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# Evaluation of the antioxidant property and effects in *Caenorhabditis elegans* of Xiangxi flavor vinegar, a Hunan local traditional vinegar*

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**Abstract:** Xiangxi flavor vinegar (XV) is one of Hunan Province's traditional fermented vinegars. It is produced from herb, rice, and spring water with spontaneous liquid-state fermentation techniques. In this study, we investigated the antioxidant property of XV by analyzing its antioxidant compounds, its free radical scavenging property in vitro and in vivo, and its effects on antioxidant enzyme activity and apoptosis in *Caenorhabditis elegans*. The results showed that XV is rich in antioxidants. In particular, ligustrazine reached 6.431 μg/ml. The in vitro 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH$^{\bullet}$), hydroxyl radical ($^{\bullet}$OH), and superoxide anion radical ($O_2^{\bullet-}$) scavenging rates of XV were 95.85%, 97.22%, and 63.33%, respectively. The reactive oxygen species (ROS) content in XV-treated *C. elegans* decreased significantly ($P$<0.01) compared to the control group. Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activities were remarkably increased ($P$<0.01) in *C. elegans* after XV treatment. In addition, XV could upregulate CED-9 protein expression and downregulate CED-3 protein expression in *C. elegans*. These results prove that XV is rich in antioxidants and scavenges radicals in vitro efficiently. XV inhibits apoptosis in *C. elegans* probably by scavenging ROS and increasing the activities of its antioxidant enzymes.

**Key words:** Vinegar; Antioxidant; Free radical scavenging property; *Caenorhabditis elegans*; Antioxidant enzyme; Reactive oxygen species (ROS); Apoptosis

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## 1 Introduction

Vinegar is a common condiment. According to the records, the production of vinegar can be traced back to 1058 BC in China (Conner and Allgeier, 1976). Currently, various types of traditional fermented vinegars are being produced locally in China, such as Shanxi aged vinegar (Shanxi Province), Zhenjiang aromatic vinegar (Jiangsu Province), Fujian Monascus vinegar (Fujian Province), and Xiangxi flavor vinegar (Hunan Province). Because of the unique fermentation techniques and the use of local materials, different local vinegars in China have their special flavors and functions.

Xiangxi flavor vinegar (XV) is mainly produced in the autonomous counties of Tujia and Miao ethnic minorities in the northwest of Hunan Province (longitude 109°10′–110°23′ E, latitude 27°45′–29°38′ N), usually referred to as Xiangxi, an area rich in natural resources. XV is made from local herbs, spring water, and rice by spontaneous liquid-state fermentation. It has a soft taste, a faint scented flavor, and a reddish brown color, and is transparent. XV has been produced for centuries, which can be partly attributed to its royal sponsorship in ancient China (Gao *et al.*, 2007).

The fermentation of XV is a spontaneous process. None of the raw materials is sterilized. The production process (Fig. 1a) can be divided into two

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parts. First, the starter Qu is produced from rice, negundo chastetree leaf (*Folium Viticis Negundo*, a herb that can be used to treat cold, dysentery, and bacteriostasis (Kong, 2011)), and spicy liao grass (*Polygonum lapathifolium* L. var. *Sali-cidolium* Sibth., a herb containing auxin for yeast and rhizopus and that can be used to treat dysentery (Pan *et al*., 2015)). Steamed rice is covered with negundo chastetree leaf and spicy liao grass at a thickness of 2–5 cm for 7 d to let the microorganisms transfer from the herbs to the rice and grow (Figs. 1b and 1c). These microorganisms include fungi (*Rhizopus*, *Monascus*, yeast, etc.) and bacteria (mainly lactic acid bacteria, *Bacillus*, and acetic acid bacteria) (Wu *et al*., 2012; Li *et al*., 2015). For storage, Qu is predried in the sun (Fig. 1d). For fermentation, Qu is powdered and mixed with steamed rice and boiled water before being placed in an urn (Fig. 1e). Second, the fermentation, carried out in the urn, can be divided into three stages: saccharification, alcohol fermentation, and acetic fermentation (Figs. 1f and 1g). The whole process takes about 2.5 years, during which old Culao (acetic fermentation residue of the last batch) is added as "vinegar seed" at the end of alcohol fermentation to initiate acetic acid fermentation (Fig. 1g).

Vinegars are reported to possess some functional properties such as antioxidant, antimicrobial, antihypertensive, and cholesterol-lowering properties (Budak *et al*., 2014). Black vinegar contains essential amino acids and polyphenols. Dietary supplementation with black vinegar not only decreases fecal triacylglycerol (TAG) and total cholesterol (TC), but

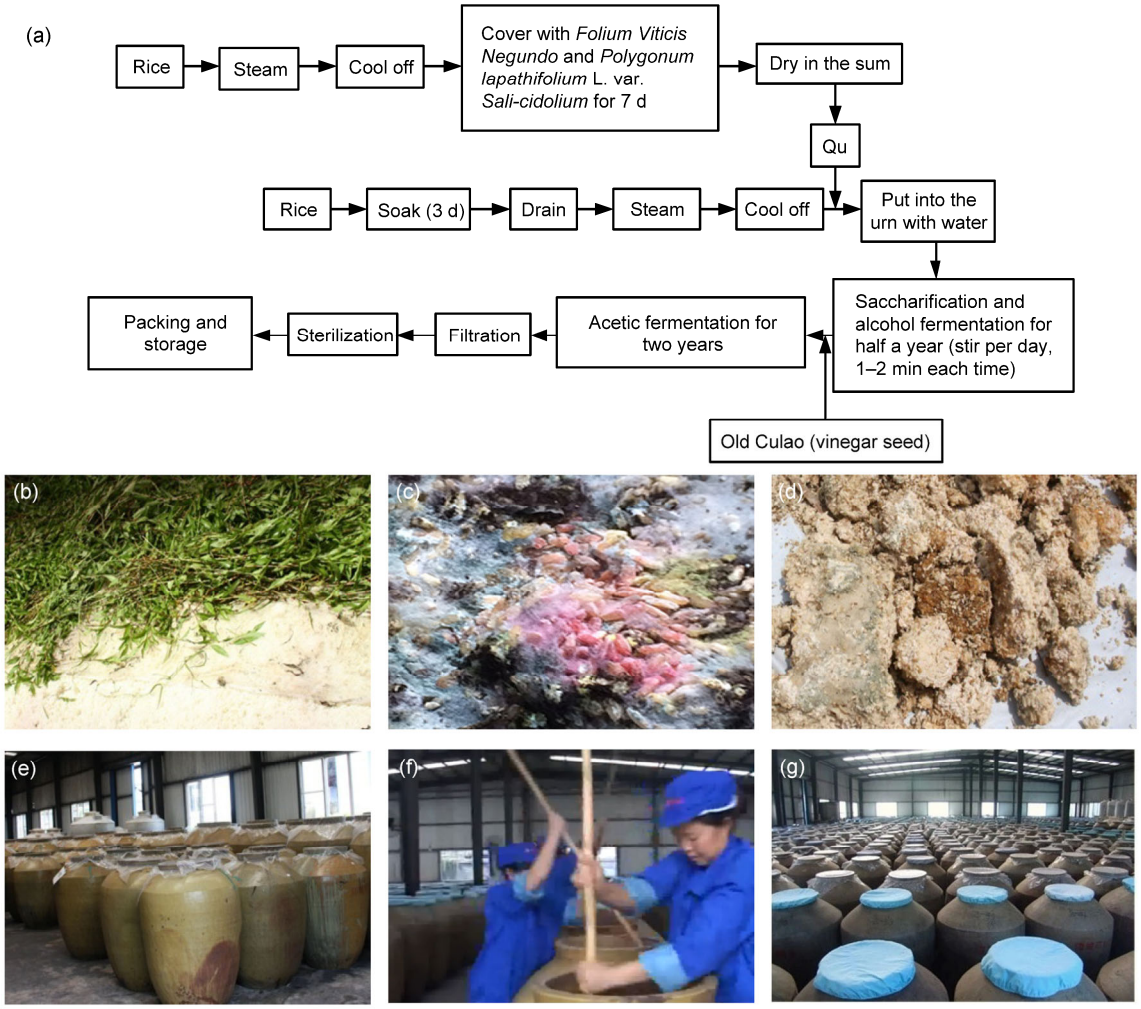


**Fig. 1 Flowchart of Xiangxi flavor vinegar production**

(a) The production process; (b) *Folium Viticis Negundo* and *Polygonum lapathifolium* L. var. *Sali-cidolium*; (c) Microorganisms transferring from herbs and growing on rice; (d) Qu after drying; (e) Putting the materials into the urn; (f) Aerobic fermentation; (g) Acetic acid fermentation

also increases the Trolox equivalent antioxidant capacity (TEAC) levels, catalase (CAT) and glutathione peroxidase (GPH-Px) activities of high-fat/cholesterol diet (HFCD)-fed hamster blood (Chou *et al*., 2015). Bertelli *et al*. (2015) studied the antioxidant activity and phenolic compounds of traditional balsamic vinegar of Modena (TBVM) and balsamic vinegar of Modena (BVM), and showed that antioxidant activity at 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) of TBVM was twice as high as that of BVM. The 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH$^{\bullet}$) scavenging activity of TBVM was 52% higher than that of BVM. Although some studies have reported that traditionally fermented Chinese XV increases superoxide dismutase (SOD) activity and removes chloasma induced by intramuscular injection of progesterone in mice (Wang *et al*., 2014b), comprehensive studies of the bioactive function of XV are not available.

The aim of this study was to evaluate the antioxidant property of XV and to define the mechanism by identifying its antioxidant compounds by high-performance liquid chromatography (HPLC). Six antioxidant substances, namely rutin, gallic acid, ferulic acid, *p*-coumaric, vanillic acid, and ligustrazine, were identified in the vinegar. The scavenging abilities of DPPH$^{\bullet}$, hydroxyl radical ($^{\bullet}$OH), and superoxide anion radical ($O_2^{\bullet-}$) in the vinegar were analyzed. Finally, the in vivo antioxidant property was tested in *Caenorhabditis elegans*.

## 2 Materials and methods

### 2.1 Sampling

XV without aging (XVWA) after fermentation and XV after 2 years of aging (XV) were collected from the Hunan Biancheng vinegar company, Xiangxi Autonomous Prefecture of Hunan Province, China. Samples were produced from late June to early July in 2012 or 2013. Both XVWA and XV were produced from the same raw materials and with the same fermentation process. Samples were collected in sterile glass bottles, immediately transported to the laboratory, and stored at −20 °C until analysis. Fermented rice vinegar (RV; Changsha vinegar factory, Changsha, China) and 3.5% (v/v) acetic acid (AA) were used for comparison with XV. The pH values of the other three samples were adjusted to that of XV.

### 2.2 Determination of antioxidant compounds by HPLC

The antioxidants were detected as previously described (Cerezo *et al*., 2008). Briefly, the samples were passed through a 0.45-μm cellulose membrane filter before being injected into a chromatograph equipped with a quaternary pump (DGP-3600RS), a degasser (SRD-3600), and a temperature-controlled automatic injector (WPS-3000TRS) from Thermo Fisher, USA. Detection was carried out by a diode array detector (DAD 3000RS) coupled to a chromatographic data system (CDS, SR13, Thermo Fisher, USA). The mobile phase was methanol and 20 mmol/L ammonium acetate (15:85, v/v). The column temperature was set at 40 °C and the flow rate was 0.8 ml/min. The peaks were identified and quantified by comparison of the retention time and peak area with known external standards. Standard rutin, gallic acid, ferulic acid, *p*-coumaric, vanillic acid, and ligustrazine were purchased from Sigma (USA).

### 2.3 Free radical scavenging

The vinegar samples were diluted to 0.20, 0.10, 0.06, and 0.05 ml/ml before assay.

#### 2.3.1 DPPH$^{\bullet}$ radical-scavenging assay

Five milliliters of 0.2 mmol/L DPPH$^{\bullet}$ radical solution, 4 ml of anhydrous ethanol, and 1 ml of the samples were added into a 25-ml colorimetric cylinder and mixed rapidly. After incubation for 30 min at room temperature, the absorbance of the mixture at 517 nm was measured using a spectrophotometer (LabTech Instruments, Beijing, China). The result was calculated as the inhibition rate according to the formula (Mishra *et al*., 2012):

$$\text{inhibition rate}=[1-(A_s-A_j)/A_c]\times 100\%,$$

where $A_s$, $A_j$, and $A_c$ are the absorbance values of the sample, blank sample, and control, respectively.

#### 2.3.2 $^{\bullet}$OH radical-scavenging assay

$^{\bullet}$OH radical was produced by the Fenton reaction (Yoon *et al*., 2001). The Fenton system used was $FeSO_4$-ethylenediaminetetraacetic acid (EDTA)-$H_2O_2$. The reaction was started by adding hydrogen peroxide. $^{\bullet}$OH oxidizes safranine and causes it to fade. The $^{\bullet}$OH radical-scavenging ability was determined by

measuring the absorbance value of safranine. The reaction mixture contained 1 ml of 0.15 nmol/L phosphate buffer solution (PBS, pH 7.4), 1 ml of safranine solution (40 μg/ml), 1 ml of 3% $H_2O_2$, 1 ml of 3.8 mmol/L $FeSO_4$-EDTA solution, and 0.5 ml of the sample. The reaction was carried out at 37 °C for 30 min. The absorbance of the reaction mixture at 520 nm was measured. The result was calculated as the inhibition rate according to the following formula:

$$\text{inhibition rate}=(A_s-A_b)/(A_c-A_b)\times 100\%,$$

where $A_s$, $A_b$, and $A_c$ are the absorbance values of the sample, blank control, and control, respectively.

2.3.3 $O_2^{\bullet-}$ radical-scavenging assay

$O_2^{\bullet-}$ was generated from a pyrogallol autoxidation reaction system (Yang *et al.*, 2010). In the system, $O_2^{\bullet-}$ will combine with an intermediate product to yield a substance with yellow color. The $O_2^{\bullet-}$ radical-scavenging ability of XV was determined by measuring the absorbance at 325 nm of the reaction mixture. The reaction system contained 4.5 ml of 50 mmol/L Tris-HCl (pH 8.2, preheated to 25 °C in a water bath for 20 min), 0.5 ml of 25 mmol/L pyrogallol, and 0.1 ml of the sample. The reaction was carried out at 25 °C in a water bath for 5 min. One milliliter of 8 mol/L HCl solution was added to stop the reaction before the absorbance measurement. The result was calculated as the inhibition rate according to the following formula:

$$\text{inhibition rate}=(A_b-A_s)/A_b\times 100\%,$$

where $A_s$ and $A_b$ are the absorbance values of the sample and blank control, respectively.

### 2.4 Culture and treatment of *C. elegans*

Wild-type *C. elegans* strain N2 and *Escherichia coli* OP50 were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, USA). Worms were cultured at 20 °C in a nematode growth medium (NGM) with *E. coli* OP50 as food source, as previously described (Strange, 2006). Synchronized worms were obtained by the lysis-centrifugation method (Wang *et al*., 2014a). Gravid hermaphrodites were lysed by a freshly prepared lysate solution (5 ml 6.4% (0.064 g/ml) sodium hypochlorite, 5 ml 1 mol/L sodium hydroxide solution). After lysis of adult worms, eggs were collected by centrifuging and washed 3–4 times with M9 buffer. The collected eggs were hatched at 20 °C for 24 h. The L1 larvae were transferred to a fresh NGM with *E. coli* OP50. L4 age-synchronized worms were obtained 3 d after synchronization.

For in vivo tests of bioactive function, 135 μl of vinegar was spread on an NGM plate. After drying, *E. coli* OP50 was added as food for the worms. L4 young adult worms were transferred on the plate and cultured at 20 °C for 48 h; M9 buffer was used as control.

### 2.5 Reactive oxygen species assay

2,7-Dichlorodihydrofluoresce in diacetate ($H_2$DCF-DA; Sigma, USA) was used as the fluorescence probe to detect reactive oxygen species (ROS) in *C. elegans* (Smith and Luo, 2003). $H_2$DCF-DA is cell-membrane-permeable. Once it enters the cell, it is converted into $H_2$DCF, which cannot penetrate the cell membrane of worms. $H_2$DCF does not fluoresce until it is oxidized to dichlorofluorescein (DCF) by ROS.

After vinegar treatment, *C. elegans* was washed by PBS-T (1× PBS containing 0.5% Tween 20) for 20 min. Worms of each group (50±2) were transferred into the wells of a 96-well microplate (WHB, Shanghai, China, 96F 1pcs) and 10 μl PBS-T and 70 μl PBS were added. Subsequently, 20 μl of 250 μmol/L $H_2$DCF-DA was added to each well. The plates were incubated at 20 °C for 30 min. The fluorescence intensity of DCF in each well was measured by a porous chemiluminescence detector (Varioskan Flash, USA). The excitation wavelength was 485 nm and the emission wavelength was 535 nm.

### 2.6 Antioxidant enzyme assay

After vinegar treatment for 48 h, 200±5 worms of each group were transferred into a 1.5-ml sterile Eppendorf tube containing 1 ml PBS. The worms were sonicated with an ultrasonic cell crusher (Vosin, Wuxi, China, BL92-IIDL). After centrifuging at 4000 r/min for 15 min, the supernatants were carefully transferred to a new sterile Eppendorf tube and incubated at 60 °C in a water bath for 15 min before a second centrifuging at 4000 r/min for 20 min. The resultant supernatant was used for enzyme assay.

The activities of GSH-Px, SOD, and CAT of *C. elegans* were determined using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

### 2.7 Determination of CED-3 and CED-9 in *C. elegans*

After vinegar treatment for 5 d, the worms were washed with M9 buffer three times. Worms of each group (200±5) were transferred into a 1.5-ml sterile Eppendorf tube with 1 ml of M9 buffer. The worms were then sonicated and centrifuged at 4000 r/min for 10 min. The supernatant and pellet were separated and stored at −20 °C until analysis.

The quantities of the apoptosis protein CED-3 and the anti-apoptosis protein CED-9 of the homogenate of vinegar-treated *C. elegans* were determined using the CED-3 assay kit and the human Bcl-2-like assay kit (RB, USA), respectively, according to the manufacturer's instructions. It has been reported that CED-9 of *C. elegans* was homologous to Bcl-2 protein, an anti-apoptosis protein in humans (Huang and Strasser, 2000). The experimental procedures were conducted according to the kit's instructions. The standard curve showed the relationship between absorbance ($A$) and CED-3 concentration ($C$) from 0 to 10 IU/L ($A$=0.202$C$+0.071, $R^2$=0.992), as well as the standard curve of absorbance and CED-9 concentration from 0 to 120 ng/ml ($A$=0.007$C$+0.54, $R^2$=0.991). The amounts of CED-3 and CED-9 in the sample were calculated according to the respective standard curve.

### 2.8 Statistical analysis

Data are presented as mean±standard deviation (SD). Statistical analysis was performed using the SPSS 19.0 software (IBM, Chicago, IL, USA). Differences in the mean values between treated samples and control were compared by $t$-test (significance at $P$<0.05).

## 3 Results

### 3.1 Antioxidant compounds in Xiangxi flavor vinegar

Six antioxidant compounds, namely rutin, gallic acid, ferulic acid, *p*-coumaric, vanillic acid, and ligustrazine, were detected in XVWA and all except rutin in XV (Table 1). None of the antioxidant was detected in AA. In comparison, four compounds were detected in RA, but not rutin and *p*-coumaric. Generally, the concentrations of the determined antioxidant compounds were higher in XV and XVWA than in RV ($P$<0.05).

The contents of antioxidant substances were different between XVWA and XV. The amounts of rutin, gallic acid, and *p*-coumaric were higher in XVWA; especially rutin was not detected in XV ($P$<0.01). Ligustrazine was much higher in XV than in XVWA ($P$<0.05), showing the accumulation of ligustrazine during aging.

### 3.2 In vitro antioxidant properties of Xiangxi flavor vinegar

The DPPH$^{\bullet}$, $^{\bullet}$OH, and $O_2^{\bullet-}$ scavenging abilities of AA, RV, XVWA, and XV are shown in Table 2. Generally, the DPPH$^{\bullet}$ scavenging abilities of XVWA and XV were much higher than those of AA and RA. The DPPH$^{\bullet}$ scavenging abilities of the basic solutions of XVWA and XV reached 66.49% and 95.85%, respectively, while those of AA and RA were only 4.88% and 15.84%, respectively. In the basic solution, the $^{\bullet}$OH scavenging rates of the four samples were higher than 45%, and those of XVWA and XV were even higher at 85%. Moreover, the scavenging rate of XV in low concentration (0.05 ml/ml) still reached 11%, which was the same as that of RV at 0.10 ml/ml concentration. Meanwhile, a higher $O_2^{\bullet-}$ scavenging ability was also determined in XVWA and XV than in AA and RV.

**Table 1 Antioxidant compounds determined in Xiangxi flavor vinegar**

| Group | Rutin (μg/ml) | Gallic acid (μg/ml) | Ferulic acid (μg/ml) | *p*-Coumaric (μg/ml) | Vanillic acid (μg/ml) | Ligustrazine (μg/ml) |
|---|---|---|---|---|---|---|
| AA | ND | ND | ND | ND | ND | ND |
| RV | ND | 0.001 | 0.067 | ND | 0.010 | 0.006 |
| XVWA | $0.665^{\Delta\Delta}$ | $5.853^{\Delta\Delta\Delta}$ | $0.191^{\Delta\Delta}$ | $0.065^{\Delta\Delta}$ | $1.449^{\Delta\Delta\Delta}$ | $3.284^{\Delta\Delta\Delta}$ |
| XV | ND | $0.500^{\Delta\Delta **}$ | $0.163^{\Delta\Delta}$ | $0.006^{\Delta **}$ | $2.725^{\Delta\Delta\Delta}$ | $6.431^{\Delta\Delta\Delta *}$ |

AA: acetic acid; RV: rice vinegar; XV: Xiangxi flavor vinegar with aging; XVWA: XV without aging; ND: not detected. Data are presented as mean±SD ($n$=3). $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, $^{\Delta\Delta\Delta}P$<0.001, compared to the RV group. $^{*}P$<0.05, $^{**}P$<0.01, compared to the XVWA group

**Table 2 Free radical-scavenging ability of Xiangxi flavor vinegar (XV)**

| Vinegar concentration | Group | DPPH$^{\bullet}$ scavenging ability (%) | $^{\bullet}$OH scavenging ability (%) | $O_2^{\bullet-}$ scavenging ability (%) |
|---|---|---|---|---|
| Basic solution | AA | 4.88±0.7$^{b}$ | 47.59±1.3$^{d}$ | 59.93±1.5$^{d}$ |
| | RV | 15.84±1.3$^{c}$ | 55.57±0.9$^{d}$ | 67.80±1.4$^{c}$ |
| | XVWA | 66.49±1.2$^{e}$ | 87.97±1.0$^{e}$ | 61.00±1.7$^{c}$ |
| | XV | 95.85±1.5$^{d}$ | 97.22±4.1$^{d}$ | 63.33±2.0$^{d}$ |
| 0.20 ml/ml | AA | 3.73±0.4$^{b}$ | 36.33±1.4$^{c}$ | 54.20±1.4$^{c}$ |
| | RV | 15.20±1.2$^{c}$ | 42.28±1.3$^{c}$ | 53.31±1.4$^{b}$ |
| | XVWA | 23.27±1.3$^{d}$ | 44.05±0.9$^{d}$ | 54.92±2.5$^{b}$ |
| | XV | 33.31±1.7$^{c}$ | 62.15±2.0$^{c}$ | 59.21±2.3$^{cd}$ |
| 0.10 ml/ml | AA | 3.46±0.9$^{b}$ | 15.82±1.6$^{b}$ | 47.41±1.3$^{b}$ |
| | RV | 13.60±1.2$^{c}$ | 11.27±1.2$^{b}$ | 52.77±1.7$^{b}$ |
| | XVWA | 14.18±0.6$^{c}$ | 19.24±2.3$^{c}$ | 52.06±1.7$^{b}$ |
| | XV | 18.66±2.2$^{b}$ | 27.47±2.4$^{b}$ | 56.89±1.4$^{cb}$ |
| 0.06 ml/ml | AA | 3.32±0.2$^{b}$ | 15.19±0.6$^{b}$ | 46.15±0.8$^{b}$ |
| | RV | 7.39±1.3$^{b}$ | 4.81±0.7$^{a}$ | 50.45±1.2$^{b}$ |
| | XVWA | 10.31±0.7$^{b}$ | 9.87±1.0$^{b}$ | 51.34±1.7$^{b}$ |
| | XV | 12.96±1.3$^{a}$ | 14.81±1.5$^{a}$ | 51.70±1.1$^{b}$ |
| 0.05 ml/ml | AA | 1.56±0.4$^{a}$ | 7.97±0.8$^{a}$ | 33.99±1.5$^{a}$ |
| | RV | 2.51±0.8$^{a}$ | 3.16±1.0$^{a}$ | 36.31±1.2$^{a}$ |
| | XVWA | 4.14±0.7$^{a}$ | 6.84±1.2$^{a}$ | 49.37±1.7$^{a}$ |
| | XV | 10.04±0.8$^{a}$ | 11.90±1.5$^{a}$ | 48.48±1.3$^{a}$ |
| *P* | AA | | * | * |
| | RV | | * | * |
| | XVWA | ** | ** | * |
| | XV | ** | ** | * |

AA: acetic acid; RV: rice vinegar; XV: Xiangxi flavor vinegar with aging; XVWA: XV without aging. Data are presented as mean±SD ($n$=3). $^{a, b, c, d, e}$ The values with different superscripts in the same group are significantly different among different concentrations. $^{*}$ $P$<0.05, $^{**}$ $P$<0.01, compared to basic solution using analysis of variance (ANOVA)

The radical-scavenging abilities of all vinegars decreased with the reduction in sample concentration. The scavenging abilities of DPPH$^{\bullet}$ and $^{\bullet}$OH were more affected by the concentrations of XVWA and XV, while the $O_2^{\bullet-}$ scavenging ability was less affected by the concentrations of XVWA and XV. This indicates that the $O_2^{\bullet-}$ scavenging abilities of XVWA and XV are stable, and $O_2^{\bullet-}$ can be effectively scavenged at a lower concentration.

### 3.3 Antioxidant properties of Xiangxi flavor vinegar in *C. elegans*

#### 3.3.1 Effects of XV on ROS in *C. elegans*

The content of ROS in *C. elegans* was estimated by DCF fluorescence intensity, and is shown as the relative level of ROS (control group set as 100 %) in Fig. 2. Compared to the control group, the three fermented vinegars RV, XVWA, and XV decreased the ROS content in *C. elegans* ($P$<0.05). The relative DCF fluorescence intensity in *C. elegans* treated with XVWA was 12.38%, which decreased approximately

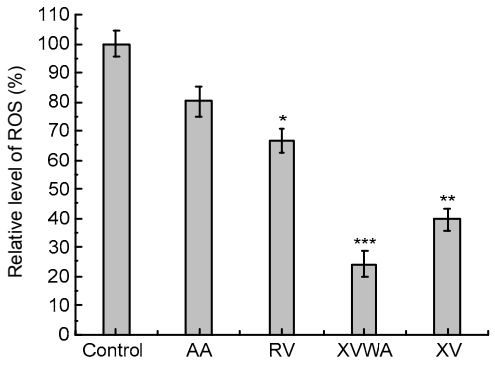


**Fig. 2 Effects of vinegars on the ROS levels in *C. elegans***
*C. elegans* was incubated with 135 μl 3.5% acetic acid (AA), rice vinegar (RV), Xiangxi flavor vinegar (XV) with aging, XV without aging (XVWA), or with an adequate volume M9 buffer solution (control) for 48 h. Individual worms of each group were transferred to a 96-well plate with 20 μl $H_2$DCF-DA in each well. The levels of ROS in different groups were determined by the change of fluorescence using a Varioskan Flash. The relative fluorescence values are presented as mean±SD of three experiments. $^{*}$ $P$<0.05, $^{**}$ $P$<0.01, $^{***}$ $P$<0.001, compared to the control group

by 87.62% and 63.80% compared to the control group and RV group, respectively. This demonstrates that XVWA diminished the ROS in *C. elegans* more effectively than the other three vinegars.

3.3.2 Effects of XV on the activities of antioxidant enzymes in *C. elegans*

The relative values of the antioxidant enzymes GSH-Px, SOD, and CAT in *C. elegans* are shown in Fig. 3 (control set as 100%). Compared to the control group, AA, RV, XVWA, and XV increased the activities of the three enzymes in *C. elegans* ($P$<0.05). RV and XVWA increased the GSH-Px activity up to 2.3 and 2.7 times compared to control ($P$<0.01), respectively. XV and AA doubled the activity of GSH-Px, but their activities were lower than that of XVWA. In addition, the three fermented vinegars increased SOD and CAT activity significantly compared to AA. XV increased the CAT activity by 3 times compared to the control group, which was 32.45% and 29.73% higher than those of RV and XVWA, respectively.

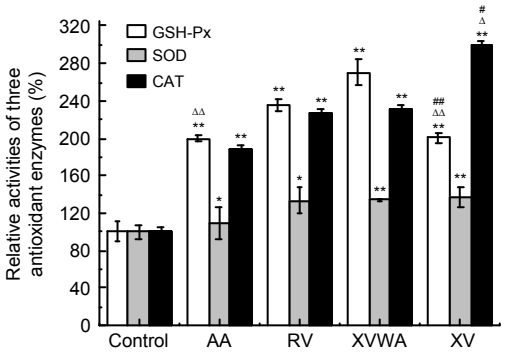


**Fig. 3 Effects of the vinegars on the activities of antioxidant enzymes in *C. elegans***

*C. elegans* was incubated with 135 μl 3.5% acetic acid (AA), rice vinegar (RV), Xiangxi flavor vinegar (XV) with aging, XV without aging (XVWA), or with an adequate volume M9 buffer solution (control) for 48 h. The levels of the antioxidant enzymes in different groups were determined by kits of GSH-Px, SOD, and CAT. The relative fluorescence values are presented as mean±SD of three experiments. $^{*}P$<0.05, $^{**}P$<0.01, compared to the control group. $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, compared to the RV group. $^{\#}P$<0.05, $^{\#\#}P$<0.01, compared to the XVWA group

### 3.4 Effects of Xiangxi flavor vinegar on apoptosis in *C. elegans*

The protein expressions of CED-3 and CED-9 in *C. elegans* after vinegar treatment are shown in Fig. 4, relative to the control (control set as 100%). The results show that all four vinegars decreased the CED-3 expression compared to the control group. CED-3 protein in *C. elegans* treated with XVWA was reduced by 46.57% and 44.73% compared with the control and RV groups, respectively. All four vinegars increased the anti-apoptosis protein CED-9 after vinegar treatment ($P$<0.01); especially, the three fermented vinegars upregulated the CED-9 expression much more than AA. Compared to RV, XVWA and XV increased the CED-9 protein by 45.48% and 30.90%, respectively ($P$<0.01). In summary, XVWA and XV downregulate CED-3 and upregulate CED-9 expression.

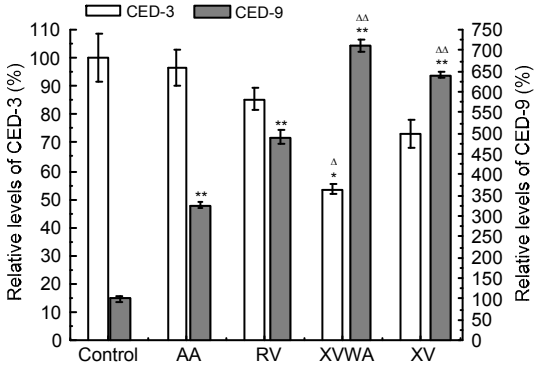


**Fig. 4 Effects of the vinegars on the CED-3 and CED-9 expression levels in *C. elegans***

*C. elegans* was incubated with 135 μl 3.5% acetic acid (AA), rice vinegar (RV), Xiangxi flavor vinegar (XV) with aging, XV without aging (XVWA), or with an adequate volume M9 buffer solution (control) for 5 d, respectively. The expression levels of CED-3 and CED-9 in different groups were determined by the ELISA kits of CED-3 and CED-9. The relative fluorescence values are presented as mean±SD of three experiments. $^{*}P$<0.05, $^{**}P$<0.01, compared to the control group. $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, compared to the RV group

## 4 Discussion

### 4.1 Antioxidant substances in Xiangxi flavor vinegar

It has been previously reported that XV has a high content of total ester, amino nitrogen, and reductive sugar (Yu *et al*., 2013), but the antioxidant property of XV was not studied. Here, six antioxidant compounds were determined in this Chinese traditional product by HPLC, and they are much greater than those in RV in both variety and concentration.

This may be due to the fact that RV is produced by pure fermentation during which the microorganisms cannot metabolize the materials completely compared to XV and XVWA. Between XVWA and XV, XVWA is higher in rutin, gallic acid, and *p*-coumaric than XV. The reason for the reduction of these three antioxidants in XV may be the fact that these substances come from Qu (Bounihi *et al*., 2014) and easily oxidized before they have accumulated sufficiently during aging.

However, ligustrazine accumulates in XV after aging. According to a previous report (Zhang *et al*., 2013), ligustrazine possesses many physiological functions like improving the function of the heart, inhibiting thrombosis, and bating blood vessels. The molecular formula of ligustrazine is tetramethylpyrazine (TMP) (Fig. 5) and ligustrazine is pyrazine produced from the Maillard reaction between reductive sugar and amino acid (Chu *et al*., 2013). At the start of the acetic acid fermentation stage, a lot of reductive sugars and amino acids produced from raw materials are metabolized by microorganisms, which can be used for the Maillard reaction. As the fermentation progresses, the content of reductive sugar decreases, indicating that the reductive sugar is the material for ligustrazine.

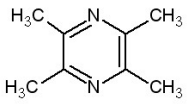

**Fig. 5 Molecular formula of ligustrazine**

Previous studies have shown that ligustrazine can not only upregulate the peroxisome proliferator-activated receptor α (*PPARα*) gene expression but also act against ischemic brain injury by scavenging $O_2^{\bullet-}$ by polymorphonuclear leukocyte (PMN) directly, in order to prevent $O_2^{\bullet-}$ from combining with NO and producing $ONOO^-$ (Zhang *et al*., 2004; Chen *et al*., 2013).

### 4.2 Antioxidant properties of Xiangxi flavor vinegar in vitro

$DPPH^{\bullet}$ is a commonly used compound to test the radical-scavenging ability of different samples because it is stable and sensitive (Dawidowicz *et al*., 2012). Hydroxyl radicals and superoxide are produced in the body. While hydroxyl can induce some oxidative damage to biomolecules and lead to cancer and other diseases, overaccumulation of superoxide kills cells and induces damage to DNA (Sakanaka and Ishihara, 2008). Radical-scavenging assay shows that XVWA and XV scavenge $DPPH^{\bullet}$, $^{\bullet}OH$, and $O_2^{\bullet-}$ effectively in vitro; especially, XV can scavenge $^{\bullet}OH$ and $O_2^{\bullet-}$ at a low concentration, showing its good antioxidant property in vitro.

### 4.3 Antioxidant properties of Xiangxi flavor vinegar in *C. elegans*

ROS are produced from cell metabolic processes and chemical contamination in the environment. Many factors affect the accumulation of ROS in the body (Sies, 2000). ROS play an important role in phagocytosis, cell division, energy metabolism, and other activities of the body. However, excessive accumulation of ROS is harmful to the body, such as promoting apoptosis and aging and inducing damage to 5-methylcytosine of DNA leading to DNA fragmentation (Back *et al*., 2012). This study showed that XVWA and XV reduced ROS to 24% and 39%, respectively, compared with the control group in *C. elegans*. In addition, XVWA and XV increase the activities of antioxidant enzymes in *C. elegans*; in particular, XV remarkably increases SOD and CAT activity ($P<0.01$). Similarly, previous studies have reported that XV significantly increases SOD and GSH-Px activity in mice (Feng, 2010). XV shows an outstanding antioxidant ability in vivo, probably because it is rich in antioxidant substances and possesses excellent $^{\bullet}OH$ and $O_2^{\bullet-}$ scavenging ability. Moreover, antioxidant enzymes with high activity in *C. elegans* can also reduce ROS.

### 4.4 Effects of Xiangxi flavor vinegar on apoptosis in *C. elegans*

There are 1090 cells in *C. elegans* in its whole life cycle. Before it grows into an adult, 131 cells will undergo apoptosis and only 959 cells remain (Strange, 2006). The number of cells in adult *C. elegans* will be stable until it becomes aged and the cells begin to undergo apoptosis. Apoptosis also promotes aging. According to a previous study (Pinan-Lucarre *et al*., 2012), the apoptosis signal pathway in *C. elegans* is similar to that in humans. The core proteins and genes of the apoptosis signal pathway are shown in Table 3. Briefly, when a cell receives the apoptosis signal, it

will upregulate the expression of EGL-1 at the transcription level. EGL-1 combines with CED-9 and changes its conformation. Because of the change of conformation of CED-9, CED-4 dimer will be released and become oligomerized, an apoptotic body that can activate *ced-3* and express CED-3, which leads to cell apoptosis (Lant and Derry, 2014).

**Table 3 Core apoptosis pathway of *C. elegans***

| Gene | Protein | Function |
|---|---|---|
| *egl-1* | EGL-1 | Inhibiting CED-9 activity |
| *ced-9* | CED-9 | Combining with CED-4 and inhibiting apoptosis |
| *ced-4* | CED-4 | Apoptotic activating factor |
| *ced-3* | CED-3 | Apoptosis protein |

In this study, we found that after XV treatment, the content of CED-3 in *C. elegans* decreased and CED-9 increased remarkably ($P<0.01$), showing that XV upregulates CED-9 expression in order to cut off the apoptosis signal pathway, so that *ced-3* is activated to express and finally inhibit apoptosis.

## 5 Conclusions

XVWA and XV are both rich in antioxidants and can scavenge radicals in vitro effectively. XVWA and XV inhibit apoptosis in *C. elegans* probably by scavenging ROS and increasing the activities of antioxidant enzymes like GSH-Px, SOD, and CAT. When XVWA and XV are compared, XV has a great antioxidative capacity and a better flavor. This study provides scientific evidence for the biofunction of XV.

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### Compliance with ethics guidelines

Run-ting HUANG, Qing HUANG, Gen-liang WU, Chun-guang CHEN, and Zong-jun LI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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## 中文概要

**题　目：湘西香醋的抗氧化特性及其对秀丽隐杆线虫作用评价**

**目　的：**评价湘西香醋体外及其对秀丽隐杆线虫（*Caenorhabditis elegans*）的抗氧化特性，并初步探讨其作用机制。

**创新点：**首次以秀丽隐杆线虫为模式生物评价湘西香醋体内抗氧化作用及其作用机制。

**方　法：**利用高效液相色谱法对配制白醋（AA）、酿造白醋（RV）、未经陈酿湘西香醋（XVWA）和湘西香醋（XV）四种食醋中抗氧化物质（芦丁、没食子酸、阿魏酸、对香豆酸、香草酸和川芎嗪）的组成和体外清除 DPPH•、•OH 和 $O_2^{\bullet-}$自由基的能力进行检测分析，并评价湘西香醋体外抗氧化特性。以秀丽隐杆线虫及突变体线虫 LD1171 为模式生物，给药后检测其体内活性氧簇（ROS）相对含量；研究体内抗氧化酶超氧化物歧化酶（SOD）、谷胱甘肽过氧化物酶（GSH-Px）、过氧化氢酶（CAT）活性，以及线虫抗氧化信号通道特征蛋白 GCS-1 表达情况；对细胞凋亡执行蛋白 CED-3 以及抗凋亡蛋白 CED-9 表达情况进行检测分析；探究食用湘西原香醋对体内生化水平的影响。

**结　论：**两种湘西香醋中抗氧化物质组成较白醋丰富，含量较高（表 1）。体外清除自由基分析结果发现湘西香醋清除自由基能力较白醋强（表 2），且湘西香醋在低浓度情况下（0.05 ml/ml）对•OH 清除率仍达到 11%。使用湘西香醋后秀丽隐杆线虫体内 ROS 含量相对于对照组有显著下降（$P$<0.01；图 2）；线虫体内 GSH-Px、SOD 和 CAT 三种抗氧化酶活性较对照组显著提高（$P$<0.05；图 3），从而能够提高其抗氧化能力。湘西香醋还能下调线虫凋亡蛋白 CED-3 的表达，并上调抗凋亡蛋白 CED-9 的表达（图 4），从而能够有效地减少线虫细胞凋亡的发生情况。

**关键词：**醋；抗氧化物质；自由基清除能力；秀丽隐杆线虫；抗氧化酶；活性氧簇；细胞凋亡