

In vitro* and *in vivo* antioxidant activities of the flavonoid-rich extract from *Flos populus

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Abstract: *Flos populi* is a well-known traditional Chinese medicine, obtained from the male inflorescence of *Populus tomentosa* Carr. or *Populus canadensis* Moench. In this study, we aimed to evaluate the antioxidant activities of *Flos populi* extract by various *in-vitro* and *in-vivo* methods. *In-vitro* results showed that *Flos populi* extract had strong antioxidant potential in terms of Superoxide radical and ABTS radical scavenging capacity, nitrogen dioxide radical inhibition and the ability to inhibit lipid peroxidation. Mice were given with *Flos populi* extract via gavage for 1 month to scrutinize the *in-vivo* antioxidant effects of *Flos populi*. The results revealed that *Flos populi* extract had markedly enhanced the levels of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), while decreased the level of malondialdehyde (MDA) in serums and livers of mice compared to CCl₄ treated group. Additionally, the flavonoids content were determined by the colorimetric method was 553.23 ± 23.45mg/g. The extract was further examined by HPLC and showed the presence of flavonoids-including quercetin (16.26±0.58mg/g), luteolin (9.97±1.07mg/g), apigenin (8.57±0.46mg/g), pinocembrin (119.71±1.05mg/g) and chrysin (16.12±0.53mg/g). The study revealed that *Flos populi* extract had antioxidant activity and could be utilized as a potential natural antioxidant.

Keywords: *Flos populi*, antioxidant activity, *in-vitro* and *in-vivo*, flavonoids, HPLC.

INTRODUCTION

Oxidative stress attributed to the risk of several chronic diseases such as atherosclerosis, inflammation, liver fibrosis, carcinogenesis disorders, circulatory system abnormalities and reperfusion injury (Mao *et al.*, 2017). Oxidative stress is defined as the production of reactive oxygen species (ROS) in the body cells and tissues in excessive amount, and antioxidant system is unable to counteract them. The reactive oxygen species (ROS) triggered in excess as a result of chemical reactions in the normal metabolic processes (especially in mitochondrial oxidative metabolism) leads to damage of important biomolecules, DNA, protein and lipids. ROS is composed of free radicals such as hydroxyl, super oxide and peroxy radical, and non radical species such as hydrogen peroxide (H₂O₂), normally produced in limited quantity and crucial for maintaining normal cell homeostasis (Chen *et al.*, 2017). The excessive and mass production of free radicals has been observed to eventuate under abnormal conditions. However, our body systems develop specific antioxidant enzymes-mediated protective mechanisms against free radical damage (Sunil and Ignacimuthu, 2011). In addition, antioxidant supplements are vital for the body to nullify the oxidative damage due to the fact that some pathological phenomena can impede the antioxidant protective mechanisms. Furthermore, these antioxidants supplements could perform a key role in treating various diseases such as Alzheimer's disease, circulatory system abnormalities, muscular degeneration and carcinogenesis (Kobayashi *et al.*, 2016).

In order to protect human health and store the foods for longer periods, antioxidants often used for food processing to lessen the production of free radicals. However, the use of synthetic antioxidants in edible products stills an issue (da Cunha *et al.*, 2013). For this purpose, more and more attention is focused on quality and safety of food additives. Recently, researches of natural sources antioxidants have gained more interest due to its growing demand by consumers. Generally, the natural antioxidants consist of many compounds including phenolic, carotenoids and nitrogen compounds. It has been demonstrated earlier that phenolic compounds are classified as simple phenols or polyphenols and the antioxidant activities of flavonoids bewitch extensive attention due to its ability to interact with ROS before cell viability is seriously affected (Ahmed *et al.*, 2016, Chen *et al.*, 2017).

Recently, *Flos populi* (belong to Salicaceae family) is extensively used as a traditional medicine in China (China, 2010). It is indigenous in China and is widely distributed because of its tolerance to a wide range of climatic conditions. Research has indicated that *Flos populi* contains flavonoids, cardiac glycoside and phenolic compounds and is used in the treatment of a variety of inflammatory diseases, as well as antidiarrheal agents in East Asian countries (Xu *et al.*, 2013, Xu *et al.*, 2014, Zhao *et al.*, 2014). In our previous study, antioxidant activities of *Flos populi* extract including 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging activity, ferric reducing ability and total oxidant capacity have been manifested (Wan *et al.*, 2014). However, *in vitro* antioxidant assays results sometimes are inconsistent

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with the results obtained from *in vivo* testing due to the fact that the antioxidant compounds are metabolized in the body (Mosele *et al.*, 2016). In view of no reports available about the *in vivo* antioxidant potential of *Flos populi* extract, the systematic research of antioxidant activity of *Flos populi* extract is of major importance.

In current study, *in vitro* antioxidant activities including ABTs radical, nitrogen dioxide radical and superoxide radical scavenging activities were scrutinized for the first time. Moreover, the ability of *Flos populi* extract to inhibit lipid peroxidation and its potential *in vivo* antioxidant activity was assessed by determining the changes in the levels of SOD GSH-Px, CAT, and MDA enzymes in both blood and liver homogenates in mice.

MATERIALS AND METHODS

Flos populi (male inflorescence of *Populus tomentosa* Carr.) was purchased from a medicinal herbs store (Anguo, Hebei Province, China) and authenticated by Associate Professor Junkai Wu (Heilongjiang University of Traditional Chinese Medicine, Harbin, China). The voucher specimen (Accession no. 1009015ch) has been deposited at the Herbarium in the College of Veterinary Medicine, Northeast Agricultural University.

The standards (quercetin, luteolin, apigenin, pinocembrin and chrysin) were obtained from the center of Pharmaceutical and Biological Products (Chinese Institute, Beijing, China). The purities of standards exceeded 98% (w/w). Pyrogallol, orchophenanthroline, Vitamin C (V_c), $K_3Fe(CN)_6$, acetic acid and DPPH (1, 1-diphenyl, 2-picrylhydrazyl) were bought from Sigma Chemical Co. (St. Louis, MO, USA). Experiments were performed with deionized water provided by Milli-Q academic water purification system (Millipore, Bedford, MA, USA). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels were determined by commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other analytical grade reagents were obtained from Hangzhou Company (Hangzhou, PR China).

Animals

BALB/c mice (20 ± 2 g weight, 8 weeks old) of both sexes were procured from the Laboratory Animal Center of Heilongjiang University of Traditional Chinese Medicine (Harbin, China). They were housed at an ambient temperature of $25 \pm 2^\circ C$ in polypropylene cages on 12 hr light-dark cycle at a humidity level of 50 - 60%. Feed and water provided ad libitum during the experiments. Mice were adapted to housing conditions for a week before experiments. The trial was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and was permitted by the Institutional Animal Ethical

Committee of Northeast Agricultural University (No. SRM-08).

Preparation of extract

The extraction was performed on the basis of our previous studies (Sheng *et al.*, 2013). In brief, *Flos populi* of 5g was extracted by condensation reflux with 100mL of 46% ethanol at $95^\circ C$ for 2.5hr. The extract was further treated with NKA-9 resin to obtain total flavonoids extract (Wan *et al.*, 2014). The extract solution was concentrated at $46^\circ C$ and dried in a vacuum oven. The dried extract was ground into powder and stored at $-4^\circ C$ for use in subsequent tests.

In vitro antioxidant properties

Total flavonoid contents was assessed in accordance with our previous studies (Sheng *et al.*, 2013). Some of the antioxidant activities (DPPH free radical, hydroxyl free radical scavenging effects, reducing power and total antioxidant capability test) of *Flos populus* extract had been evaluated in our previous study (Wan *et al.*, 2014). So we examined the radicals such as superoxide, ABTs, nitrogen dioxide, and inhibition of lipid peroxidation.

Superoxide radical scavenging assay

The superoxide radical scavenging activity of the purified flavonoids was carried out according to previous procedure with minor modifications (Huang *et al.*, 2011). In brief, 4.5mL 50mM Tris-HCl buffer (pH 8.2) was heated up to 20 min at $37^\circ C$. Then 2mL distilled water, 2ml of sample solutions of different concentrations and 0.5mL pyrogallol (25mM) were added to stimulate the reaction. After 6 min, 1.0mL HCl (10mM) was added to stop reaction. The absorbance of the reaction was measured at 325 nm. V_c was taken as positive controls. The experiments were performed thrice and the average is calculated. The percentage of scavenging rate was determined by the following formula:

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2) / A_0] \times (1)$$

Where A_0 (the absorbance distilled water instead of sample), A_1 (absorbance of the sample), and A_2 (absorbance of the sample only, Tris-HCl buffer instead of pyrogallol solution).

ABTS radical scavenging assay

The ability of sample of different concentrations to scavenge the ABTS radical cation was determined in accordance with the literature procedure with minor modifications (Suzek *et al.*, 2016). 7mM ABTS and 2.45mM potassium persulfate were reacted at room temperature for 16 hr to produce a solution of ABTS radical cation ($ABTS^+$). The solution was then diluted with 80% ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. Absorbance at 734 nm was recorded. ABTS radical scavenging activity was evaluated by the following formula:

Table 1: Regression parameters and precision of HPLC for the five compounds

Compounds	Regression equation	Correlation coefficient	Linear range (µg/mL)
Quercetin	$y=6 \times 10^7 x - 328010$	0.9990	9.6~86.4
Luteolin	$y=6 \times 10^7 x + 32740$	0.9991	17.1~154.08
Apigenin	$y=5 \times 10^7 x + 62543$	0.9991	10~90.72
Pinocembrin	$y=5 \times 10^6 x - 17309$	0.9993	8~72
Chrysin	$y=6 \times 10^7 x - 229876$	0.9990	11.84~106.56

Table 2: Effects of *Flos populi* extract on serous level of SOD, GSH-Px, MDA and CAT on CCl₄ treated oxidative stress in mice.

Treatment	SOD (U/mL)	GSH-Px (U/mL)	MDA (nmol/mL)	CAT (U/mL)
Normal control group	79.16±4.62	82.3±6.64	3.18±0.29	77.06±6.27
CCl ₄ treated	50.91± 5.93	51.44± 4.52	8.21 ±1.09	47.03±2.85
Extract (150mg/kg)+CCl ₄ treated	55.03±2.87 ⁺⁺	61.02±3.02 ^{***++}	7.11 ± 0.99 ⁺⁺	55.59±4.61 [*]
Extract (300mg/kg)+CCl ₄ treated	66.68±6.5 ^{**}	71.7± 3.69 ^{**}	6.72 ± 1.45 ⁺	59.83±2.67 ^{**}
Extract (600mg/kg)+CCl ₄ treated	71.84± 2.4 ^{**}	74.72±3.05 ^{**}	5.86 ± 0.99 ^{**}	63.49±7.32 ^{**}
V _c (50mg/kg) + CCl ₄ treated	70.69± 5.38 ^{**}	75.52±3.16 ^{**}	5.43 ± 0.76 ^{**}	62.12±4.03 ^{**}

Values were expressed as mean ± SD (n = 6), and evaluated by one-way AVONA. Different were considered to be statistically significant if $p < 0.05$. ^{*} $p < 0.05$ compared to CCl₄ treated group. ^{**} $p < 0.01$ compared to CCl₄ treated group. ⁺ $p < 0.05$ compared to V_c (50mg/kg) + CCl₄ treated group. ⁺⁺ $p < 0.01$ compared to V_c (50mg/kg) + CCl₄ treated group.

Table 3: Effects of *Flos populi* extract on hepatic level of SOD, GSH-Px, MDA and CAT on CCl₄ treated oxidative stress in mice.

Treatment	SOD (U/ mg protein)	GSH-Px (U/ mg protein)	MDA (nmol/mg protein)	CAT (U/ mg protein)
Normal control group	206.13±17.92	124.49±8.35	3.12±0.61	212.53±10.2
Control + CCl ₄ treated	152.07± 8.43	74.26±8.35	13.22 ± 2.48	143.44±11.23
Extract(150mg/kg)+CCl ₄ treated	165.05±7.25	83.03±9.76 ⁺	10.88±1.09 ⁺⁺	151.92±6.21
Extract(300mg/kg)+CCl ₄ treated	168.64±5.15 [*]	87.14±6.12 [*]	9.92±1.12 ^{**}	153.72±8.68
Extract(600mg/kg)+CCl ₄ treated	175.96±8.46 ^{**}	98.55±8.58 ^{**}	7.77±2.42 ^{**}	166.92±6.44 ^{**}
V _c (50mg/kg) + CCl ₄ treated	171.49±10.58 [*]	96.34±8.18 ^{**}	7.5 ±1.53 ^{**}	161.34±9.72 ^{**}

Values were expressed as mean ± SD (n = 6), and evaluated by one-way AVONA. Different were considered to be statistically significant if $p < 0.05$.

^{*} $p < 0.05$ compared to Control + CCl₄ treated group. ^{**} $p < 0.05$ compared to Control + CCl₄ treated group. ⁺ $p < 0.05$ compared to V_c (50mg/kg) + CCl₄ treated group. ⁺⁺ $p < 0.01$ compared to V_c (50mg/kg) + CCl₄ treated group.

Scavenging activity (%) = $(A_3 - A_1) / A_3 \times 100$ (2)
where A_5 is the absorbance of distilled water alone, A_1 is the absorbance of sample of different concentrations.

Nitrogen dioxide radical inhibition assay

Nitrogen dioxide radical inhibition was determined by the previous method (Atala et al., 2013). In brief, the extract and Vitamin C were dissolved with 46% ethanol to prepare different sample solutions (20-100 µg/mL). The reaction mixture containing NaNO₂ solution (10µg/mL, 1mL) and sample solution (1mL) was incubated at 37°C for exactly 20 min. Then, the amino benzene sulfonic acid solution (0.4%, 1mL) and N-Naphthylethylenediamine Dihydrochloride solution (0.2%, 0.5mL) were added to stimulate the reaction for 15 min, and absorbance measured at 540 nm. Vitamin C was used as positive control. The percentage of inhibition was calculated using Eq. (1).

Inhibition of lipid peroxidation in mice liver homogenate

The thiobarbituric acid method was employed to estimate the inhibition effect of *Flos populi* extract on lipid peroxidation (Sunil and Ignacimuthu, 2011). In this method, reaction mixture containing 0.2ml of *Flos populi* extract (200-1000µg/mL), 1mL of 1% liver homogenate (each 100mL homogenate solution contains 1g mice liver), 50µL of FeCl₂ (0.5mM) and H₂O₂ (0.5mM) was incubated at 37°C for 60 min; Then the Incubation mixtures were treated with 1ml of trichloroacetic acid (15%) and 1ml of thiobarbituric acid (0.67%). The total volume was then made up to 4mL with distilled water and the mixture was kept in a water bath at 100°C for 15 min. The absorbance of the organic layer was recorded at 532 nm. The percentage inhibition of lipid peroxidation was determined using Eq. (1).

In vivo antioxidant properties

After accustomed to conditions for one week, the mice were randomly divided into six groups (6 mice/group); normal control group, negative group (CCl₄ treated), positive control group, low-dose group, middle-dose group and high-dose group. The normal control group received 1% CMC (10mL/kg bw/day, p.o.). Other groups were administered simultaneously with CCl₄-olive oil mixture (1:1, 2mL/kg body weight, s.c.) on alternate days after 30 min of administration of the 1% CMC (10mL/kg body weight/day, p.o.), *Flos Populi* extract (150, 300, 600 mg/kg body weight/day, p.o.), Vitamin C (50mg/kg body weight/day, p.o.). The different doses were administered once daily for 30 consecutive days.

Twenty four hours after the last dose of CCl₄, mice were sacrificed humanely by cervical dislocation. Blood samples were centrifuged at 4000 rpm at 4 °C for 10 min to collect the serum. The livers were dissected out from each animal, washed carefully and homogenized. In order to remove cellular debris, the homogenate was centrifuged again at 4000 rpm for 10 min at 4 °C and the supernatant was collected for analysis. Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were measured in total blood samples and liver homogenates using commercial kits came from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Briefly, GSH-Px activity was measured on the basis of the reaction of GSH and 5, 5'-dithio-bis-(2-nitrobenzoic acid). SOD activity was measured based on the inhibition of hydroxylamine oxidation by the superoxide radicals generated in the xanthine-xanthine oxidase system. The data obtained were expressed as units per milliliter (U/ml) in serum or units per milligram of protein (U/mg protein) in liver. The activity of CAT was tested on the basis of the rate of H₂O₂ reduction (Bensouici *et al.*, 2016). The MDA levels were determined in total blood samples according to the method described by Vittorazzi *et al.* (2016) (Vittorazzi *et al.*, 2016). The principle of the assay is based on the reaction of MDA with thiobarbituric acid (TBA) and forming a pink colored compound with a maximum absorbance at 532 nm. The data obtained were expressed as nmole per milliliter (nmol/mL) in serum or nmole per milligram of protein (nmol/mg protein) in liver. The protein content in the liver supernatants was determined by the Lowry method using bovine serum albumin as the standard (Suganthi and Devi, 2016).

HPLC analysis

For the qualitative and quantitative analysis of the flavonoid compounds, the dried sample and five standards were dissolved in HPLC grade MeOH (1mg/ml) and filtered with 0.45µm membrane (Lab Instrument Co., Ltd.) before injection. The SHIMADZU HPLC system equipped with LC-10ATVP binary pump, UV detector

SPD-10AVP, a CTO-10ASVP column oven and a N300 workstation was used for quantitative analysis. A C₁₈ column (150×4.6mm, 5µm, Diamonsil, Dikma Technologies, China) was used for chromatographic separation. The mobile phase was 0.1% formic acid-methanol and the flow rate was 1mL/min. 20µL was the injection volume and column temperature was kept at 25°C. The solvent gradient elution schedule was as follows: 0-23 min, 65% B; the solvent gradient was increased to 90% B at 23-24 min and it was maintained at 90% B for 10 min; 34-35 min, 90%-65% B; 35-50 min, 65% B. Quantification was performed using the external standard method with means of a five-point calibration curve. The correlation coefficient and linear range of the five compounds are listed in table 1.

STATISTICAL ANALYSIS

The results were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan test and compared with CCl₄ and Vc treated group. The results are presented as the mean ± standard deviation (SD). All analyses were performed using the SPSS 17.0 software package. Statistical significance was determined at a value of *p* < 0.05.

RESULTS

In vitro antioxidant properties

Superoxide radical scavenging assay

As represented in fig.1a, the IC₅₀ values of superoxide anion radical scavenging capacity for *Flos populi* extract and Vc were 3.268 and 0.066mg/mL, respectively. It also showed that the scavenging ability of *Flos populi* extract on the superoxide anion was less efficient than Vc. However, the dose-effect relationship between content of *Flos populi* extract and superoxide anion radical scavenging ability was remarkable. These results indicated that *Flos populi* extract was capable of relieving the oxidative injury.

ABTS radical scavenging assay

ABTS^{•+} could generate a blue-green radical cation if it was oxidized. Some antioxidant can react with ABTS radical, which lead to fading of the mixture solution. As Figure 1b described, both of the tested sample and Vc showed dose-dependent activities. Moreover, *Flos populi* extract at high concentration can get higher activity than Vc. The IC₅₀ of Vc and *Flos populi* extract on ABTS radical are 0.049 and 0.343mg/mL, respectively. These findings showed that *Flos populi* extract could scavenge ABTS radical.

Nitrogen dioxide radical inhibition assay

The scavenging activity of nitrogen dioxide radical by the *Flos populi* extract and Vc were represented in fig. 1c. The *Flos populi* scavenging activity and Vc increased

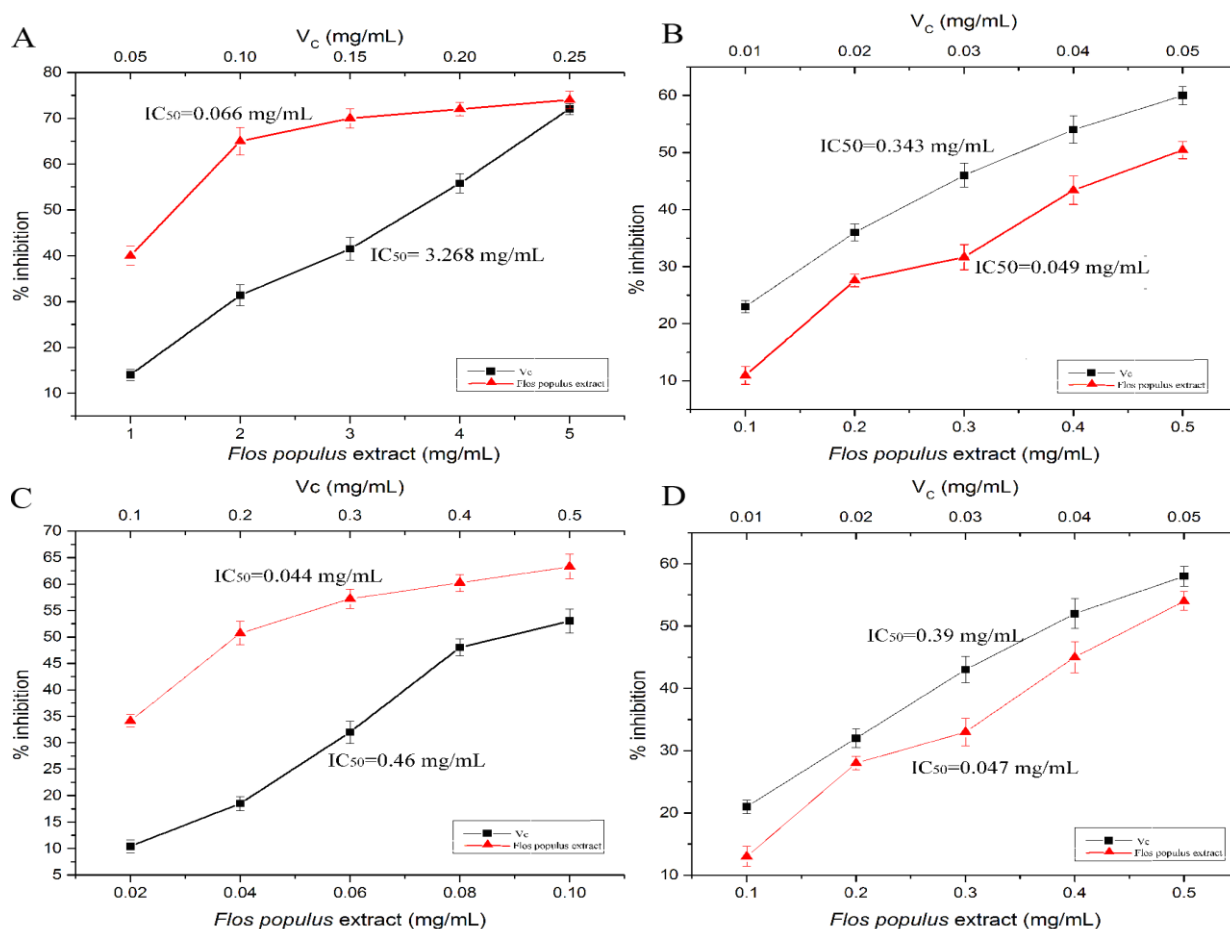


Fig. 1: Scavenging effects on (A) Superoxide radical, (B) ABTs radical, (C) nitrogen dioxide radical and (D) inhibition of lipid peroxidation of *Flos Populi* extract and Vc. Values are presented as means $\pm$ SD (n = 3).

with increasing doses, which had a similar trend to ABTS radical scavenging assay. The IC_{50} values of *Flos populi* and Vc were 0.46 and 0.044 mg/mL, respectively. Hence, it is suggested that *Flos populi* extract had nitrogen dioxide radical scavenging ability.

Inhibition of lipid peroxidation in mice liver homogenate

The data showed that *Flos populi* extract can neutralize the cell membrane damage by inhibiting lipid peroxidation. The dose-effect relationship between content of *Flos populi* extract and inhibition activity is shown in fig. 1d. The IC_{50} of Vc and *Flos populi* extract on lipid peroxidation are 0.047 and 0.39 mg/mL, respectively. The data revealed that *Flos populi* extract had remarkable ability to inhibit lipid peroxidation in a dose-dependent manner.

Antioxidant activity of flos populi in vivo

As shown in table 2, the MDA levels in the treatment groups (*Flos populi* extract administration or Vc administration) markedly increased compared with the MDA level in normal control group, however, the MDA levels in serum decrease with the treatment of *Flos populi* extract in a dose-dependent manner along with exposure

of CCl_4 compared with CCl_4 treated alone group. Although MDA level decreased dose-dependently, but the differences were not statistically significant for 150 mg/kg and 300 mg/kg *Flos populi* extract compared with the MDA level in CCl_4 treated group ($p > 0.05$). However, significant decrease ($p < 0.01$) was seen in the doses of 600 mg/kg for *Flos populi* extract and 50 mg/kg for Vc group compared with CCl_4 treated alone group. Moreover, there is no marked difference noted in the MDA at a dose of 600 mg for *Flos populi* extract compared with Vc group. In terms of liver MDA levels, the trend is similar to the blood MDA levels (table 3). The differences were not statistically significant between the dose of 150 mg/kg extract treated group and CCl_4 treated group ($p > 0.05$), whereas 50 mg/kg of Vc, 300 and 600 mg/kg of *Flos populi* extract administration caused significant decrease ($p < 0.01$) compared with CCl_4 treated group.

Effects of *Flos populi* extract and Vc on the activities of SOD, CAT and GSH-Px displayed in tables 2-3. The data indicated that CCl_4 administration caused a notable reduction in the SOD, CAT and GSH-Px levels, while Administration of *Flos populi* extract could partially restore the activities of these enzymes. Administration of *Flos populi* extract at a dose of 150 mg/kg increased the

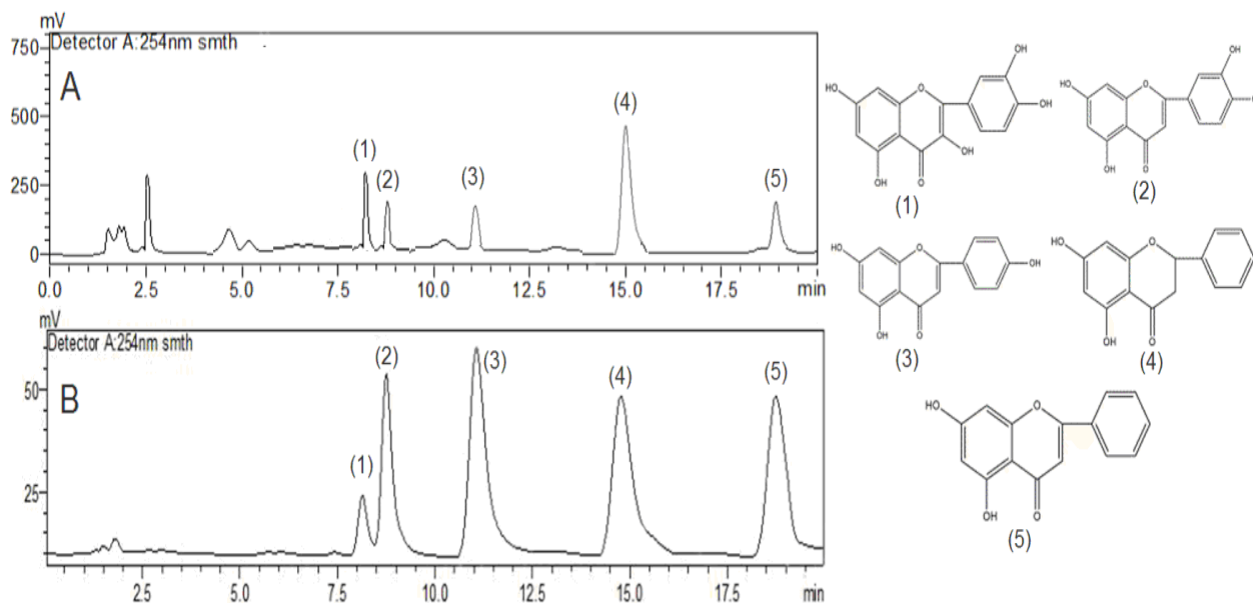


Fig. 2: HPLC chromatograms of sample solution of (A) *Flos Populi* extract and (B) standard solution of five chemical reference standards. (1) quercetin, (2) luteolin, (3) apigenin (4) pinocembrin and (5) chrysin.

SOD activity in total blood and liver tissues samples compared with that in CCl_4 treated group ($p > 0.05$). While, 300 and 600 mg/kg of *Flos populi* extract causes significantly increase ($p < 0.01$) in SOD activity. In 150, 300 and 600 mg/kg-treated groups of *Flos populi* extract, GSH-Px and CAT activities in blood were markedly increased ($p < 0.05$ or 0.01). GSH-Px enzyme and CAT activities in liver tissues enhanced (statistically not significant) in 150 mg/kg-treated groups ($p > 0.05$) and markedly increased in 600 mg/kg-treated groups (0.01). A similar increase has been noted in GSH-Px enzyme activity of 600 mg/kg *Flos populi* extract group and Vc-treated group. Hence, it is suggested that *Flos populi* extract manifested strong antioxidant activity and the results are in agreement with the *in vitro* test results.

Total flavonoids content (TFC) and HPLC analysis

TFC ($553.23 \pm 23.45\text{mg/g}$) in the *Flos populi* extract was tested based on our previous study (Wan, Sheng *et al.*, 2014). Moreover, the extract was analysed for their specific composition by HPLC. As can be seen in table 1, these calibration curves had good linear regression ($R^2 = 0.9990-0.9993$). The above results indicated that the method was reliable. The quantitative results revealed the existence of flavonoids-including quercetin ($16.26 \pm 0.58\text{mg/g}$), luteolin ($9.97 \pm 1.07\text{mg/g}$), apigenin ($8.57 \pm 0.46\text{mg/g}$), pinocembrin ($119.71 \pm 1.05\text{mg/g}$) and chrysin ($16.12 \pm 0.53\text{mg/g}$) (fig. 2).

DISCUSSION

Superoxide anion is the most common free radical which could react with macromolecules (e.g., DNA) and lead to

tissue damage. These radicals could be produced by pyrogalllic acid directly under alkaline conditions *in vivo*. Lots of active compounds were able to scavenge superoxide anion radicals due to its ability to reduce auto-oxidation of pyrogalllic acid (Cheong *et al.*, 2015). The ability of scavenging ABTS radical was often used to evaluate total antioxidant power of extract or purified compounds due to its simple, fast, reliable and inexpensive properties (Luo *et al.*, 2011). It has been previously reported that the oxidative degradation of lipids (lipid peroxidation) proceeded by a free radical chain reaction mechanism. The free radicals could "steal" electron/electrons from cell membrane, lead to cell injury (Goyal *et al.*, 2017). Therefore, the antioxidant activities of *flos populi* *in vitro* were evaluated firstly.

In vitro antioxidant assays often used for the evaluation of antioxidant capability of natural products, however, it has been noticed that the *in vitro* results often could not agree with *in vivo* findings due to bioavailability (Luo *et al.*, 2011). Generally, lots of antioxidant potential of natural products was attributed to their rich flavonoid and polyphenolic compounds. However, some adverse factors including low absorption in organism, short half-life in plasma, and its extensive metabolism in liver and intestine reduce the capability of dietary flavonoids *in vivo*. So appraisal *in vivo* antioxidant activity potential of *Flos populi* extract is incredibly necessary. *In vivo* antioxidant potential of *Flos populi* extract was studied in mice under elevated oxidative stress. Therefore, the increasing doses of 150, 300 and 600 mg/kg body weight, p.o. were used to assess the dose-depended response of *Flos populi* extract. SOD, GSH-Px and CAT are referred as primary

antioxidant enzymes which act mutually to play a major role in preventing cells against ROS (Jiang, Wang *et al.*, 2017). Generally, SOD can transform superoxide radicals into hydrogen peroxide by catalytic reaction. Hydrogen peroxide is harmful to the body as it has active oxygen, while GSH-Px and CAT detoxifies hydrogen peroxide into water and oxygen. To assess the *in vivo* antioxidant potential of *Flos populi* extract, the activities of antioxidant enzymes (SOD, GSH-Px and CAT) were examined due to its involvement in detoxification (Jiang *et al.*, 2017). In addition, MDA is a key by-product of lipid per oxidation leading to oxidative stress. So the amount of MDA was calculated to determine lipid per oxidation level (Almeida *et al.*, 2014).

As a result, *Flos populi* extract enhanced SOD, GSH-Px, CAT activities and reduce MDA production in serum and liver tissues, indicated that *Flos populi* extract had significant potential to protect health and reduce the risk of diseases. Surprisingly, the efficient dose of *Flos populi* extract was more than 10 times higher (600mg vs. 50mg), but *Flos populi* extract was a crude extract, and Vc is a refine product. Interestingly, *Flos populi* extract used for diabetic patients in Chinese folk medicine is probably safe for use.

A DNA protective capacity of apigenin, luteolin and quercetin against free radicals generated by H₂O₂ had already been reported (Romanova *et al.*, 2001). Moreover, it has been demonstrated that chrysin, apigenin and luteolin had the protective effect against tert-butyl hydroperoxide (tBHP)-induced oxidative stress. They have the ability to up regulate HO-1, GCLC, and GCLM gene expression level (Huang *et al.*, 2013). While, Pinocembrin could decrease neuronal nitric oxide synthase, myeloperoxidase, and superoxide dismutase activities both *in vitro* and *in vivo* (Kapoor, 2013), and Chrysin has a protective capacity in rats against free radical-mediated oxidative stress (Sathivelu *et al.*, 2009). Since, these reports proved that the five flavonoid compounds have strong and efficient *in vitro* and *in vivo* antioxidant properties. Thus, we concluded from our results that these active compounds may be the effective substances for antioxidant capacity of *Flos populi*.

CONCLUSIONS

The results have clearly exhibited that *Flos populi* extract had protective effects against *in vitro* free radical generation. *Flos populi* extract significantly elevated the antioxidant enzymes (SOD, CAT and GSH-Px), but decline the level of malondialdehyde (MDA) of mice. Notably, *Flos populi* extract exhibited similar antioxidant activity *in vivo* with Vc at 10th times higher dose (600 mg/kg group). The pronounced antioxidant activity of *Flos populi* extract was possibly due to its high flavonoids

content. Taken together, these results demonstrated that the extract of *Flos populi*, which is rich in antioxidant flavonoids, could be utilized in food and therapeutics.

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