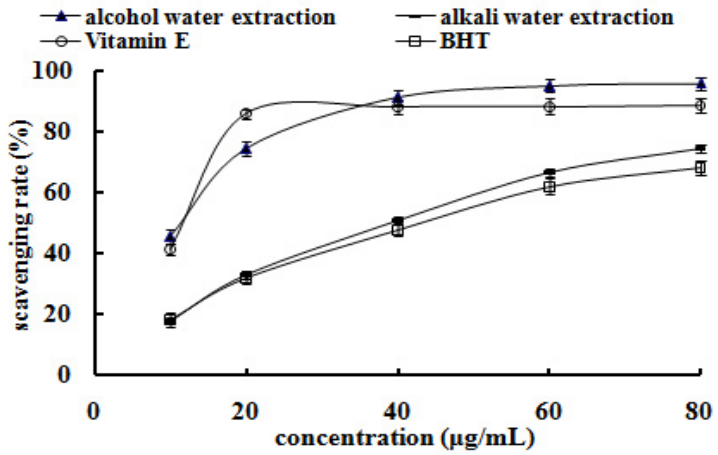


**Fig. (1).** Comparison of the scavenging ability of flavonoids by different extraction methods on DPHH• radical.

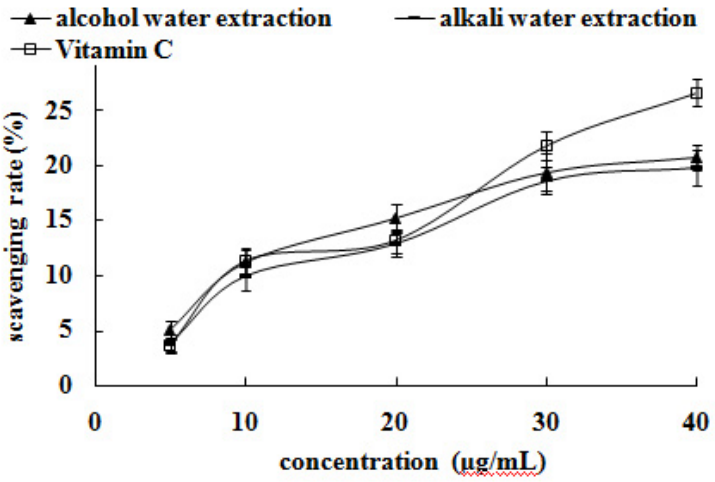


**Fig. (2).** Comparison of the scavenging ability of flavonoids by different extraction methods on Nitrite.

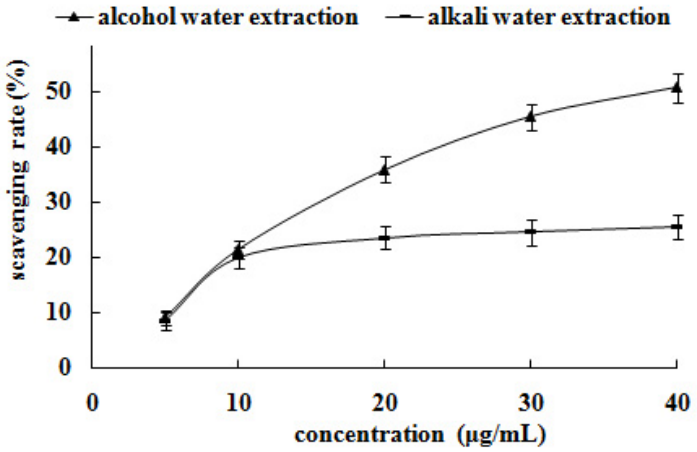


**Fig. (3).** Comparison of the scavenging ability of flavonoids by different extraction methods on Superoxide Anion.

extracts obtained by alcohol water extraction method are better than those obtained by alkali water extraction method.

#### *3.2.4. Hydroxyl Radical Scavenging*

$H_2O_2$ and $Fe^{2+}$ were mixed for Fenton reaction to generate the hydroxyl free radical with very high reaction activity. The hydroxyl free radical could be effectively captured by salicylic acid and generated the colored material. But if the material with the scavenging action was added, it would compete with salicylic acid so as to reduce the ferrous products.

The hydroxyl free radical scavenging rates are shown in Fig. (**4**).

As can be seen in Fig. (**4**), when concentration is low, alkali extracts, Vc and BHT have similar ability in scavenging hydroxyl radicals. Extracts obtained by alcohol water extraction method are better than those obtained by alkali water extraction method. When concentration is little high, they have similar effects in scavenging hydroxyl radicals, and better effects than Vc, $V_E$ and BHT. When the concentration is very high, both of them are lower than VE and BHT, but better than Vc.

### 3.3. The Effects on K562 Cell Viability *in Vitro*

MTT method was widely used to determine cell survival rate in the screening of antitumor drugs *in vitro*. The chemical name of MTT was 1,1-diphenyl-2-trinitrobenzene hydrazine and its effective component tetrazolium blue could react with mitochondrial succinate dehydrogenase in the living cells, then it was deoxidized to the water-fast blue crystal-formazan and precipitated in the cells. However, the dead cells had no such function. According to the characteristic of MTT, the dyed formazan had the maximum absorption peak at 570 nm after dissolving with DMSO. Its absorbance was used to calculate the cell survival rate.

The extracts obtained by different methods all have the effects on K562 cell viability *in vitro*, the results are shown in Fig. (**5**).

As can be seen from Fig. (**5**), within the concentration range of experimental concentration (40 ~ 200 μg / mL),

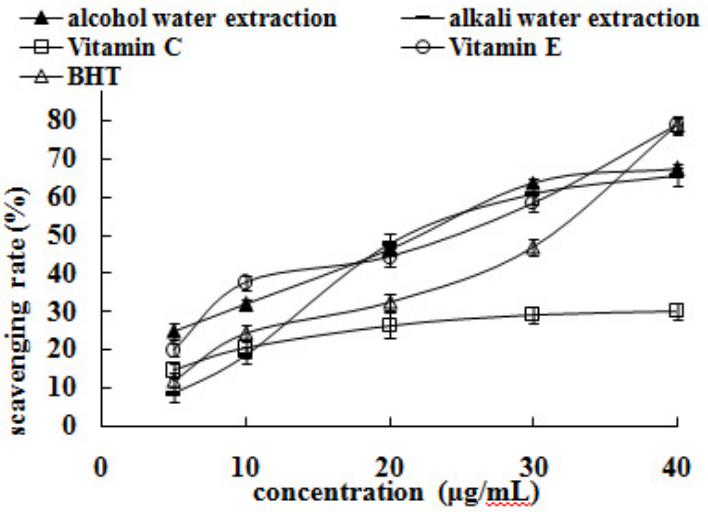


**Fig. (4).** The Hydroxyl Radical scavenging ability of Vc, $V_E$, BHT and flavonoids by different extraction methods.

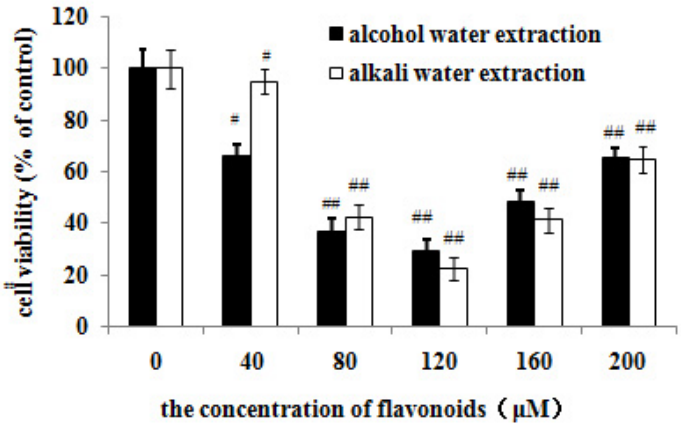


**Fig. (5).** Cytotoxicity of the flavonoids on K562 cells by different extraction methods.

extracts obtained by alcohol water extraction method and alkali water extraction method have little difference in inhibiting the growth of K562 cells, their inhibiting effects increase firstly and then decrease with the increase of concentration, and reached the strongest point at 120 μg/mL; the strongest inhibition rate are 70.6% and 77.7% respectively.

K562 cells were treated with different flavonoids extracts at various total flavonoids concentrations for 72 h and cell viability was determined by MTT assay. Data were shown as mean ± SD (n=6). #$p$<0.05, ## $p$<0.01 compared with the control.

## CONCLUSION

Generally, the extraction of flavonoids is based on similarity and compatibility principle, using a certain concentration of ethanol as an extraction solvent. Flavonoids are weakly acidic and soluble in alkali water solution, and onionskin has very few proteins, polysaccharides, and other substances that can be greatly affected by pH. Alkali extraction and acid precipitation method can also be used for the extraction.

Both the total flavonoids obtained from onionskin by alcohol water and alkali water extraction method have good biological activity, and their antioxidant activity is not weaker than the commercially available common antioxidants; in most cases, they have similar biological activity; in terms of antioxidant activity, the flavonoids obtained by alcohol water extraction method are slightly better than those obtained by alcohol water extraction method; the alkali water extraction method is simple without any alcohol consumption, and the product purity is high. Although the yield rate of flavonoids obtained by alkali water extraction method is lower than those obtained by alcohol water extraction method, it still has certain significance in industrial production.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

## ACKNOWLEDGEMENTS

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