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Characterisation and free radical scavenging activities of novel red pigment from *Osmanthus fragrans*' seeds

Yingming Pan [a,*], Zhiren Zhu [a], Zhili Huang [a], Hengshan Wang [a,*], Ying Liang [b], Kai Wang [a], Qian Lei [a], Min Liang [a]

[a] School of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 541004, PR China
[b] The Eighth Department, Guilin University of Electronic Technology, Guilin 541004, PR China

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ABSTRACT

A novel red pigment (RP) was isolated from *Osmanthus fragrans*' seeds. The optimised experimental parameters of extraction obtained with a four-factor at three-level orthogonal array experimental design $L_9(3^4)$ were ethanol concentration, temperature, pH and extraction time as 90%, 78 °C, 2.5 and 40 min, respectively. A yield of 34.6 ± 2.2 g/100 g was obtained under optimised conditions. The red pigment directly from *O. fragrans*' seeds can be dissolved in alkaline, acidic waters solutions and hydrophilic organic solvents in common use. The colour of a water solution of RP changed with pH. RP was stable to heat in the temperature range of 25–100 °C. Physical and chemical properties of RP revealed that the red pigment was also stable in the presence of Na_2SO_3, NaCl, amino acid, organic acid, sugar, starch or metal-ions (such as Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Al^{3+}, Mg^{2+} and Na^{+}), but was bleached by strong oxidants ($KMnO_4$, $K_2Cr_2O_7$ and NaOCl). Subsequently, free radical scavenging activities of RP were assessed using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals using a new resonance scattering spectral method. RP showed an excellent DPPH radical scavenging activity and was superior to butylated hydroxytoluene (BHT), and exhibited quite a strong concentration-dependent inhibition of hydroxyl radical at low concentrations compared with ascorbic acid and quercetin. When the concentration of RP was 0.03 μg/ml, the scavenging percentage of hydroxyl radical reached 92.3%. Salidroside was isolated as an active principle.

1. Introduction

Edible pigments are divided into synthetic and natural (Zhang, Zhan, Su, & Zhang, 2006). Colourants, widely used in the food industry, are important for appeal, a major criterion of identification, indicator of quality and freshness, and a determinant of consumer acceptability, market size and value (Gouveia et al., 2005). In the past, synthetic pigments were used indiscriminately to manipulate food colour. However, synthetic colourants are hardly nutrient, and have been blamed for allergenic and intolerance reactions (Wang, Pan, Tang, & Huang, 2006). Therefore the current trend is to substitute natural colourants for synthetic colourants (Britton, 1999; Es-Safi, 2004).

Osmanthus genus plants, distributed mainly in China, are generally renowned for their colour, fragrance, posture and charm. Therefore, the splendid *Osmanthus* culture was generated. Research on *Osmanthus* genus focuses mainly on *Osmanthus fragrans*. *O. fragrans*, belonging to the family *Osmanthus* genus, is a horticultural ornamental plant. *O. fragrans*, as a flower native to China, is cultivated extensively and has long been prized for its abundant sweet-scented flowers (Liu & Xiang, 2003). It is especially valued as an additive for tea and other beverages and also as an economic and valuable plant in the east. *O. fragrans*' extracts are of high value and accordingly are used in only the most expensive perfumes and flavours. In the previous research, the melanin isolated from *O. fragrans*' had been comprehensively studied in our laboratory (Wang et al., 2006). However, the red pigment extracted from *O. fragrans*' seeds has not been reported.

In the present work, experiments were carried out in order to evaluate the physical and chemical properties of the red pigment derived from *O. fragrans*' seeds. Furthermore the free radical scavenging activities of RP were studied using DPPH and hydroxyl radicals using a new resonance scattering spectral method.

2. Materials and methods

2.1. Materials

In these experiments, mature *O. fragrans*' seeds were collected from Guilin city, China, and transferred immediately to the laboratory where they were manually peeled and subjected to RP

* Corresponding authors. Tel.: +86 773 5846279; fax: +86 773 5803930.
E-mail address: panym2004@yahoo.com.cn (Y. Pan).

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extraction. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) and synthetic antioxidant butylated hydroxytoluene (BHT) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals were purchased from China National Medicine Group Shanghai Corporation (Fuzhou Road, Shanghai City, China). All chemicals used were of analytical grade.

2.2. Equipment and apparatus

The following instruments were used: TU-1901 spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing, China); the Rayleigh scattering and synchronous fluorescence (SF) spectra were recorded with a model RF-535.5 spectrofluorophotometer (Shimadzu, Kyoto, Japan), using the synchronous scanning technique that the excited wavelength λ_{ex} was equal to the emission wavelength λ_{em} ($\lambda_{ex} = \lambda_{em}$), a model of TU-1901 dual beams spectrophotometer (Puxi Com., Beijing, China) were used for recording the absorption spectra; RE-52AA rotavapour (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China); DZF-1B vacuum drier (Shanghai Yuejin Medical Instrument CO., LTD, Shanghai, China); SHB-βA water-circulation multifunction vacuum pump (Zhengzhou Great Wall Scientific Industry and Trade CO., LTD, Zhengzhou, China).

2.3. Optimization of RP extraction technology by orthogonal array design

Optimization of RP extraction technology by orthogonal array design was according to the method of Taguchi and Konishi (1987). Orthogonal array design is a popular method for optimising experimental parameters. To obtain the optimised experimental parameters, a four-factor at three-level orthogonal array experimental design $L_9(3^4)$ was adopted. The factors studied and the assignments of the corresponding levels are listed in Table 1. Based on the experimental results of the previous univariate design, the ranges for ethanol concentration, temperature, pH and extraction time were set as 60–90%, 40–78 °C, 1.0–4.0 and 10–40 min, respectively. The extraction of 1.00 g material was accomplished with 10 ml volume extraction vessel. The absorbance was determined at λ_{max} (visible light area).

According to the Taguchi design $L_9(3^4)$, nine tests were performed. The analytical results are listed in Table 2. The sum (K) and average (k) of the extract absorbance in each test were calculated. The values of $|k_{max} - k_{min}|$ in Table 2 indicate the effect of ethanol concentration, pH, extraction time and temperature. Thus, temperature was the major factor affecting the extraction, whilst ethanol concentration, pH and extraction time had a less obvious influence. In order to obtain the maximum K_i or k_i values, ethanol concentration, temperature, pH and extraction time were chosen as 90%, 78 °C, 2.5 and 40 min, respectively.

Isolation of RP was performed under optimised extraction condition. First, the raw materials of *O. fragrans*' seeds were washed with running water at a volume ratio of 1:50 (raw materials/water) for 5 min followed by eliminating solid matter. Wet peels of *O. fragrans*' seeds were dried in a DZF-1B vacuum drier at 30 °C and 0.07 Mpa. Then the dried peels were ground (max particle size 0.4 mm) and 10 g of ground material were extracted with 100 ml of 90% ethanol, which pH was adjusted to 2.5 with hydrochloric acid, for 40 min at 78 °C. Subsequently, extraction solution was filtered and evaporated using a RE-52AA rotavapour at 60 °C and a SHB-βA water-circulation multifunction vacuum pump. The residue of red pigment was lyophilised to yield 3.46 ± 0.22 g.

Table 1
Assignment of the levels to factors

Level	Ethanol concentration (%)	Temperature (°C)	pH	Extraction time (min)
1	60	40	1.0	10
2	75	60	2.5	25
3	90	78	4.0	40

Table 2
The $L_9(3^4)$ matrix associated with the analytical results

Number	Ethanol concentration (%)	Temperature (°C)	pH	Extraction time (min)	Absorbance
1	1	1	1	1	0.027 ± 0.002
2	1	2	2	2	0.264 ± 0.005
3	1	3	3	3	0.224 ± 0.009
4	2	1	2	3	0.144 ± 0.007
5	2	2	3	1	0.123 ± 0.003
6	2	3	1	2	0.311 ± 0.004
7	3	1	3	2	0.078 ± 0.001
8	3	2	1	3	0.300 ± 0.007
9	3	3	2	1	0.275 ± 0.003
K1	0.515	0.249	0.638	0.425	
K2	0.578	0.689	0.683	0.653	
K3	0.653	0.810	0.425	0.668	
k1	0.172	0.083	0.213	0.142	
k2	0.193	0.230	0.228	0.218	
k3	0.218	0.270	0.142	0.223	
$\lvert k_{max} - k_{min}\rvert$	0.046	0.187	0.086	0.081	

2.4. Characterisation of RP

The physical and chemical characteristics of RP were measured according to standard procedures (Nicolaus, 1968; Paim, Linhares, Magrich, & Martin, 1990; Prota, 1992; Wang et al., 2006), including ultraviolet–visible absorption spectrum, solubility in water and common organic solvents, oxidised bleaching by $KMnO_4$, $K_2Cr_2O_7$, NaOCl and reduced by Na_2SO_3, stability in different temperature and chemicals such as salt, sugar, amino acid, organic acid, starch, metal-ion and so on.

2.5. Assessment of antioxidant properties of RP

2.5.1. Scavenging activity on DPPH radical

To evaluate the free radical scavenging activity, RP was allowed to react with a stable free radical, 2,2′-diphenyl-1-picrylhydrazyl radical (Brand, Cuvelier, & Berset, 1995; Pan et al., 2007, 2008; Blois, 1958). RP solution (0.2 ml) in 95% ethanol at different concentrations (0.2, 0.5, 0.8, 1.2 mg/ml) was added to 8 ml 0.004% (w/v) solution of DPPH in 95% ethanol. The reaction mixture was incubated at 28 °C. The scavenging activity on DPPH radical was determined by measuring the absorbance at 515 nm each 10 min until the reaction reached the steady state. The antioxidant activity was expressed as a percentage of scavenging activity on DPPH radical: SC% = [1-(absorbance of sample)/(absorbance of control)] × 100%. The control contains all reagents except the extract. The DPPH radical scavenging activity of BHT (0.5 mg/ml) was also assayed for comparison. IC_{50} value (μg compound ml^{-1}) is the effective concentration at which DPPH radical were scavenged by 50%. All tests were performed in triplicate and the results were centred.

2.5.2. Scavenging percentage of hydroxyl radical on RS method

The hydroxyl radical scavenging activity of RP was estimated using the method of Liang, Zhou, and Jiang (2006). A certain volume of the HCl–NaAc buffer solution, KI solution (0.020 mol/l), certain volume of Fe(II) solution and certain volume of H_2O_2 standard solution were piped in a 10 ml graduated tube, then added rhodamine B (RhB, 1.50×10^{-4} mol/l) and mixed. The mixed solu-

tion was diluted to 5 ml with water and mixed thoroughly. The resonance scattering spectra was obtained by using the synchronous scanning technique in a model RF-535.5 spectrofluorophotometer. The I_s which presents the RS intensity for the system containing H_2O_2 and scavenger, I presents the RS intensity for the system containing H_2O_2 and I_b presents the RS intensity in the absence of H_2O_2 were measured at 420 nm. The percent inhibition (P) could be calculated as $P\ (\%) = [(I - I_s)/(I - I_b)] \times 100\%$. The hydroxyl radical scavenging activities of quercetin and ascorbic acid were also assayed for comparison. All tests were performed in triplicate and the results were centred.

2.6. Isolation of active compound

RP was partitioned with methanol and divided into the methanol soluble fraction and the methanol insoluble fraction. The methanol soluble fraction showed the strong activity antioxidant activity. The active compound (Fig. 1) was separated and purified from the methanol soluble fraction by rechromatograph on a silica gel column using $CHCl_3$/MeOH (8:1, v/v) as eluent. Its detailed spectral data from ^{1}H NMR, ^{13}C NMR and MS agreed well with the reported compound, salidroside (tyrosol 8-O-β-glucopyranoside) (Han, Zhang, Wei, Cao, & Ito, 2002).

White needle crystal; mp. 158–159 °C; ^{1}H NMR (500 MHz, MeOD): δ 2.86 and 3.19 (2H each, t, J = 7.2 Hz), 3.22–3.37 (4H, m), 3.60–3.74 (2H, m), 3.88 (2H, d, J = 10.4 Hz), 4.05 (1H, dt, J = 10.4, 3.8 Hz), 4.30 (1H, d, J = 7.8 Hz), 4.56 (1H, s), 6.72 (2H, d, J = 8.4 Hz), 7.08 (2H, d, J = 8.4 Hz); ^{13}C NMR (125 MHz, MeOD): δ 155.4, 129.5, 114.8, 103.0, 76.8, 76.6, 73.8, 70.7, 70.4, 61.5, 35.0; FAB-MS: m/z 301 $[M + H]^+$.

2.7. Statistical analysis

All experimental results were centred at using three duplicated measurements of mean ± SD. Analysis of variance was performed by ANOVA procedure. Duncan's new multiple-range test was used to determine the differences of means. P values <0.05 were regarded as significant and P values <0.01 as very significant.

3. Results and discussion

3.1. Spectroscopic analysis of RP

Fig. 2 shows the ultraviolet–visible absorption spectrum (200–800 nm) of RP solution. RP exhibits strong optical absorbance in a wide spectral range. The absorption spectrum of RP shows a typical peak at 217.0 nm and 287.5 nm in the ultraviolet area, whilst 535.5 nm in the visible light area. In the following experiments, the absorbance was measured at 535.5 nm.

3.2. Solubility of RP

The red pigment displayed a good solution both in alkaline and acidic water. Furthermore, it also was soluble in hydrophilic organic solvents such as acetone, methanol and ethanol, whereas insoluble in lipophilic organic solvents such as petroleum, diethyl ether, ethyl acetate and n-hexane, and was slightly dissolved in chloroform. Shown in Table 3, solvents also exerted important effects on the colour and maximum absorption wavelengths (λ_{max}) of RP.

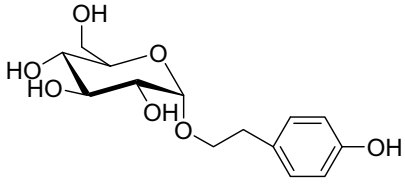

Fig. 1. The structure of salidroside.

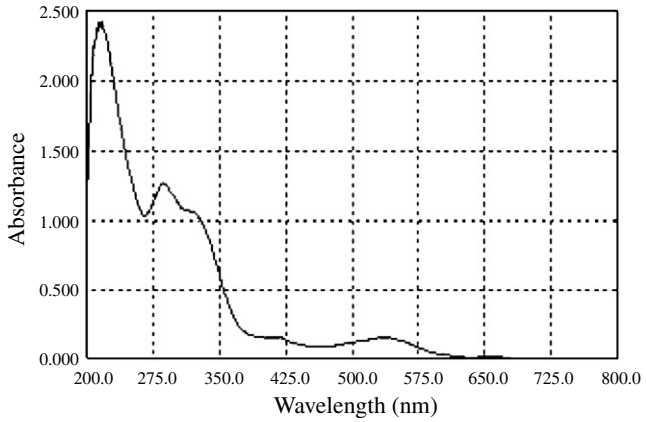

Fig. 2. Ultraviolet–visible absorption spectrum of RP from *Osmanthus fragrans*' seeds.

3.3. Effect of pH on RP

Table 3 shows that when RP was dissolved in water with different pH, different colours were assumed and slight change in the maximum absorption wavelengths (λ_{max}). RP was red colour at pH 1–5, and the red colour became light with pH increase. It displayed henna colour at pH 7–10, bottle green colour above pH 10, and the green colour became dark with pH increase. Due to the effect of pH, RP are known to be at the stable state in acidic condition. Its property of changing colour with pH will help in the food processing and other applications.

3.4. The stability of RP

The effect of temperature on RP stability was determined to ascertain the potential use of RP as a natural colorant. When RP solutions were in heat of 25–100 °C for 2.5 h, the red colour of RP was almost unchanged. It revealed that the pigment was stable to temperature.

Natural pigments can generally be oxidised or reduced to alter their chemical structures. In this study, the oxidative bleaching to RP with $KMnO_4$, $K_2Cr_2O_7$ and NaOCl was investigated. The absorbance of RP gradually diminished with the increased concentration of $KMnO_4$, $K_2Cr_2O_7$ and NaOCl. It illuminated that RP could be bleached by these oxidants. The effect of reducer, Na_2SO_3, was also investigated. The absorbance of RP solution has no obvious change in the presence of 200 mg/l Na_2SO_3 after 48 h. The results showed that RP was stable to Na_2SO_3.

Table 3
Effect of pH and solvent on colour and λ_{max} of RP

Solvents or pH	Colour	λ_{max} (nm)
Water, pH 1	Sanguine	539.5
Water, pH 2	Red	539.5
Water, pH 3	Red	538.5
Water, pH 5	Red	538.5
Water, pH 7	Henna	538.5
Water, pH 10	Light henna	538.5
Water, pH 12	Green	535.5
Methanol	Red	528.0
Ethanol	Red	537.0
Acetone	Henna	524.0

The absorbance of RP solution had no obvious change when added sodium chloride. And the absorbance of RP almost unchanged when mixed with ions, such as Ca^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Al^{3+}, Mg^{2+} and Na^{+}, amino acid, organic acid, sugar or starch. It illuminated that RP solution was stable to these chemicals.

In summary, RP is a stable pigment. The colour value of RP was $E^{1\%}_{1\,cm}$535.5.0 nm = 44.6, which was far higher than the colour value of turnip red pigment ($E^{1\%}_{1\,cm}\lambda_{max} = 4.0$), and similar to the colour value of the general pigment ($E^{1\%}_{1\,cm}\lambda_{max} = 45.0$) (Zhang, Sun, Chi, Lai, & Wang, 1998).

3.5. Free radical scavenging activities of RP

3.5.1. Scavenging activity on DPPH radical

The DPPH radical assay could determine the radical scavenging activities of an antioxidant by measuring of a decrease in the absorbance of DPPH at 515 nm and this assay possesses the advantage of rapid, facile and commercially available (Gülçin, 2006). A concentration-dependent assay was carried out with RP and the results are presented in Fig. 3. These results provide a direct comparison of the antioxidant activity with BHT. RP possessed significant scavenging activity on the DPPH radical and acted as an antioxidant. The scavenging effect was increased with increasing concentration and reaction time. RP showed a higher scavenging activity than BHT at concentrations of 0.8 and 1.2 mg/ml. At the same concentration (0.5 mg/ml), RP exhibited a DPPH scavenging activity of 35.13%, 38.38%, 41.78%, 44.96% and 47.65%, whilst BHT showed 20.94%, 30.97%, 38.88%, 43.83% and 47.61%. Hence, at concentration of 0.5 mg/ml, the scavenging activity of RP was better than that of BHT during the first 50 min, and close to BHT at 50 min. Furthermore, scavenging activity of RP reached a very high degree within 10 min, BHT was distinctly slower than that of RP in scavenging DPPH radical.

3.5.2. Scavenging activity on hydroxyl radical

In present study, hydroxyl radical scavenging activity of RP was measured by a new resonance scattering spectral method of Liang et al. (2006) from Fenton reaction. In the reaction system of this method, the main reaction process as,

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \dot{O}H + OH^-$$

$$\dot{O}H + I^- \rightarrow OH^- + I_2$$

$$I_2 + I^- \rightarrow I_3^-$$

$$nRh^+ + nI_3^- \rightarrow (Rh - I_3)_n$$

Based on these reaction and resonance scattering effect of $(Rh - I_3)_n$ at 420 nm, this method could be employed to determined hydroxyl radical scavenging activity.

According to this procedure, the scavenging percentage of RP on the hydroxyl radical was investigated. Fig. 4 shows that RP exhibited quite a strong concentration-dependent inhibition of hydroxyl radical at low concentration compared with ascorbic acid and quercetin. When the concentration of RP was 0.03 μg/ml, the scavenging percentage reached 92.3%.

3.6. Free radical scavenging activity of salidroside

The results are normalised and expressed as EC_{50} values. EC_{50} of salidroside (81.54 μg/ml) on DPPH present were significantly ($P < 0.01$) lower than that of BHT (446.18 μg/ml). Generally, the lower IC_{50} value shows the higher scavenging activity. Thus, salidroside shows strong free radical scavenging activity.

4. Conclusions

There has been much interest in the development of new natural colourants for use in the food industry, which is apparently due to strong consumer demand for more natural products. The production of the pigment is increasing at a rate of 10% every year in the international market (Wang et al., 2006).

In this study, the red pigment was isolated firstly from *O. fragrans*' seeds by orthogonal test. The optimising experimental parameters of extraction obtained with a four-factor at three-level orthogonal array experimental design $L_9(3^4)$ were ethanol concentration, temperature, pH and extraction time as 90%, 78 °C, 2.5 and 40 min, respectively. A yield of 34.6 ± 2.2 g/100 g was obtained in optimised condition. The colour value of RP was $E^{1\%}_{1\,cm}$535.5 nm =

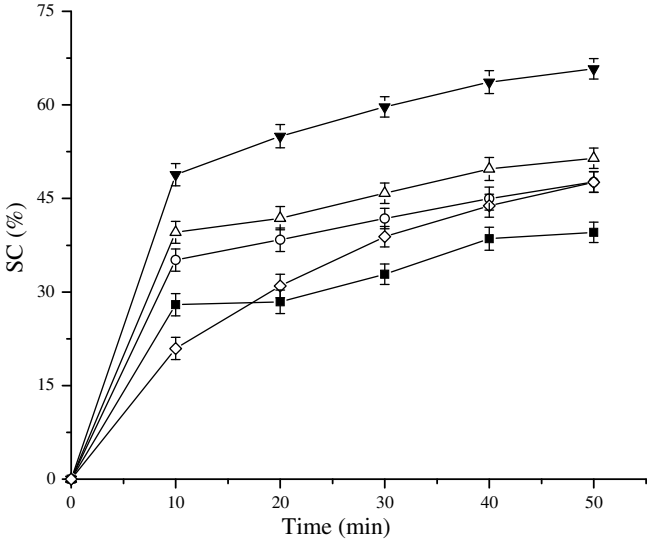

Fig. 3. DPPH free radical scavenging activity of RP and BHT. 0.2 mg/ml RP (■), 0.5 mg/ml RP (○), 0.8 mg/ml RP (Δ), 1.2 mg/ml RP (▼) and 0.5 mg/ml BHT (◇). SC% (percentage of scavenging activity on DPPH radical) = [1-(absorbance of sample)/(absorbance of control)] × 100. Results are mean ± SD of three parallel measurements. $P < 0.01$, when compared to the control.

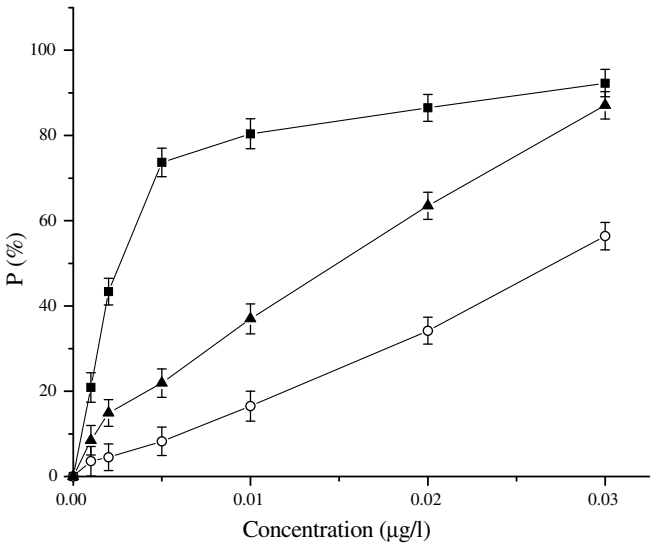

Fig. 4. Scavenging activity on hydroxyl radical of RP at pH 4.95, 3.00×10^{-3} mol/l KI, 4.00×10^{-5} mol/l Fe^{2+}, 3.00×10^{-5} mol/l RhS and 6.48 μmol/l H_2O_2 (OR = 5, $S = 2$). RP (■), Quercetin (○), Ascorbic acid (▲). P% (percent inhibition) = $[(I - I_s)/(I - I_b)] \times 100\%$. Results are mean ± SD of three parallel measurements. $P < 0.01$, when compared with the control.

44.6, which was similar to the colour value of general pigment ($E^{1\%}_{1\,cm}\lambda_{max} = 45.0$).

No evident influence of sodium chloride as food additives on the pigment stability was observed. RP was not resistant to chemical oxidization, therefore, oxidizers should be avoided when refining, processing and using of the pigment. The red colour was almost unchanged when RP solution was heated in heat condition of 25–100 °C for 2.5 h. RP exhibited a good solution both in alkaline and acidic water. Furthermore, it also was soluble in hydrophilic organic solvents and hardly soluble in lipophilic organic solvents. The colour of RP changed with pH, red colour at pH 1–5, henna colour at pH 7–10, and green colour above pH 10. Most inspected metal-ions hardly affected pigment stability. The pigment was stable to amino acid, organic acid, sugar and starch.

RP also showed an excellent 2,2′-diphenyl-1-picrylhydrazyl radical scavenging activity and was superior to BHT. The hydroxyl radical scavenging activity based on resonance scattering effect of $(Rh - I_3)_n$ at 420 nm was also investigated and RP exhibited quite a strong concentration-dependent inhibition of hydroxyl radical at low concentration compared with ascorbic acid and quercetin. When the concentration of RP was 0.03 μg/ml, the scavenging percentage reached 92.3%. Salidroside was isolated as an active principle.

In a broad sense, the good characteristics of RP give it potential for use by the food processing industry as an additive. Considering its excellent free radical scavenging capability, it can be also used as an antioxidant.

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