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Full Length Research Paper

Free radical scavenging activities of pigment extract from *Hibiscus syriacus* L. petals *in vitro*

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The macroporous resin adsorption method was used to purify the pigment from *Hibiscus syriacus* L. petals. Through the comparison of the adsorption and desorption rates of six types of macroporous resins including AB-8, S-8, NKA-9, DM-130, D101 and 860021 to the pigment, 860021 resin was selected as the most appropriate resin to purify the pigment. The antioxidant capacities of the pigment extract (PE) were evaluated through *in vitro* experiments using hydroxyl radical scavenging assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and lipid peroxidantion (LPO) inhibition capacity assay induced by Fe^{2+}-H_2O_2. Total flavonoid content (TFC) of PE was determined using the colorimetric methodology and total phenolic content (TPC) using Folin–Ciocalteu reagent. PE produced significant antioxidant activity. In addition, PE demonstrated higher TFC and TPC of 63.4±1.8 mg rutin equivalents/g and 172.6±2.4 mg gallic acid equivalents/g, respectively. This study suggests that *H. syriacus* L. petal can be used potentially as a source of natural antioxidants.

Key words: Macroporous resin, *Hibiscus syriacus* L., pigment, free radical.

INTRODUCTION

Free radicals containing one or more unpaired electrons are highly unstable species, which can damage other molecules by getting electrons from them (Lien et al., 2008; Perry et al., 2000). In living organism, free radicals including hydroxyl radical, superoxide anion radical, peroxide radical are generated as the medium of energy supply, detoxification, chemical signal and immunity. But during the excessive metabolism, they cause extensive oxidative damage to cells leading to aging, cancer, neurodegenerative disorders, liver cirrhosis, atherosclerosis, diabetes, inflammation and other human diseases (Halliwell, 2009; Lien et al., 2008; Liu et al., 2008). More and more epidemiological reports associate diets on fruits and vegetables with reduced risk of heart disease and other chronic diseases. An important factor to reduce these diseases is more consumption of antioxidants, including Vitamin C and E, carotenoids and phenolic compounds. It is reported that phenolic compounds are natural substances in plants with potent *in vitro* antioxidant ability and may help to protect cells against oxidative damage caused by free radicals.

Many phenolic compounds have the stronger antioxidant activities by scavenging hydroxyl radical (Sun et al., 2010; Zhang et al., 2011), superoxide anion radical (Rehecho et al., 2010; Sun et al., 2010) and inhibiting lipid peroxidantion (Costa et al., 2011; Tai et al., 2011) through the conjugated rings and hydroxyl groups hydrogenation or complexing with oxidizing species. Macroporous resins can be used to adsorb and purify compounds based on the differences in molecular weight, polarity and size, which leads to differences in affinity for the resin. And many types of macroporous resins have been used to purify phenolic compounds (Chi et al., 2011; Liu et al., 2010; Zhou et al., 2011) in the research and commerce for economic and environmental reasons. *Hibiscus syriacus* L. is widely distributed all around the

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Abbreviations: PE, Pigment extract; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **LPO,** lipid peroxidation; **TPC,** total phenolic content; **TFC,** total flavonoid content; **MDA,** malondialdehyde.

Table 1. Physical properties of the several macroporous resins.

Type	Surface area (m^2/g)	Average pore diameter (Å)	Particle diameter (mm)	Polarity
AB-8	480~520	130~140	0.315~1.25	Weak-polar
S-8	100~120	280~300	0.315~1.25	Polar
NKA-9	250~290	150~165	0.3~1.25	polar
DM-130	500~550	90~100	0.3~1.25	Moderate-poplar
D101	400~600	100~120	0.2~0.6	Non-polar
860021	≥450	90	0.25~0.84	Weak-polar

world as ornamental and green plants (Sung et al., 1998). Furthermore, it is also a medicinal plant used as antipyretic, antihelminthic and antifungal agent in the orient (Chen and Chen., 1993; Li, 2000; Hsu et al., 1986; Huang, 1993; Xu et al., 2000). There have been some reports on the active constituents of its stem and root bark (Kwon et al., 2003; Yun et al., 1998, 1999) or its buds and flowers (Barbara et al., 1973; Yun et al., 2001). However, there are few investigations into the antioxidative activity of the pigment from its petals. As we know, the main components of flower pigments are phenolic compounds, in particular, flavonoids and anthocyanin. Therefore, the objective of this study was to screen the most appropriate macroporous resin for pigment purification, and to evaluate the antioxidant activities of PE using hydroxy radical scavenging assay, DPPH radical scavenging assay and LPO inhibition assay. The results of this study will provide useful information for exploiting its potential commercial application as a source of natural antioxidants.

MATERIALS AND METHODS

The blooming flowers of *H. syriacus* L. were picked from Xinxiang Xinzhong Street (China). The petals were picked out by hands, aired between 35 and 40℃ in an electro-thermostatic blast oven for 48 h, milled into powder in a shredder, orderly. The powder was kept in a brown glass flask at room temperature for use.

Preparation of crude pigment

Briefly, the powder was mixed with 50% ethanol (V/V) as 1:50 (g/mL). The mixture was ultrasonicated intermittently for 90 min at 40℃, and then filtered with filter paper. The ethanol concentration of the filtrate was adjusted to 80% with 95% ethanol. The undissolved substances were removed by centrifugation (2750 g for 10 min). The crude pigment concentrate was obtained in a rotary vacuum evaporator at 40℃.

Adsorption and desorption tests of macroporous resin on pigment

Macroporous resins including AB-8, S-8, NKA-9, D101, DM-130 and 860021 were purchased from Chemical plant of Nankai University (Tianjin, China) or Shandong Lukang Record Pharmaceutical Company Limited (Jining, China). Their physical properties are summarized in Table 1. In order to remove the monomers and porogenic agents trapped inside the pores during synthesis process, the resins were pretreated as follows. First, it was soak in 95% ethanol for 8 h, then wash to no white turbidity adding 3-fold volume water, and the ethanol was rinse with deionized water. Secondly, it was soak in about 4-fold volume 1mol/L HCl for 8 h, then wash to neutral with deionized water. Thirdly, it was soak in about 4-fold volume 1mol/L NaOH solution for 8 h and wash to neutral with deionized water. The adsorption tests (Fu et al., 2007, 2005; Wang et al., 2008) of pigment on macroporous resins were performed as follows: 2.000 g macroporous resin (blotted up by filter paper) was put into a 250 mL conical flask with a lid, 40 mL PE solution (A=1.202) was added. Then the flasks were shaken (130 r/min) for 8 h at room temperature. Absorbance of the solution was determined at 532 nm against the deionized water. Adsorption rate and adsorption capacity were calculated according to the following equations:

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

$$Qa = (A_0 - A_1) \times V / W$$

Where, *Ar* is the adsorption rate (%) of the resin under adsorption equilibrium; *Qa* is the adsorption capacity; A_0 is the absorbance of PE before adsorption; A_1 is the absorbance of PE after adsorption; *V* is the volume of the initial PE and *W* is the weight of the resin. The process of desorption was carried out as follows. After reaching adsorption equilibrium, 1.000 g macroporous resins (washed by deionized water and dried by filter paper) were desorbed with 20 mL ethanol-water solution (50, 70 and 95%, respectively) in the conical flask (250 mL). The flasks were shaken (130 r/min) for 4 h at room temperature. Then the absorbance of the solution was determined. Desorption rate was calculated as the following equation:

$$Dr(\%) = \frac{A_d \cdot V_d}{Q_c \cdot W_1} \times 100$$

Where, *Dr* is the desorption rate (%); A_d is the absorbance of pigment solution after desorption; V_d is the volume of desorption solution; Q_c is the capacity of corresponding resin and W_1 is the weight of resin.

Preparation of PE

The crude pigment concentrate was adsorbed with 860021 macroporous resin, and desorbed with 70% ethanol. The following procedure was to concentrate the effluent in a rotary vacuum evaporator at 40℃. The purple powdery PE was obtained directly.

Determination of antioxidant activity

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by the salicylic acid method (Smirnoff and Cumbes, 1989) with some modifications. Briefly, the pigment powder was dissolved in double distilled water at 0.1, 0.2, 0.4, 0.6, 0.8 or 1.0 mg/mL. A 1.0 mL pigment solution (sample) was mixed with 1.0 mL of 9 mmol/L salicylic acid, 1.0 mL of 9 mmol/L $FeSO_4$ and 1.0 mL of 9 mmol/L H_2O_2. The reaction was activated by adding H_2O_2 and the reaction mixture was incubated for 60 min at 37°C in a water bath. After incubation, the absorbance of the mixtures was measured at 510 nm using a ultraviolet/visible (UV/Vis) spectrophotometer. The control group does not contain pigment solution. The self group does not contain H_2O_2. The blank group absents the pigment solution and the salicylic acid. The ascorbic acid was used as the positive control compound. Hydroxyl radical scavenging activity was calculated as the following equation.

$$Sa(\%)=\left(1-\frac{A_{samlpe}-A_{self}-A_{blank}}{A_{contral}}\right)\times 100$$

Where, *Sa* is the scavenging activity of tested sample (%); A_{sample} is the absorbance of the tested sample; A_{blank} is the absorbance of the blank; A_{self} is the absorbance of the selves and $A_{control}$ is the absorbance of the control.

DPPH free radical scavenging assay

The scavenging activity of the pigment on the DPPH radical (Sigma, USA) was assayed by the method described in the literatures (Chan et al., 2007; Conforti et al., 2008; Liu et al., 2008; Zhang et al., 2011) with a minor modification. Briefly, a 2×10^{-4}mol/L of DPPH ethanol solution was prepared. A 1.0 mL different concentration of sample was added to 2.0 mL of DPPH solution. The mixture was incubated for 60 min at 25°C in the dark. Then the discolorations were measured at 517 nm against ethanol. The ascorbic acid was used as the positive control compound. Percent of sample on DPPH was calculated as the following equation.

$$Sa(\%)=\left(1-\frac{A_{sample}-A_{self}}{A_{contral}}\right)\times 100$$

Where, *Sa* is the scavenging activity of the tested sample (%); A_{sample} is the absorbance of 1.0 mL sample and 2.0 mL DPPH; A_{self} is the absorbance of 1.0 mL sample and 2.0 mL ethanol; $A_{control}$ is the absorbance of 1.0 mL double distilled water and 2.0 mL DPPH.

Assays of LPO in the rat liver homogenate

The inhibition of LPO was determined by quantification malondialdehyde (MDA) decomposed from the lipid peroxide according to the method described in the literatures (Chen et al., 2008; Li et al., 2003; Mee et al., 2001) with some modifications. For the *in vitro* studies, the 10% (w/v) rat liver homogenate was first prepared. Briefly, the male Sprague Dawley (SD) rats (Experimental Animal Center, Xinxiang medical University, China) aged 10~12 weeks, with body mass of 300 to 350 g were sacrificed by cervical dislocation. The rat liver was removed, washed with ice-cold saline, homogenized in 9-fold volume ice-cold saline and then centrifuged at 1000 g for 15 min. The suspension was the 10% rat liver homogenate used to determine LPO. A 0.5 mL of the suspension was mixed with 0.2 mL different concentrations of sample, 50 □L $FeSO_4$ (9 mmol/L) and 100 □L H_2O_2 (0.1mol/L). The mixture was adjusted to a final volume of 1.0 mL with saline and then incubated for 60 min at 37°C. The reaction was stopped by adding 1.5 mL trichloroacetic acid (TCA, 10%, w/v), and then mixed with 2.0 mL 2-thiobarbituric acid (TBA, 0.67%, w/v). Then, the mixture was heated in a boiling water bath for 20 min and centrifuged at 2750 g for 10 min. The supernatant was subjected to analysis by a UV/Vis spectrophotometer at 532 nm against saline. The blank group contained all the reagents but the sample group. The inhibition percent of LPO of pigment was calculated as the following equation.

$$Ir(\%)=\left(1-{A_{sample}}\Big/{A_{blank}}\right)\times 100$$

Determinations of TFC and TPC

TFC was determined by a colorimetric method (Bao et al., 2005; Piccolella et al., 2008) with some modification. A 0.5 mL PE or rutin (Sigma, USA) standard solution was added to a 10 mL volumetric flask. Then 60% (V/V) ethanol was added up to the volume of 5 mL and mixed with 0.3 mL of $NaNO_2$ (5%, W/V). After 6 min, 0.3 mL of $AlCl_3$ (10%, W/V) was added. The mixture was mixed and kept for another 6 min at room temperature, and then 4.0 mL NaOH (1.0 mol/L) was added. The reaction mixture was diluted with 0.4 mL double distilled water and kept for 15 min. The increase in absorbance was read at 510 nm against the blank (without pigment or rutin). The flavonoid content was expressed as rutin equivalents in milligrams per gram of dry basis, using a standard curve generated with rutin. TPC was estimated by the Folin-Ciocalteu method (Bao et al., 2005; Conforti, 2008; Huber and Rupasinghe, 2009) with a little modification. Briefly, 1.0 mL PE solution (0.5 mg/mL) was mixed with 0.5 mL Folin-Ciocalteau reagent and 2.5 mL Na_2CO_3 (7.5%, W/V) (added 2 min after the Folin-Ciocalteau reagent) in a 10 mL volumetric flask. The absorbance was determined at 760 nm against the blank (without pigment) after incubation for 2 h at 25°C. TPC of the PE was expressed as milligrams per gram of gallic acid equivalents of dry basis, using a standard curve generated with gallic acid.

Statistical analysis

All the experiments were carried out three times and the three repeated samples were employed in colorimetric assays. Data were shown in means values ± standard deviations and then analyzed using Origin 7.5. Statistical analyses were performed using the student's *t*-test. Differences were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Adsorption and desorption effects of macroporous resin on the pigment

Many factors can affect the resin adsorption properties. In general, when the resin with the approximate polarity to the adsorbed molecules, larger specific surface area and suitable aperture, it will have better adsorption effect. As shown in Table 2, the adsorption capacity and adsorption

Table 2. Adsorption and desorption effects of the macroporous resins.

Type	Adsorption rate (%)	Adsorption volume	Desorption rate (%)		
			50%	70%	95%
AB-8	80.25±1.27	19.58±0.05	36.77±0.33	61.08±1.21	54.34±0.93
S-8	59.59±1.31	14.54±0.06	55.84±1.26	89.41±0.56	77.03±0.77
NKA-9	39.43±0.65	9.62±0.07	35.76±1.99	66.94±1.36	56.55±0.76
DM-130	61.56±0.94	15.02±0.08	51.13±0.72	87.88±0.97	77.76±0.82
D101	45.41±0.60	11.08±0.12	45.13±1.91	81.59±1.71	70.40±0.49
860021	74.67±1.20	18.26±0.04	67.03±0.37	91.13±0.81	79.96±0.87

Data are means ± SD (n = 3).

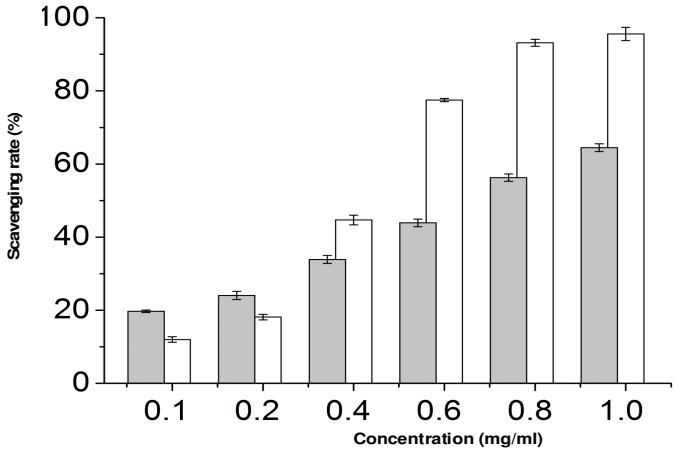

Figure 1. Effects of PE (gray bar) and ascorbic acid (white bar) on hydroxyl radical. Data are means ± SD (n = 3).

rate of AB-8 and 860021 resins toward the pigment were higher than those of other resins, with adsorption rate of 80.25 and 74.67%, respectively. In order to find more suitable desorption solvent, different concentrations of ethanol were used as the eluent in the desorption tests. The desorption rate of different concentrations of ethanol on all types of macroporous resin increased when the concentration changed from 50 to 70%, but decreased when the concentration increased from 70 to 95%. In any concentration solvent, the resin 860021 had better desorption rate than other resins. Taking all factors into consideration, the resin 860021 was selected as the purification resin and 70% ethanol as the desorption solvent.

Hydroxyl radical scavenging activity

The hydroxyl radical is the most active in the reactive oxygen species, and it can cause most damage to the organism. Hydroxyl radicals can react with variety of molecules such as proteins, polypeptides, nuclear acids and lipids, to cause biomolecules oxidative damages and cell necrosis or mutations (Balaban et al., 2005; Klaunig and Kamendulis, 2004; Shi et al., 2004). As shown in Figure 1, PE had a scavenging activity toward hydroxyl radicals in a dose-dependent manner. The scavenging activity of PE was obviously higher than that of the ascorbic acid when the concentration was below 0.2 mg/mL. But the scavenging activity was relatively lower than that of the ascorbic acid when the concentration was over 0.4 mg/mL. The IC_{50} value of PE is 0.718 mg/mL whereas that of the ascorbic acid is 0.393 mg/mL.

DPPH radical scavenging activity

DPPH radical is a stable nitrogen-centered on free radical with an unpaired electron. Its solution appears deep violet and shows a strong absorbance at 517 nm. Substances, which make its color lighter and absorbance descendent can be considered as antioxidants and therefore radical scavengers (Brand-Williams et al., 1995). Now, it is well accepted that DPPH radical is used to analyze the scavenging potential of compounds. As shown in Figure 2, PE showed appreciable free radical scavenging activity, which was weaker than ascorbic acid in the same concentrations. There was a dose-effect relationship with the increasing concentration. The IC_{50} value of PE to DPPH radical was 76.8 □g/mL, also it was higher than

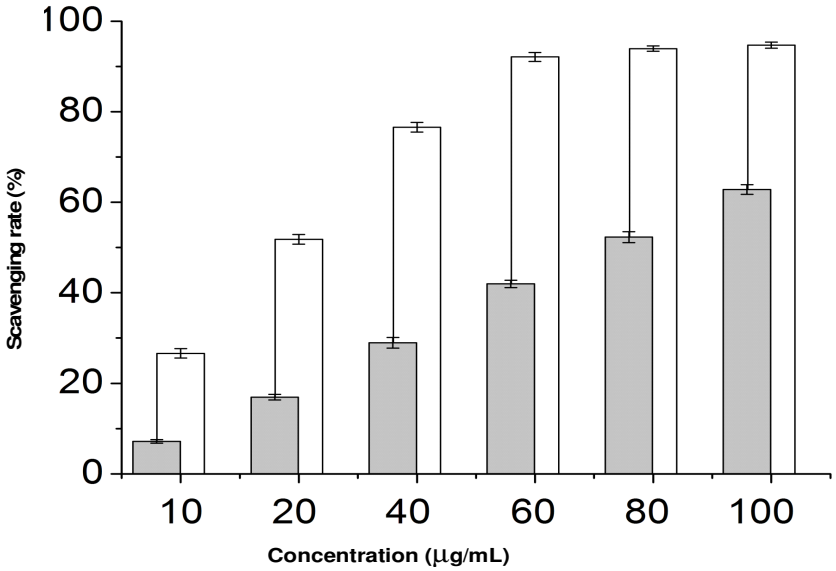

Figure 2. Effects of PE (gray bar) and ascorbic acid (white bar) on DPPH radical. Data are means ± SD (n = 3).

Table 3. Effects of PE on liver LPO induced by Fe^{2+}-H_2O_2.

Concentration (μg/mL)	A_{532}	Inhibition (%)
Control	0.967±0.019	-
15	0.462±0.021	52.22±2.36
30	0.457±0.015	52.74±1.81
60	0.440±0.012	54.50±1.53
120	0.428±0.009	55.74±1.27
240	0.414±0.013	57.19±1.59

Data are means ± SD (n = 3).

the ascorbic acid (IC_{50}=20.8 μg/mL).

Determination of capability of LPO inhibition

LPO was initiated from the process of the unsaturated fatty acids oxidation deterioration induced by free radicals, such as superoxide, hydroxyl radicals, etc., and other reactive oxygen species. Low concentrations of LPO are found in tissues in the normal physiologic conditions. But under the oxidative stress conditions, LPO can harm the cells by inactivating the enzymes and receptors in membrane, depolymerizing deoxyribonucleic acid (DNA) and proteins cross-link. Furthermore, several LPO by products can damage other bio-molecules (Koh et al., 1997; Marnett, 2002; Costa et al., 2011). As shown in Table 3, PE showed significant antioxidant activity in LPO and its inhibition activity was presented in a concentration-dependent manner. The inhibition rate of LPO was 52.22% even at a concentration of 15 μg/mL.

Total flavonoid and phenolic contents

Phenolic compounds are responsible for the antioxidant activity of plant materials due to their redox properties. And the phenolic hydroxyl group helps them to work as reducing agents, hydrogen donors and singlet oxygen quenchers (Chua et al., 2008). Many reports indicated that antioxidant activities were in accordance with TPC (Lachman et al., 2010; Rehecho et al., 2010; Sheng et al., 2011; Xue et al., 2009) or TFC (Rehecho et al., 2010; Zhang et al., 2011). Total phenols and flavonoids were expressed in terms of gallic acid and rutin equivalents per gram. PE was rich in phenols and flavonoids. TFC and TPC of PE were estimated to be 63.4±1.8 mg rutin

equivalents/g and 172.6±2.4 mg gallic acid equivalents/g, respectively. This also explains why the PE has strong antioxidant activity.

Conclusion

The present research indicates that extracting pigment from *H. syriacus* L. can be performed by macroporous resins, and PE is antioxidative *in vitro*. The pigment contains higher TCP and shows efficient radicals scavenging activity on hydroxyl, DPPH and LPO. Especially, PE has strong antioxidant ability to hydroxyl radical in low concentration. Moreover, a more detailed study between the component of PE and the antioxidant activity *in vivo* needs to be carried out in future. Like carthamin, curcumin, □-renieratene and other pigments in the market, the pigment from *H. syriacus* L. might also be employed as a food colorant and pharmaceuticals.

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