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Antioxidant and cytotoxic activities of various fractions of ethanol extract of *Dianthus superbus*

Jian-Oing Yu [a,*], Zhi-Xiong Liao [a], Jia-Chuan Lei [b], Xian-Ming Hu [a]

[a] *College of Pharmacy, Wuhan University, Wuhan 430072, China*
[b] *Renmin Hospital, Wuhan University, Wuhan 430060, China*

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Abstract

This study examined the antioxidant and cytotoxic activities of various fractions (prepared by using solvents of varying polarity) of ethanol extract of *Dianthus superbus* (DS). The antioxidant activities of various fractions were evaluated in terms of their free radical-scavenging activity. The total phenolics and reducing powers of the fractions were measured. The cytotoxic activities of the fractions on three human malignant cell lines were also investigated. Among all fractions, the ethyl acetate fraction (EE-DS), which had the highest phenolic content and the strongest reducing power, exhibited the strongest antioxidant and cytotoxic activities.

Keywords: *Dianthus superbus*; Antioxidant; Cytotoxic; Active fraction

1. Introduction

Reactive oxygen species (ROS), such as superoxide radical and hydroxyl radical, are generated in many redox processes. ROS can easily react with other molecules, such as protein, DNA and lipids (Klaunig, Xu, Bachowski, & Jiang, 1997), and induce oxidative damage to biomolecules. This damage may cause ageing, heart disease, stroke, arteriosclerosis, diabetes, cancer and other many diseases (Aruoma, 1994; Kehrer, 1993). ROS are continuously produced during normal physiological events, and removed by antioxidant defence mechanisms (Halliwell, Gutteridge, & Cross, 1992). In pathological conditions, ROS are overproduced and result in lipid peroxidation and oxidative stress. In recent years, one of the areas which has attracted a great deal of attention is antioxidants in the control and prevention of those diseases in which oxidative damage has been implicated. Plant extracts containing flavonoids, tannins and other phenolic constituents have shown antioxidant potential (Banerjee, Dasgupta, & De, 2005; Salah et al., 1995).

The incidence and mortality rates of cancer still rank high in the worldwide population. Currently, chemotherapy is still the standard treatment method, but survival rates of cancer patients have been little increased. Scientists have begun to search for new antitumor agents from natural products, including plants, microorganisms and marines. Many Chinese herbs have been discovered to be potential sources of antitumor drugs (Vickers, 2002). Because different components in a herb may have synergistic activities or buffering toxic effects, mixtures or extracts of herbs might have more therapeutic or inhibitory activity than the herbs alone (Cheng et al., 2004; Li, Motwani, Tong, Bornmann, & Schwartz, 2000).

Dithanus superbus L. (DS), which is widely distributed in China, is commonly used as a traditional Chinese medicine, as a diuretic and anti-inflammatory agent, in the treatment of urinary infections, carbuncles and carcinoma of the oesophagus (Wang, Tan, Zhou, & Wu, 1998). It was also used as a health beverage. As far as our literature survey could ascertain, little research on the bioactive fraction of

* Corresponding author. Tel.: +86 27 66044555; fax: +86 27 87669560.
E-mail address: jq_yu@126.com (J.-O. Yu).

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DS has been reported. The aim of the present study is to investigate the antioxidant and antitumor potential of various fractions of an ethanol extract of DS.

2. Materials and methods

2.1. Materials

The herb of DS was collected in Hubei province (China) in August 2004. Voucher specimens have been deposited at the herbarium of the College of Pharmacy, Wuhan University, China. Human cervix carcinoma cell lines (HeLa) and human liver carcinoma cell lines (HepG2 and Bel-7402) were purchased from the China Center for Type Culture Collection. RPMI 1640 medium, Minimum Essential Medium (MEM) and fetal calf serum (FBS) were obtained from GIBCO-BRL (Grand Island, New York, USA). Pyrogallol, trichloracetic acid (TCA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 10-phenanthroline, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) and potassium hexacyanoferrate [$K_3Fe(CN)_6$] were purchased from the Sigma Chemical Co. (St. Louis, USA). All other chemicals and solvents were of analytical grade.

2.2. Plant material, extraction and fractionation

The whole plant (1.0 kg) was extracted three times with 95% ethanol. The extract was concentrated under reduced pressure to yield an ethanol extract. Then the ethanol extract was suspended in deionized water and partitioned sequentially with petroleum ether, ethyl acetate and 1-butanol. Each fraction was concentrated under reduced pressure to yield a petroleum ether fraction (PE-DS), ethyl acetate fraction (EE-DS), 1-butanol fraction (BE-DS) and a remainder (of water) fraction (WE-DS).

2.3. Assay of DPPH radical-scavenging activity

0.5 ml sample solution (in methanol) was added to 2.5 ml of 0.2 mM DPPH radical solution (in methanol). Finally, the total volume of the reaction mixture was adjusted to 5.0 ml by adding 100 mM Tris–HCl buffer (pH 7.4). The contents were mixed vigorously and allowed to stand at 20 °C for 30 min (Negi, Chauhan, Sadia, Rohinishree, & Ramteke, 2005) . The absorbance was read at 517 nm.

2.4. Assay of superoxide anion-scavenging activity

Superoxide anion-scavenging activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a slightly modified method of Heo, Park, Lee, and Jeon (2005). 0.1 ml sample solution (4 mg/ml) and 2.8 ml of 50 mM Tris–HCl buffer (pH 8.2) were added to a freshly prepared 0.1 ml solution of 60 mM pyrogallol (dissolved in 10 mM HCl). The inhibition rate of pyrogallol auto-oxidation was measured at 325 nm. Absorbance of each extract was recorded at every 0.5 min interval for 5 min.

2.5. Assay of hydroxyl radical-scavenging activity

The scavenging activity for hydroxyl radicals was measured, with the Fenton reaction, by a slightly modified method (Zhao, Xiang, Ye, Yuan, & Guo, 2006). Reaction mixture contained 0.5 ml of 7.5 mM $FeSO_4$, 0.5 ml of 7.5 mM 1,10-phenanthroline, 2.5 ml of 0.2 M phosphate buffer (pH7.8), 0.5 ml of 30 mM H_2O_2 and 0.5 ml of sample solution. The reaction was started by adding H_2O_2. After incubation at room temperature for 5 min, the absorbance of the mixture at 536 nm was measured.

2.6. Determination of total phenolics

The concentration of phenolics in various fractions was determined by the method of Negi, Jayaprakasha, and Jena (2003) and results were expressed as gallic acid equivalents. Five milligrams of each dried fraction were mixed with a 10 ml mixture of acetone and water (6:4 v/v). An aliquot of the samples was mixed with 1.0 ml of 10-fold diluted Folin–Ciocalteu reagent and 2 ml of 10% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 760 nm.

2.7. Reducing power assay

The reducing power of each fraction was evaluated according to Oyaizu (1986). A sample solution (2.5 ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50 °C for 20 min; 2.5 ml of 10% TCA was added to the mixture and it was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated reducing power.

2.8. Cytotoxicity assay

2.8.1. Cell lines and culture

The cell lines were grown and maintained in a humidified incubator at 37 °C and in a 5% CO_2 atmosphere. MEM supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin were used for cell culture of HeLa and HepG2 cells. RPMI 1640 medium, supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin, was used as the culture medium for Bel-7402 cells.

2.8.2. MTT assay

The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondria and cell viability (Lau et al., 2004). Briefly, after being harvested from culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. For HepG2, 1×10^4 cells were incubated in 96-well plates containing 100 μl of the growth medium

per well. For HeLa and Bel-7402, 5×10^3 cells were seeded per well. Cells were permitted to adhere for 24 h, and then treated with various fractions dissolved in medium for 48 h; 20 μl of 5 mg/ml MTT in phosphate buffered saline (PBS) were added to each well and the plate was incubated at 37 °C for 4 h. The medium was removed, and 100 μl of DMSO were then added to each well. After incubation at 37 °C for 10 min, absorbance at 570 nm of the dissolved solutions was measured by a microplate ELISA reader (Thermo Labsystems). The absorbance of control cells (treated with 0.1% DMSO) was considered as 100%.

3. Results

3.1. The scavenging activity for DPPH radicals

DPPH radical, a stable free radical, has been widely used to evaluate the radical-scavenging ability of antioxidants. The DPPH radical-scavenging activity of each fraction was assayed. As shown in Fig. 1, all factions quenched DPPH radicals to different degrees with increased activities at higher concentrations. Among all fractions tested, EE-DS showed stronger activity than did other fractions with an IC_{50} of 1.25 mg/ml, followed by WE-DS with an IC_{50} of 1.84 mg/ml. PE-DS showed a relatively low inhibitory effect with an IC_{50} of 5.19 mg/ml. IC_{50} was the concentration of the fraction inhibiting 50% of free radicals.

3.2. The scavenging activity for superoxide anion radicals

Superoxide anion-scavenging activity of each fraction was measured using the pyrogallol auto-oxidation system and the results were expressed as inhibitory rate of the superoxide productivity. As shown in Fig. 2, EE-DS showed prominent superoxide-scavenging effects; other fractions exhibited slight effect.

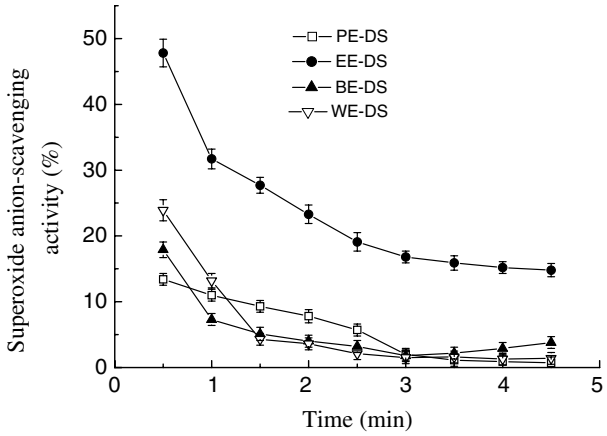

Fig. 2. Superoxide anion-scavenging activities of various fractions from *Dianthus superbus* (DS) measured using the pyrogallol auto-oxidation system. Concentration of each fraction was 4 mg/ml. Each value represents mean ± SD ($n = 3$).

3.3. The scavenging activity for hydroxyl radicals

Scavenging activity of each fraction against hydroxyl radical was investigated using the Fenton reaction and the results were expressed as an inhibition rate. As shown in Fig. 3, each fraction exhibited concentration-dependent scavenging activity against hydroxyl radicals. Among all fractions tested, EE-DS showed the strongest activity, followed by BE-DS. PE-DS showed the weakest *scavenging* effect against hydroxyl radicals.

3.4. The total phenolics

The phenolic contents in different fractions varied significantly. EE-DS had the highest phenolic content, followed by BE-DS and WE-DS. PE-DS had the lowest phenolic content (Fig. 4).

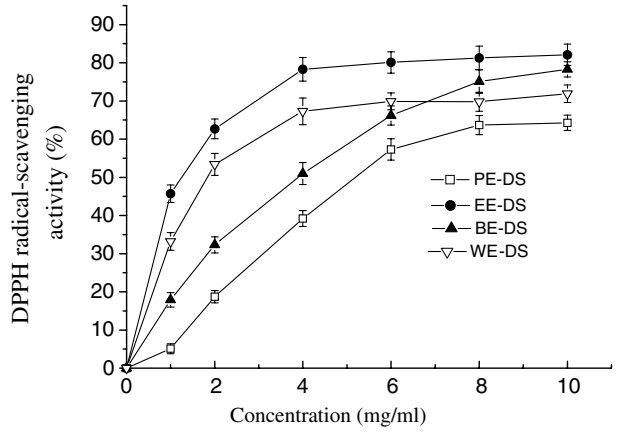

Fig. 1. DPPH radical-scavenging activities of various fractions of *Dianthus superbus* (DS) at different concentrations. Each value represents mean ± SD ($n = 3$).

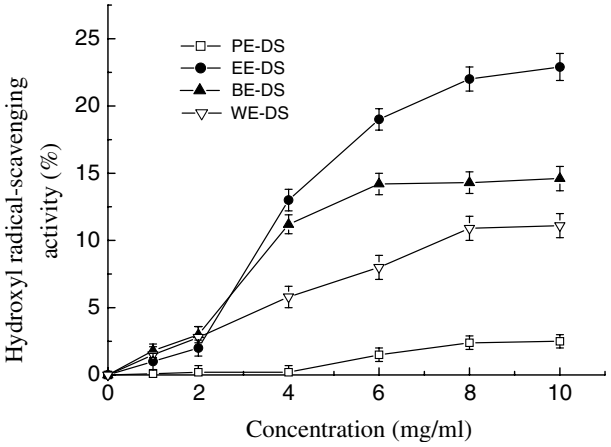

Fig. 3. Hydroxyl radical-scavenging activities of various fractions from *Dianthus superbus* (DS) at different concentrations measured with the Fenton reaction. Each value represents mean ± SD ($n = 3$).

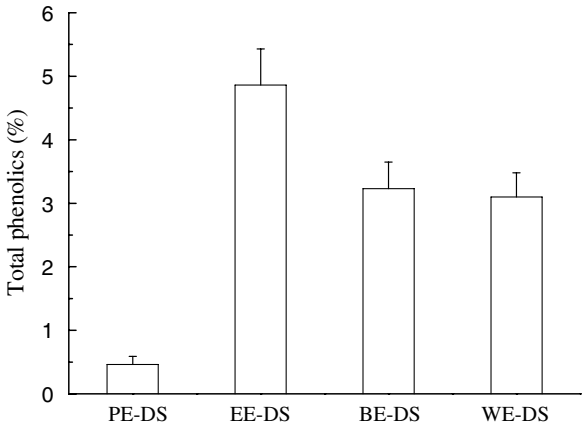

Fig. 4. Total phenolic contents of various fractions from *Dianthus superbus* (DS). Each bar was mean ± SD ($n = 3$).

3.5. The reducing power

In order to examine the reducing power of each fraction, the Fe^{3+} to Fe^{2+} reduction in the presence of various fractions was investigated. As showed in Fig. 5, EE-DS, BE-DS and WE-DS exhibited strong reducing power for Fe^{3+} in a dose-dependent manner. Among all fractions, EE-DS had the highest activity. However, PE-DS displayed little reducing power at the experimental concentrations.

3.6. Cytotoxic activity on cancer cells

The cytotoxicity of each fraction for three human cancer lines, after 48 h exposure at dosages from 5 to 200 μg/ml, is shown in Table 1. Among all fractions, EE-DS (IC_{50} 20 ~ 36 μg/ml) had the greatest cytotoxicity, followed by PE-DS, whereas WE-DS at 200 μg/ml showed slight cytotoxicity on all cell lines tested.

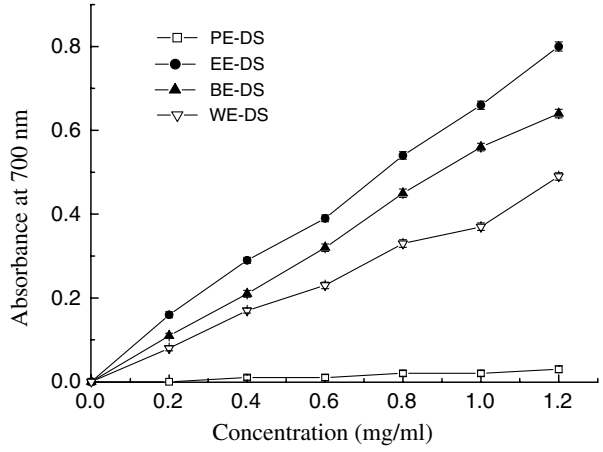

Fig. 5. Reducing powers of various fractions from *Dianthus superbus* (DS) at different concentrations by spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformation. Each value represents mean ± SD ($n = 3$). High absorbance at 700 nm indicates high reducing power.

Table 1
Concentration producing 50% growth inhibition (IC_{50})[a] of various fractions from *Dianthus superbus* (DS) on the three cell lines

Cell lines	IC_{50} (μg /ml)			
	PE-DS	EE-DS	BE-DS	WE-DS
Bel-7402	155 ± 2.1	35.6 ± 0.9	>200	>200
Hep G2	126 ± 2.3	22.6 ± 1.0	167 ± 3.2	>200
HeLa	138 ± 3.0	20.5 ± 0.8	183 ± 3.5	>200

[a] IC_{50} values were expressed as the mean ± SD ($n = 3$), determined from the results of MTT assay.

4. Discussion

The antioxidant activities of the various fractions of ethanol extract of DS were assayed by using several test systems. Recent investigations have shown differences between the test systems for the determination of antioxidant activity (Schlesier, Harwat, Böhm, & Bitsch, 2002). It is recommended to use at least two methods. In this study, we used several methods. DPPH radical is a free radical donor, which has been widely used to evaluate the free radical-scavenging effect of natural antioxidants (Jao & Ko, 2002; Matsukawa et al., 1997). All fractions of ethanol extract of DS showed scavenging activity for DPPH radical. Superoxide anion is formed in viable cells during several biochemical reactions and its effect can be magnified because it produces other types of free radicals and oxidizing agents that can induce cell damage (Heo et al., 2005; Lui & Ng, 1999).Our results showed that EE-DS exhibited prominent superoxide-scavenging effects. Hydroxyl radical is also very reactive and can be generated in biological cells through the Fenton reaction. EE-DS also showed the strongest scavenging activity against hydroxyl radical.

Among all fractions, EE-DS showed the strongest scavenging activity against free radicals. EE-DS also had the highest phenolic content and the strongest reducing power. It appears that antioxidative activity may have a correlation with phenolic content and reducing power.

We also evaluated the potential cytotoxic fractions of ethanol extract of DS. Among solvent fractions, EE-DS was found to be more cytotoxic on three cells than were the other fractions.

5. Conclusion

In summary, we investigated antioxidant and cytotoxic activities of various fractions of ethanol extract of DS. Among various fractions, EE-DS exhibited powerful antioxidant and cytoxic activities. EE-DS may be an active fraction for the treat and prevention of some diseases. Therefore, it has potential for further investigations, including elucidation of active components and evaluating its *in vivo* mechanism of action.

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