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Antioxidant Activity and Flavones Compounds Contents of Tartary Buckwheat Seedlings Extract

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Flavonoids extract of tartary buckwheat seedlings was prepared by ultrasound-assisted extraction and AB-8 macroporous resin purification. High performance liquid chromatography analysis showed that flavones compounds such as rutin, quercetin and kaempfenol were the primary characteristic compounds in the flavonoids extract. The flavonoids extract and flavone compounds of tartary buckwheat seedlings exhibited strong free radical scavenging activity, especially against hydroxyl radical and DPPH radical, along with superoxide radical, which could be related to the reducing power and metal iron chelating activity of flavone compounds in tartary buckwheat seedlings. The radical scavenging activity and lipid peroxidation inhibiting activity of the flavone compounds decreased in the same order as following: quercetin > rutin > kaempfenol. This work has gathered experimental evidences on the great potential of tartary buckwheat seedlings as an easily accessible health food, natured antioxidant for its high content of flavonoids, low cost and very short cultivated period. This work has gathered experimental evidences on the great potential of tartary buckwheat seedlings as an easily accessible health food, natured antioxidant.

Key Words: Tartary buckwheat seedlings, Flavones compounds, High performance liquid chromatography, Antioxidant activity.

INTRODUCTION

Flavonoids, abundant in fruits, vegetables, medicinal plants, have received the greatest attention[1-3]. They can interact with various biological systems and have shown to be a highly effective antioxidant and less toxic than synthetic antioxidants, such as BHA or BHT[4]. They can also inhibit lower density lipoprotein oxidation effectively and are a kind of antiinflammatory, hypolipidemic and hypoglycemic[5-7].

Buckwheat, belonging to fagopyrum of polygonaceae, is widely planted in China and many other countries of the world[8]. The commonly planted species of buckwheat are common buckwheat and tartary buckwheat[9]. Consumption of tartary buckwheat may be beneficial owing to its high content of flavonoids[10,11]. Recently, tartary buckwheat seedling, as a kind of sprout vegetable, is extensive used in China and Japan as a natural food to promote good health[12-14]. Though studies have found that buckwheat extract has a strong antioxidative activity and could reduce the risk of age-dependent diseases[15-17], the effect of flavones compounds in tartary buckwheat seedling has not been well studied so far[18]. Therefore it is significant to study flavones compounds and bioactivities of tartary buckwheat seedling.

In the present work, the flavonoids extract of tartary buckwheat seedling was prepared by ultrasound- assisted extraction and macroporous resin purification. The antioxidant activities were evaluated by six systems. The content of total flavonoids in the extract and tartary buckwheat seedling was also determined, high performance liquid chromatography (HPLC) was employed for the analysis of flavones compounds in the extract. The experimental results will be helpful to the chemical and pharmacological understanding of tartary buckwheat seedling.

EXPERIMENTAL

Tartary buckwheat seedling, the seedling of *Fagopyrum tataricum* (L.) Gaertn, cultivated for 17 d, was from LongHai Mountain of Luoping, Yunnan province of China and identified by Dr. Yang Jingsong, Key Lab of National Medicine Supported Jointly by State Ethnic Affairs Commission and Ministry of Education. Tartary buckwheat seedling was dried and powdered to be less than 0.5 mm before experiment. Rutin and 1,1-biphenyl-2-picryl-hydrazil radical (DPPH) were purchased from sigma Chemicals Co. (St. Louis, USA); quercetin and kaempferol were from National Institude for Control of Pharmaceutical and Biological Products (Beijing, P.R. China); AB-8 macroporous resin was purchased from the Chemical Plant of NanKai University. Other chemicals were purchased from China National Medicine Group Shanghai

Corporation (Shanghai, PR China). Microporous membrane (Shanghai Wanzi Shiye Co. Ltd., Shanghai, P.R. China). HPLC-grade methanol and phosphoric acid were products of Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical. Deionized water was purified by a Milli-Q gradient system(Millipore, Bedford, MA, USA).

AS10200AD ultrasound cleaning bath (Tianjin Autoscience Instrument Co. Ltd., Tianjin, P.R. China) with 330 × 270 × 290 cm internal dimensions, a volume of 10.0 L and 4 transducers was used in the experiment. The temperature was controlled and maintained at 45 ± 1 ºC by circulating external water from a thermostated water bath; R-200 rotary evaporator (Buchi, Switzerland); Shimadzu high performance liquid chromatography system SPD-IOAT series (Shimadzu Corp., Japan), Prominence CBM-20A, Quaternary pump LC-10 Atvp, autosampler SIL-IOAP, column heater G1316A and SPD-10 Avp detector, Waters XterraTM MS C_{18} column (150-3.9 nm, 5 µm); UV-2000 UV-VIS spectrophotometer (Unico (Shanghai) Instrument Co. Ltd., Shanghai, P.R. China).

Extraction and purification of flavonoids: Ultrasound-assisted extraction[19] was employed to obtain crude extract. 10 g dried tartary buckwheat seedling powder was soaked in a 200 mL 75 % (v/v) ethanol solution for 2 h and then placed in ultrasound bath and sonicated at 45 ºC for 40 min at 60 kHz and 320 W input power. The solution of crude extract was filtered off through 0.45 µm microporous membrane.

This crude extract was further purified by AB-8 macroporous resin column (400 × 2.5 cm i.d.) to obtain flavonoids extract. The filtrate was extracted with 50 mL of petroleum ether to get rid of fats oils and chlorophyll. And then poured into the AB-8 macroporous resin column until the solution was absorbed completely. The column was washed by enough distilled water to remove carbohydrates and then washed by 95 % (v/v) ethanol to elute flavonoids. The eluate was collected and concentrated with a rotavapour to obtain flavonoids extract. The extract was dried by a vacuum dryer and then redissolved in ethanol to a concentration of 100.0 mg mL^{-1}, the solution was stored in a amber colour air-tight container at 4 ºC prior to further use.

Determination of total flavonoids content: The total flavonoids content was determined by the colourimetric method described by Kaijv *et al*.[20] with slightly modifications. 0.3 mL $NaNO_3$ solution (5 %, w/v), 0.6 mL $AlCl_3$ solution (10 %. w/v) and 2 mL NaOH solution (1 mol/L) were added to 1 mL diluted sample solution. The final volume was adjusted to 10.0 mL with deionized water. The mixture was allowed to stand for 5 min. The absorption was measured at 507 nm against the mixture without sample solution as a blank. The content of total flavonoids was expressed as rutin quivalents (mg rutin g^{-1}) through the calibration curve of rutin (A = 9.27c-0.0119, r^2 = 0.9993).

HPLC analysis: The HPLC column was operated at 25 ºC with a flow rate of 0.5 mL min^{-1} and the detection wavelength was 354 nm. Mobile phase A and B were methanol and 0.5 % (v/v) phosphoric acid solution, respectively. The sample injection volume was 10.0 µL. The gradient elution program: 0-15 min, 35-42 % A; 16-40 min, 42 % A.

The sample solution of flavonoids extract and the standard compound solutions of rutin, quercetin and kaempfenol were prepared with methanol and filtered with 0.45 µm microporous film, the filtrates were used directly in chromatographic analysis.

Assay of hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity was assayed by the deoxyribose method[21]. The reaction mixture contained 0.5 mL of 2-deoxyribose (2.8 mmol L^{-1}) in potassium phosphate buffer (50 mmol L^{-1}, pH 7.4), 0.2 mL of premixed ferric chloride (0.1 mmol L^{-1}), 0.1 mL EDTA (0.1 mmol L^{-1}) solution (1:1; v/v), 0.1 mL of H_2O_2 (0.2 mmol L^{-1}) and 0.1 mL sample solution at different concentrations. The reaction was triggered by adding 0.1 mL ascorbate (0.3 mmol L^{-1}) and incubated for 1 h at 37 ºC. The extent of deoxyribose degradation was measured by the thiobarbituric acid method[22]. 1 mL thiobarbituric acid (1 % w/v) and 1 mL trichloroacetic acid (2 % w/v) were added to the mixture and heated in a water bath at 100 ºC for 20 min. The absorbance was measured at 532 nm. The absorbance of the control was determined by replacing the sample solution with ethanol. The percentage scavenging of hydroxyl radical was calculated as follow:

$$\text{Scavenging activity (\%)} = [(A_{517\ (control)} - A_{517\ (sample)}) / A_{517\ (control)}] \times 100$$

Assay of superoxide radical scavenging activity: Superoxide radical scavenging activity was assayed by riboflavin-light-nitro blue tetrazolium chloride monohydrate (NBT) system[23]. 0.5 mL of phosphate buffer (50 mmol L^{-1}, pH 7.4), 0.3 mL riboflavin (20 mmol L^{-1}) and 0.1 mL NBT (0.50 mmol L^{-1}) were added to 1.0 mL sample solution at different concentrations. Reaction was started by illuminating the reaction mixture with a fluorescent lamp. The absorbance was measured at 560 nm after 20 min incubation. The absorbance of the control was determined by replacing the sample solution with ethanol. The percentage scavenging of superoxide radical was calculated by following formula:

$$\text{Scavenging activity (\%)} = [(A_{560\ (control)} - A_{560\ (sample)}) / A_{560\ (control)}] \times 100$$

Assay of' DPPH radical scavenging activity: DPPH radical scavenging activity was measured by the method of Shimada *et al*.,[24]. 1.0 mL DPPH solution (0.1 mmol/L, dissolved in ethanol) was added to 3 mL sample solution at different concentrations. The absorbance was measured at 517 nm after 30 min. The DPPH concentration in the reaction system was calculated from the following calibration curve: Absorbance = 0.0036 × [DPPH] (r^2 = 0.9545). Lower absorbance of the reaction system indicates higher DPPH radical scavenging activity.

Assay of lipid peroxidation inhibition: Lipid peroxidation inhibitiing activity was assayed by the method of Duh *et al*.[25] with slight modifications. 300 mg lecithin was added to 30 mL phosphate buffer (50 mmol L^{-1}, pH 7.4). 0.5 mL and sonicated to dissolve with the ultrasonic cleaner in ice water bath for 2 h, this solution was incubated with 0.5 mL sample solution at different concentrations in the presence of 0.2 mL $FeC1_3$ (1 mmol L^{-1}) and 0.2 mL ascorbic acid (1 mmol L^{-1}) at 37 ºC for 1 h. The reaction was terminated by addition of 500 µL trichloro acetic acid (200 %, w/v) and 500 µL thiobarbituric acid (10 %, w/v) and then the solution was heated at 100 ºC for 15 min. The absorbance of the malondialdehyde (MDA)-TBA complex was measured at 532 2 nm. The absorbance of

the control was determined by replacing the sample solution with ethanol. The percentage inhibiting of lipid peroxidation was calculated as follow:

$$\text{Inhibition } (\%) = [(A_{532\ (control)} - A_{532\ (sample)}) / A_{532\ (control)}] \times 100$$

Reducing power assay: The Fe^{3+} reducing power was determined by the method of Oyaizu[26] with slight modifications. 0.75 mL sample solution at various concentrations was mixed with 0.75 mL of phosphate buffer (0.2 mol L^{-1}, pH 6.6) and 0.75 mL of potassium hexacyanoferrate $[K_3Fe(CN)_6]$ (1.0 %, w/v), followed by incubating at 50 ºC in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of trichloroacetic acid (TCA) solution (10 %) and then centrifuged at 3000 rpm for 10 min. 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride ($FeCl_3$) solution (0.1 %, w/v). After 10 min, the absorbance at 700 nm was measured and as the reducing power. Increasing absorbance at 700 nm of the reaction system indicates the increasing reducing power.

Fe^{2+} chelating activity assay: The determination of chelating activity for ferrous ion (Fe^{2+}) was based on the decrease in the maximal absorbance of Fe^{2+}-ferrozine complex according to previously reported methods[27]. 1.6 mL of deionized water, 0.05 mL $FeCl_2$ (2.0 mmol L^{-1}) and 0.1 mL ferrozine (5 mmol L^{-1}) were added to 0.5 mL of test sample dissolved in ethanol. Ferrozine reacted with the divalent iron to form stable magenta complex species after 10 min at room temperature. The absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The absorbance of the control was determined by replacing the sample solution with ethanol. The chelating activity for Fe^{2+} was calculated as:

$$\text{Chelating activity } (\%) = [(A_{562(control)} - A_{562(sample)}) / A_{562(sample)}] \times 100$$

RESULTS AND DISCUSSION

Content of total flavonoids and HPLC analysis: The standard compound and flavonoids extract were analyzed by HPLC. The peaks of analytes were identified by comparing the retention time of the peaks with those of the standard references eluted under the same HPLC conditions mentioned above and the content of rutin, quercetin and kaempfenol in flavonoids extract was calculated from the corresponding calibration curve (Table-1).

TABLE-1
STANDARD CURVE AND LINEARITY RANGE OF STANDARD REFERENCES

	Calibration equation (r^2)	Linear range (g L^{-1})
Rutin	$Y = 2.2 \times 10^7X+97.2$ (0.9993)	0.041-0.45
Quercetin	$Y = 4.6 \times 10^7X+106.2$ (0.9996)	0.022-0.25
Kaempfenol	$Y = 1.9 \times 10^7X+112.2$ (0.9994)	0.022-0.28

The HPLC chromatograms were shown in Fig. 1, the spectra of compounds **1-3** were found to be completely the same as that of rutin (10.8 min), quercetin (17.7 min) and kaempfenol (22.9 min), respectively, indicating that primary constituents in flavonoids extract might be rutin, quercetin and kaempfenol, which was further confirmed by the test of standard addition.

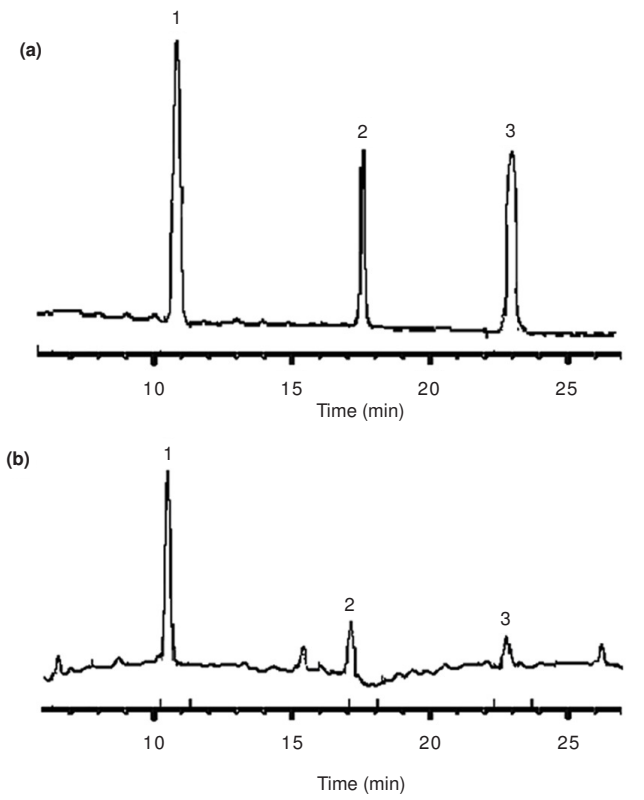

Fig. 1. HPLC chromatogram of standard references (a) and sample (b) 1. rutin, 2. quercetin, 3. kaempfenol

The contents of total flavonoids, rutin, quercetin and kaempfenol were shown in Table-2. Total flavonoids in tartary buckwheat seedling, crude extract and flavonoids extract was 31, 247 and 717.2 mg/g, respectively. The present work showed that flavonoids content in tartary buckwheat seedling (31 mg g^{-1}) was much higher than that of common buckwheat seedling (10.6 mg g^{-1})[28]. Total flavonoids in flavonoids extract was almost three times than that in crude extract, indicating that AB-8 macroporous resin give the best purification efficiency to flavonoids owing to its high surface area, optimum average pore diameter and appropriate surface functional residues[29,30].

HPLC analysis showed that the predominant flavone was found to be rutin amounting to 438.7 mg g^{-1}, followed by quercetin 94.0 mg g^{-1} and kaempfenol 64.9 mg g^{-1} (Table-2).

Antioxidative activity: The antioxidative activity of flavonoids extract, rutin, quercetin and kaempfenol was evaluated by four different assay systems: hydroxyl radical, superoxide radical and DPPH radical scavenging and lipid peroxidation inhibiting. IC_{50} values (The concentration required to scavenge free radical or to inhabitant lipid peroxidation by 50 %) were calculated from the regression equations prepared from the concentration and the percentage scavenging of radical or percentage inhibiting of lipid peroxidation.

The effect of flavonoids extract, rutin, quercetin and kaempfenol on the percentage scavenging of hydroxyl radical, superoxide radical, DPPH radical and the percentage inhibiting of lipid peroxidation was showed in Figs. 2a-d, respectively. The values of IC_{50} were showed in Table-3.

TABLE-2
CONTENTS OF TOTAL FLAVONOIDS, RUTIN, QUERCETIN AND KAEMPFENOL (n = 5)

	Content (mg g^{-1})	RSD (%)	Added (mg g^{-1})	Found (mg g^{-1})	Recovery (%)
Total flavonoids in TBS	31.0	8.3			
Total flavonoids in crude extract	247.0	2.2			
Total flavonoids in flavonoids extract	717.2	3.4			
Rutin in flavonoids extract	438.7	5.1	200.0	714.1	111.8
Quercetin in flavonoids extract	94.0	4.6	100.0	181.0	93.3
Kaempfenol in flavonoids extract	64.9	6.7	100.0	160.6	97.4

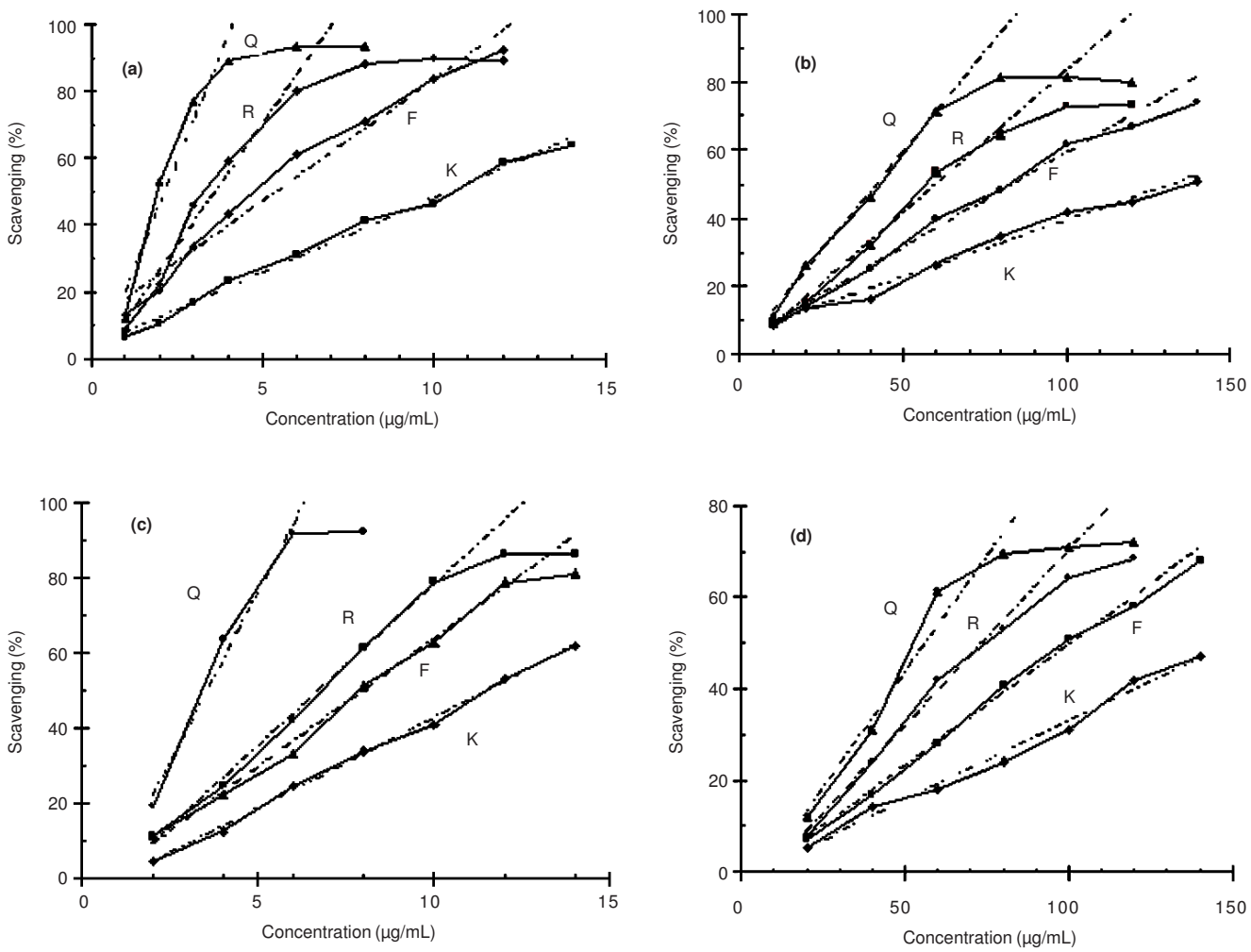

Fig. 2. Effect of flavonoids extract (F), rutin (R), quercetin (Q) and kaempfenol (K) on the % scavenging of hydroxyl radical (a), superoxide radical (b), DPPH radical (c) and the % inhibiting of lipid peroxidation (d). (n = 3)

TABLE-3
IC_{50} VALUES OF FREE RADICAL SCAVENGING OR LIPID PEROXIDATION INHIBITING (μg mL^{-1}) (n=3)

	Hydroxyl radicalg	Superoxide radical	DPPH radical	Lipid peroxidation
Flavonoids extract	5.43	83.64	8.00	100.93
Rutin	3.75	60.23	6.73	73.86
Quercetin	1.76	42.14	3.55	56.53
Kaempfenol	10.43	132.52	11.52	149.52

Among the reactive oxygen species, hydroxyl radical is extremely reactive and highly damaging species in biological system. Fig. 2a shown that hydroxyl radical scavenging activity of flavonoids extract was increased markedly with the increase of concentration of flavonoids extract and exhibited a strong dose effect relation between the concentration and the percentage scavenging to hydroxyl radical, with an IC_{50} value of 5.43 μg mL^{-1} (Table-3).

Compared with hydroxyl radical, superoxide radical is a weak oxidant, but it gives rise to the generation of powerful and dangerous hydroxyl radical as well as single oxygen, both of them contribute to the oxidative stress. Flavonoids extract was found to be an effective scavenger of superoxide radical (Fig. 2b) with an IC_{50} value of 83.64 μg mL^{-1} (Table-3) and also exhibited a strong concentration dependent scavenging to superoxide radical. DPPH, commonly used for the assessment of antioxidant potency *in vitro*, is a stable nitrogen-centered free radical and a foreigner to biological system. Flavonoids extract quenched DPPH radical effectively (Fig.

2c) and the percentage scavenging for DPPH radical was proportional to the concentration of flavonoids extract and the IC_{50} value of flavonoids extract was 8 μg mL^{-1} (Table-3).

Lipid peroxidation plays an important role in oxidative stress in biological system. Furthermore, several toxic byproducts from the peroxidation can damage other bio-molecules. As Fig. 2d and Table-3 shown, flavonoids extract inhibited lipid peroxidation in a dose dependent manner, with an IC_{50} value of 100.93 μg mL^{-1}.

The experiment results suggest that the flavonoids extract of tartary buckwheat seedling is a powerful scavenger of the hydroxyl radical, superoxide radical and DPPH and can inhibit lipid peroxidation effectively (Fig. 2a). Where, rutin, quercetin and kaempfenol also exhibited strong scavenging effect on hydroxyl, superoxide and DPPH radical and inhibiting effect on lipid peroxidation as Figs. 1a-b shown, their scavenging and inhibiting effects were in a dosage effect, similar to flavonoids extract of tartary buckwheat seedling. These results imply that the excellent antioxidative activity of tartary buckwheat seedling can be attributed to its high content of rutin, quercetin and kaempfenol.

Table-3 showed that the IC_{50} value for the scavenging of hydroxyl radical, superoxide radical and DPPH radical decreased in the same order as following: kaempfenol > rutin > quercetin. Meanwhile, the IC_{50} value for the inhibiting of lipid peroxidation was as follows, in decreasing order: kaempfenol > rutin > quercetin. A lower IC_{50} value indicates greater antioxidative activity, the results suggest that quercetin shows the best antioxidant potential, followed by rutin and kaempfenol.

Reducing power: Fig. 3 depicted the reducing power of flavonoids extract, rutin, quercetin and kaempfenol compared with BHA as standards. The reducing power of all samples increased with increasing their concentration. For a dose of 1 mg, the reducing powers (absorbance at 700 nm) of flavonoids extract, rutin, quercetin, kaempfenol and BHA were 2.89, 3.47, 4.36, 1.89 and 3.32, respectively. In general, the reducing power observed as follows, in decreasing order: quercetin > rutin > BHA > flavonoids extract > kaempfenol.

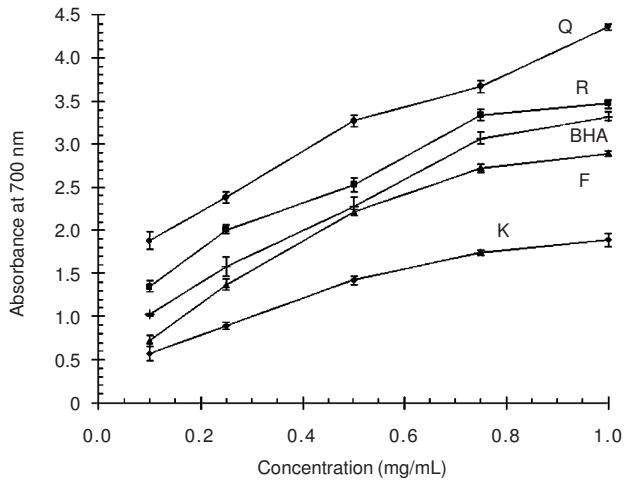

Fig. 3. Reducing power of flavonoids extract(F), rutin (R),quercetin (Q), Kaempfenol (K) and BHA. Each value is the mean ± SD (n = 3)

Reducing power has been reported to be concomitant with antioxidant activity and related to the electron transfer ability[31-34]. Flavone compounds appear to function as good electron and hydrogen atom donors and may serve as a reductone[24,35]. It exerts antioxidant activity by converting free radicals into more stable products and terminating the free radical chain reaction. Therefore, the antioxidant activity of flavonoids extract might mainly be the result of its reducing power.

Chelating ability for ferrous ion: Ferrous ion can generate hydroxyl radical through Fenton reaction, which may induce lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. It was reported that chelating agents of ferrous ion can reduce the redox potential and stabilize the oxidized form of ferrous, thereby reducing the concentration of ferrous ion in lipid peroxidation[25], they are effective as secondary antioxidants[36]. So, ferrous ion chelating ability is significant in antioxidative activity assay.

The chelating activity of flavonoids extract for ferrous ion was assayed by inhibiting of the formation of red-coloured ferrozine and ferrous complex. Fig. 4 shows the chelating ability of flavonoids extract, rutin, quercetin and kaempfenol, compared with EDTA as standard. The percentage of chelating ability of flavonoids extract, rutin, quercetin, kaempfeno and EDTA for 100 μg/mL ferrous ion were found as 42.0, 50.2, 62.4, 25.4 and 97.4 %, respectively, indicating that flavonoids extract is an effective ferrous iron chelator, which could be also attributed to its high content of flavones compounds, such as rutin, quercetin and kaempfenol.

The antioxidant effect of polyphenols compound such as flavonoids has been correlated with their iron chelating properties[37]. Fig. 4 revealed that flavonoids extract demonstrated the ability for iron binding though to a smaller extent compared to EDTA, implying that its action as peroxidation protector may be partially related to its iron binding capacity.

Conclusion

The determination of total flavonoids and HPLC analysis show that flavone compounds, such as rutin, quercetin and kaempfenol, are the primary characteristic compound of tartary buckwheat seedling. The extract and flavone compounds of tartary buckwheat seedling exhibit strong antioxidant activity, they are effectively free radical scavenger and inhibitor of lipid peroxidantion, which could be related to the reducing power and metal iron chelating activity of flavone compounds in the extract of tartary buckwheat seedling.

Studies were developed concerning the bioactivity of buckwheat, indicating that buckwheat extract has a strong antioxidative activity. But relevant studies on tartary buckwheat seedling are still limited, more detailed chemical and pharmacological understanding of tartary buckwheat seedling is necessary. This work has gathered experimental evidences on the great potential of tartary buckwheat seedling as an easily accessible health food, natured antioxidant and excellent dietary source of bioactive compounds for its high content of flavonoids, low cost and very short cultivated period.

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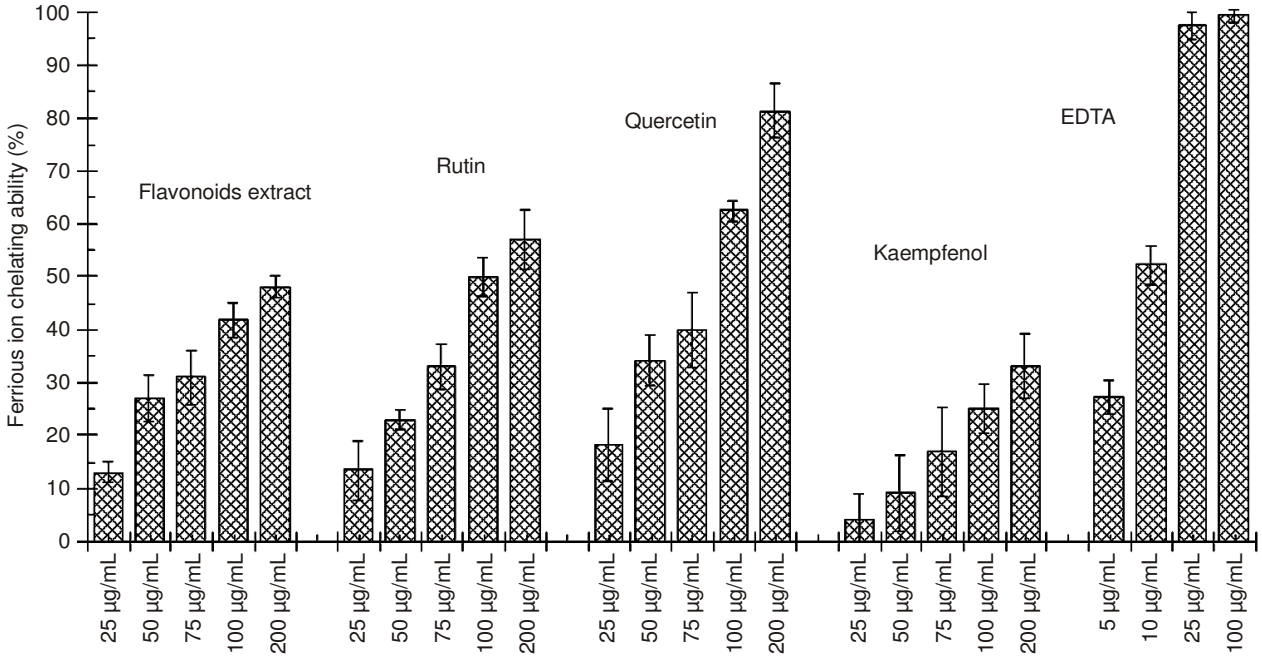

Fig. 4. Ferrous ion chelating ability (%) of flavonoids extract, rutin, quercetin, Kaempfenol and EDTA. Each value is the mean ± SD (n = 3)

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