

**Evaluation of anti-oxidant capacity of root of *Scutellaria baicalensis* Georgi, in
comparison with roots of *Polygonum multiflorum* Thunb and *Panax ginseng* CA Meyer**

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Running title: *Radix Scutellaria baicalensis* Georgi's anti-oxidant capacity

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Abstract: In Chinese communities, regular consumption of Chinese-medicated diets (CMD) (usually in the form of soup) is a traditional practice to promote health and prevent diseases development. The overall improvement of health conditions is believed to be correlated with the anti-oxidant potentials of these herbs. Huangqin, roots of *Scutellaria baicalensis* Georgi (Lamiaceae), is one of the herbs commonly used in CMD. In this study, the anti-oxidant capacities of Huangqin extracts (water, ethanol and ether extracts) were evaluated and compared with commonly used CMD herbs, Heshouwu, roots of *Polygonum multiflorum* Thunb (Polygonaceae) and Renshen (or Ginseng), roots of *Panax ginseng* CA Meyer (Araliaceae). The anti-oxidant capacities were measured by using both cell-free assay [ferric reducing/anti-oxidant power (FRAP)] and biological methods [2,2'-azobis-(2-amidinopropane) (AAPH)-induced haemolysis assay and H₂O₂-induced cell damage on H9C2 cells]. Additionally, the total phenolic content was measured using Folin-Ciocalteu methods. Water extract of Huangqin was found to have the highest anti-oxidant activities compared with the ethanol and ether extracts evaluated. A positive relationship between the anti-oxidant effects and total phenolic contents of extracts was demonstrated. This shows that Huangqin could be an effective dietary anti-oxidant that can be consumed regularly as a functional food for the prevention of oxidants/free radicals-related diseases.

Keywords: Chinese-medicated diet; anti-oxidant activity; ferric reducing/anti-oxidant power (FRAP); total phenol content; *Scutellaria baicalensis* Georgi; *Polygonum multiflorum* Thunb; *Panax ginseng* CA Meyer

Introduction

Mammals are constantly subjected to various oxidative stress challenges. Oxidative stress occurs when the generation of reactive oxygen species (ROS) overwhelms the cellular anti-oxidant defences (Duarte and Jones, 2007; Sies, 1997). In fact, aging and chronic disease development are believed to be related to the damaging effects of ROS (Beckman and Ames, 1998; Loeb et al., 2005).

In the context of Traditional Chinese Medicine (TCM), functional foods and medicines are believed to have similar origins but of different uses. A regular consumption of Chinese-medicated diets (CMD) is considered as an essential part of TCM practices to improve/enhance human health and to prevent or even cure diseases (Guo et al., 2008). It is important to note that the dosage of a medicinal herb depends on its intended use. In CMD a low dose is used while a higher dose will be prescribed for therapeutic purposes. Many medicinal herbs contain a large quantity of isoflavones which possess anti-oxidant properties (Liao et al., 2008). A high anti-oxidant potential of these herbs is widely accepted by the general public, as there is a common belief that regular consumption of natural food/herbs

with high anti-oxidant power can help to slow down the aging process as well as the progression of chronic diseases. It has been suggested that the beneficial effects of CMD are mainly attributed to their anti-oxidant effects (Ohkoshi et al., 2009).

Huangqin, the roots of *Scutellaria baicalensis* Georgi (Lamiaceae), is widely included in the prescriptions for some chronic diseases such as cancer, hepatitis and hypertension (Li et al., 2004). It is a relatively economical herb and commonly used in CMD. So far, the anti-oxidant property of Huangqin has not been evaluated systematic by both chemical and biological methods. In fact, for the complicated composition of medicinal herbs, in this study more than one assays were used to give a complete anti-oxidant profile of Huangqin.

In this study, the anti-oxidant capacity of Huangqin extracts obtained using water, ethanol or ether were investigated and compared using chemical and biological methods. The total phenolic content of different extracts was measured as well. For comparsion, two well-knwon but expensive CMD herbs, Heshouwu (roots of *Polygonum multiflorum* Thunb (Polygonaceae)) and Renshen (also known as Ginseng, roots of *Panax ginseng* CA Meyer (Araliaceae)) were also evaluated. Since these two herbs are commonly used for anti-aging purpose and believed to have high antioxidant level (Choi, 2008; Zhong, 2009).

Materials and Methods

Reagents

2,4,6,-tripyridyl-s-triazine (TPTZ) (Fluka, Switzerland), Folin-Ciocalteu's reagent (BDH, United Kingdom), gallic acid (Advanced Technology and Industrial Co. Ltd, Hong Kong), ascorbic acid (Panreac Quimica SA, Spain) and butylated hydroxytoluene (BHT) (Acros Organics, USA). Celltiter 96 aqueous MTS reagent powder was obtained from Promega Chemicals Co. (USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were obtained from Gibco (USA). Folin-Ciocalteu phenol reagents were obtained from Merck (Germany). All other reagents were of analytical grade (Lab-Scan analytical Sciences, Thailand).

Extraction procedures

All herbs (listed in Table 1) used in this study were provided by the Laboratory of Traditional Chinese Medicine of the Institute of Modern Chinese Medicine, Institute of Materia Medica of The Hong Kong Polytechnic University. The voucher specimens were stored in dark at room temperature (~20°C) in air-tight plastic bags in Dr. Peter H.F. Yu's laboratory (Department of Applied Biology and Chemical Technology, the Hong Kong Polytechnic University).

Herbs were cut into small pieces, freeze-dried (24-48 hr), weighed and grounded into fine powder using a blender. Powders were passed through 12-mesh sieve before subjecting to extraction procedures using different solvents (water, ether or ethanol), as illustrated in

Figure 1. For each sample, powder (1 g) was extracted (3 times) with each solvent (20 mL) by gentle shaking (2 hr in dark) (260 rpm at 37°C). Then, the mixture was centrifuged (2735 ×g, 10 min) and the supernatants obtained from three extractions (using the same solvent) were pooled together before freeze dried (for water extracts) or in rotary evaporator (at 60°C) (for ether and ethanol extracts). Extracts obtained were stored in desiccators (in dark, 4°C) before commencing the assays. In the chemical assays, the extracts were re-dissolved (4 mL) in the corresponding extraction solvent. In the biological tests, extracts were re-dissolved (4 mL) in phosphate buffered saline (PBS) with 1 % (v/v) dimethyl sulfoxide. Solutions of ascorbic acid and gallic acid (served as control and standard, respectively) were dissolved in distilled water.

Total phenolic content assay

The total phenolic content in the herbal extracts was measured using the Folin-Ciocalteu method (Singleton et al., 1999). Total phenolic content of each extract was expressed as gallic acid equivalents (GAE) per gram of herb used.

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay is a direct measurement of the “total anti-oxidant power” of a substance which employs anti-oxidants as reductants in redox-linked colorimetric reactions (Benzie and

Strain, 1996). Details of the modified procedures were reported previously by our group (Chan et al., 2008).

Inhibition of AAPH-induced haemolysis

The water-soluble azo compound, AAPH (Cayman, USA), is a free radical generator. Free radicals generated by AAPH attack polyunsaturated fatty acids of the red blood cell (RBC) membrane and cause haemolysis. In this study, heparinized whole blood (from pig) was collected, centrifuged (625 ×g, 10 min) and the RBC pellet obtained was washed three times with PBS (composition: 137 mmol/L NaCl; 2.7 mmol/L KCl; 8.1 mmol/L Na₂HPO₄; 1.5 mmol/L KH₂PO₄). The pellet was re-suspended into a suspension (20 % v/v), and 600 µL of RBC suspension was mixed with 600 µL extract. Then, the mixture was incubated (rolling, 22 rpm) (37°C) for 10 min before adding 300 µL AAPH (400 mM, in PBS) into the medium (37°C, rolled at 22 rpm). After 2 hr incubation, 100 µL of the mixture was added into ice-cold PBS (1.25 mL) or water (1.25 mL, to induce complete RBC haemolysis i.e. 100% haemolysis), and centrifuged (385 ×g, 10 min). In controls, all procedures performed were the same except the sample (600 µL) and AAPH (300 µL) in PBS were replaced with PBS (900 µL). In AAPH group, sample (600 µL) was replaced with PBS. The absorbance of supernatant was measured (540 nm) using a micro-plate reader (Bio-Rad, USA). Percentage

haemolysis = $\frac{Abs_{Sample}}{Abs_{Water}} \times 100\%$, where Abs_{Sample} is the absorbance of the resultant supernatant

from extract-treated RBC whereas $\text{Abs}_{\text{Water}}$ is the absorbance of the resultant supernatant from water-treated RBC (100% haemolysis means all RBC were lysed).

Protective effects of herbal extract on H_2O_2 -induced damage of H9C2 cells

The anti-oxidant effects of different extracts were evaluated by measuring the ability of H9C2 cells to resist H_2O_2 -induced cell damage. H9C2 cells were maintained in low glucose (5.5 mM) DMEM supplemented with FBS (10%, v/v), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) in culture plate at 37°C in a humidified incubator (5% CO_2). Cells were fed every 2 days and sub-cultured once they reached 70-80% confluence. On day 1, 3,000 cells were seeded per well of 96-well plates (Falcon). On day 2, cells were treated with different extracts or vehicle for 48 hr at 37°C in a humidified incubator (5% CO_2). Then, each well was further incubated with fresh medium plus H_2O_2 (5.5 mM) for 24 hr. At the end of incubation, the culture medium was changed with fresh medium and MTS solution (10 μl) was added and incubated for another 2 hr. Results were expressed as the percentage growth inhibition with respect to untreated cells (controls).

Statistical analysis

Data were expressed as means \pm standard error of mean (S.E.M.), and n denotes the number of replications for each data point. Comparison of parameters among different groups was

made with one-way analysis of variance, followed by Newman-Keul's test for multiple comparisons among means. Correlation analysis was performed using linear regression and the correlation coefficient (r) was determined. Statistical significance of r was evaluated using *t* test by giving a null hypothesis (H_0) of $r = 0$. If H_0 holds, $t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$ approximately follows the *t* distribution with degrees of freedom equal to $n-2$, where *n* represents the sample size. In all cases, $p < 0.05$ means that there is a significant difference/relationship between two groups/variables. All statistical analysis tests were performed using GraphPad Prism 4.02 for Windows (GraphPad Software, USA).

Results and discussion

Total phenol content

In this study, CMD herbs (Huangqin, Hesouwu and Renshen) were extracted using three common solvents: water, ethanol and ether. In this study, Folin-Ciocalteu method (Cespedes et al., 2008) was employed to estimate the phenolic components present in herbal extracts. In Huangqin, a comparable amount of total phenolic content was detected, irrespective of the extraction solvent used (water, 3.85 ± 0.07 mg GAE/g; ethanol, 3.65 ± 0.07 mg GAE/g; ether, 3.45 ± 0.06 mg GAE/g) (Figure 2). A similar amount of total phenolic content was detected in water extracts of Heshouwu and Renshen, and the total phenolic content measured in ethanol and ether extracts of Heshouwu and Renshen was only 8-13 % of that detected in water

extract of the respective herb (Figure 2). Nonetheless, the sum of total phenolic content obtained from three extraction methods was highest in Huangqin (Figure 2).

Ferric reducing/antioxidant power assay

The total anti-oxidant capacities of the herbal extracts were determined and compared using FRAP methods. The FRAP methods measure the anti-oxidant effects/reducing ability of a substance present in the medium. It has been successfully utilized to measure anti-oxidants present in different samples such as plasma and botanicals (Guo et al., 2008; Koutelidakis et al., 2009). In this study, water extract of Huangqin (0.25 g herb/ml) was found to have the highest FRAP value ($71933.34 \pm 3355.39 \mu\text{mol/g}$) as compared with the water extracts of two herbs evaluated (Heshouwu (0.25 g herb/ml), $41650.00 \pm 6677.58 \mu\text{mol/g}$; Renshen (0.25 g herb/ml), $1624.33 \pm 81.56 \mu\text{mol/g}$) (Figure 3). Our results demonstrated that extract obtained from water (the most polar) extraction has the highest FRAP value followed by ethanol (less polar than water) extract and the lowest FRAP was detected in extract obtained using ether (the least polar solvent) extraction methods. In addition, it is important to point out that the anti-oxidant potential of ascorbic acid (0.1 $\mu\text{g/ml}$, a commonly used anti-oxidant) was only ~16% of the water extract of Huangqin (Figure 3) which is similar to the ether extract of Huangqin. Thus, our results strongly suggest that water extract of Hunagqin could be utilized as a powerful herbal-origin anti-oxidant. More importantly, our recent study has shown that

there is a positive correlation between anti-oxidant properties and anti-cancer activities of herbal extracts of TCM formulae with reported anti-cancer therapeutic effects (Li et al., 2007). Hence, it is tempting to suggest that a regular consumption of Huangqin (e.g. Huangqin-containing soup) can provide beneficial anti-oxidant properties which may be useful in retarding the progression of and/or even treating ROS-related diseases.

As discussed above, Huangqin extract contains a greater total sum of phenolic compounds than those in Heshouwu and Renshen. The major polyhydroxy phenols of Huangqin are baicalin, baicalein and wogonin (Wang et al., 2007). In addition, phenolic compounds possess diverse biological activities such as anti-carcinogenic and anti-atherosclerotic activities (Guo et al., 2008). These therapeutic effects are probably related to their anti-oxidant activities of polyhydroxy phenols. In fact, electron paramagnetic resonance study clearly demonstrated that baicalin and baicalein, but not wogonin, scavenged hydroxyl radicals, DPPH radicals and alkyl radicals (Hamada et al., 1993). Thus, it is tempting to speculate that flavonoids (baicalin and baicalein) are probably associated with the anti-oxidant activities of Huangqin extract. Compare with Heshouwu and Renshen, Huangqin could be a promising candidate for providing herb-origin antioxidants as extracts of Huangqin containing a higher amount of total phenolic components (Figure 2). However, the discrepancy between the FRAP value and the total phenolic content of Huangqin need to be determined. By the same token, we cannot rule out the possibility that some phenolic

compounds extracted may not possess anti-oxidant properties.

Inhibition of AAPH-induced haemolysis

It is agreed that the complicated composition of medicinal herbs makes it impossible to estimate the anti-oxidant potency of individual sample with a single analytical method (Ferreira et al., 2009; Koleva et al., 2002). Thus, different methods have been developed for measuring the antioxidant activity of natural products. In addition to the aforementioned cell-free methods, biological tests were adopted to evaluate the protective effects, if any, of extracts of Huangqin, Heshouwu and Renshen against AAPH-induced haemolysis. It is well documented that AAPH-induced haemolysis assay is an excellent and reliable method (Takebayashi et al., 2007). In this assay, AAPH decomposes and generates alkyl radicals which are converted to highly reactive peroxy radicals and cause haemolysis (Banerjee et al., 2008; Savitha et al., 2005).

In this assay, the effects of different herbal extracts on the rate of haemolysis were evaluated (Figure 4). Without AAPH, a negligible haemolysis response (close to 0%) was observed. However, addition of AAPH to RBC suspension resulted in ~80% haemolysis after 120 min incubation, and a 100% haemolysis was achieved at 180 min (Figure 4A). Our results clearly demonstrated that irrespective of the extraction solvents used, Huangqin (0.5 g herb/ml) ameliorated AAPH-evoked RBC haemolysis (Figure 4B-D). In contrast, water

extract of Heshouwu demonstrated a greater inhibition of AAPH-induced haemolysis as compared with extracts obtained using ethanol and ether (Figure 4B), both of which caused a similar magnitude of inhibition of AAPH-elicited RBC haemolysis (Figure 4C). On the other hand, extracts of Renshen demonstrated differential inhibition effects on AAPH-induced haemolysis. Our results illustrate that the water extract of Renshen possesses the greatest inhibition which is followed by the ethanol extract, and the ether extract was the least effective (Figure 4D). Taken together, our results suggest that the water-soluble anti-oxidants found in CMD herbs (especially Huangqin) are effective scavengers of peroxy radicals by directly inhibiting haemolysis.

Protective effects on H₂O₂-induced H9C2 cell damage

The protective effects of different extracts of a particular “organ-like” system was also evaluated by using H9C2 cardiomyoblasts as our model which is a clonal cell-line derived from embryonic heart ventricle (Kimes and Brandt, 1976) and retains physiological properties of adult cardiomyocytes. Under oxidative stress conditions, H9C2 cells respond in a similar manner to myocytes in primary cultures or isolated heart preparations (Su et al., 1999). Thus, any protective effects offered by the herbal extracts in H9C2 cells reflect the therapeutic efficacy in a more complex and diverse cell populations such as myocardium.

In this study, both water and ethanol extracts of Huangqin demonstrated the greatest

protective effects in preventing H₂O₂-induced H9C2 cell damage (i.e. a higher cell viability was recorded) (Table 2). Although it is relatively smaller in magnitude, the ether extract of Huangqiu also possesses a certain degree of cell protecting effects (Table 2). In contrast, only the water and ethanol extracts, but not the ether extract, of Heshouwu managed to provide protective effects against H₂O₂-induced loss of H9C2 cell viability (Table 2). However, none of the Renshen extracts managed to protect H9C2 cells from H₂O₂-mediated loss of cell viability.

In the H₂O₂-induced H9C2 cell damage assay, various extracts were added into the culture media for 48 hr and removed before being incubated in H₂O₂ (24 hr). Thus, the observed protective effect of the extracts can be considered as the ability to enhance H9C2 cells' ability to handle oxidative stress. In the case of the AAPH-induced haemolysis, CMD herbal extracts were present throughout the experimental period so the protective effect should be the combined effects of the extracts on enhancing RBCs' oxidative stress handling power and chemically removing free radicals. Therefore, the discrepancy of cellular protective effects of different extracts of individual herb as well as among different herbs examined can be explained by the different working principles of two assays.

Correlation between the antioxidant capacity and total phenolic content

Phenolic compounds are major antioxidants found in plants and medicinal herbs (Larson,

1988). We therefore tested a hypothesis that there is a correlation between the anti-oxidant effects and total phenolic content. Scatter plots analysis of anti-oxidant activities and total phenolic contents were performed (Figure 5). Interestingly, there was a significant positive correlation between the FRAP value and the total phenolic content ($r = 0.7183$, $p < 0.001$) (Figure 5A). Thus, our results were in line with previous studies in which the phenolic compounds play a significant role in providing anti-oxidant capacities in medicinal herbs (Aljadi and Kamaruddin, 2004; Li et al., 2008). More importantly, to the best of our knowledge, the present study is the first study demonstrating a positive relationship between the ability of herbal extract to resist AAPH-induced haemolysis and the total phenolic content ($r = 0.9750$, $p < 0.001$) (Figure 5B) as well as a significant correlation between the maintenance of cell (H9C2 cells) viability in response to H_2O_2 challenge and the total phenolic contents of the herbal extract ($r = 0.7974$, $p < 0.001$) (Figure 5C). Hence, our results strongly suggest that phenolic compounds found in herbs play a crucial role in protecting cells by counteracting the damaging/detrimental effects of ROS which are important in aging as well as the development of diseases.

Conclusion

In this study, three common medicinal herbs Huangqin, Heshouwu and Renshen were evaluated. Water extract of Huangqin was demonstrated to have the highest anti-oxidant

activities as estimated using cell-free chemical methods and cell-based biological assays. The anti-oxidant and cellular protective effects of the water extract of Huangqin are related to highest levels of phenolic components measured. Thus, Huangqin can be considered as an economical natural source of dietary antioxidant which can be used in the prevention of diseases caused by free radicals generation *in vivo*. However, additional analytical and biochemical investigations are needed to identify the active components existing in the water extract of Huangqin.

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Figure legends:

Figure 1. Extraction sequences of dried Chinese medicinal herbs.

Figure 2. Folin-Ciocalteu analysis of the herbal extracts. Data are expressed as means \pm S.E.M., n = 3. A: water extract, B: ethanol extract, C: ether extract, GAE: gallic acid equivalents.

Figure 3. FRAP (ferric reducing/anti-oxidant power) assay of the herbal extracts. Data are expressed as means \pm S.E.M., n = 3. A: water extract, B: ethanol extract, C: ether extract, D: ascorbic acid.

Figure 4. Comparisons of AAPH-induced RBC haemolysis of control (A), water (B), ethanol (C) and ether (D) herbal extracts. Data are expressed as means \pm S.E.M., n = 3.

Figure 5. Correlation of the anti-oxidant capacity and total phenolic contents of medicinal herbs. Antioxidant capacities were measured by FRAP assay (A), ability to resist AAPH-induced haemolysis (B) and cell viability of H9C2 cell under H₂O₂ challenge (C), respectively. GAE: gallic acid equivalents.

Tables

Table 1

List of medicinal herbs used in this study.

Chinese name	Scientific name	Family	Part of the herb used	Voucher number
Huangqin	<i>Scutellaria</i> <i>baicalensis</i> Georgi	Lamiaceae	Root	CW07-1
Heshouwu	<i>Polygonum</i> <i>multiflorum</i> Thunb	Polygonaceae	Root	CW07-2
Renshen	<i>Panax ginseng</i> CA Meyer	Araliaceae	Root	CW07-3

Table 2

Protective effects of water, ethanol and ether extracts of different herbs on H₂O₂-induced H9C2 cells damage.

Samples		Cell viability (%)
Control	- H ₂ O ₂	96.67 ± 7.26***
	+ H ₂ O ₂	40.33 ± 5.93
Huangqin	Water extract	92.33 ± 2.19***
	Ethanol extract	90.67 ± 1.76***
	Ether extract	67.00 ± 2.08**
Heshouwu	Water extract	75.33 ± 4.48***
	Ethanol extract	63.33 ± 2.40*
	Ether extract	58.67 ± 2.40
Renshen	Water extract	57.33 ± 4.84
	Ethanol extract	41.67 ± 4.81
	Ether extract	40.67 ± 4.26

Data are expressed as means ± S.E.M., n = 3. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 compared to the Control (+ H₂O₂) group.

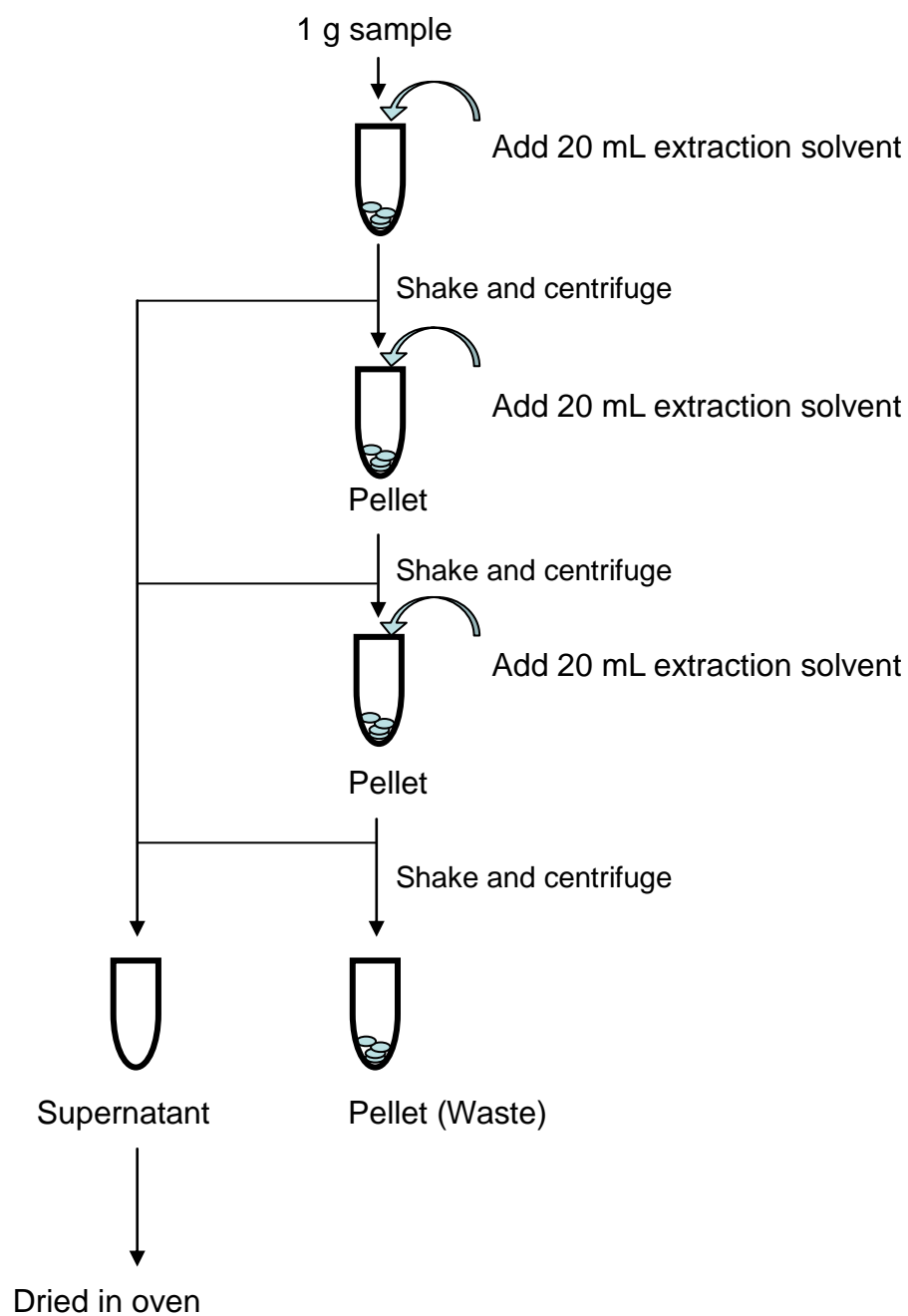


Figure 1.

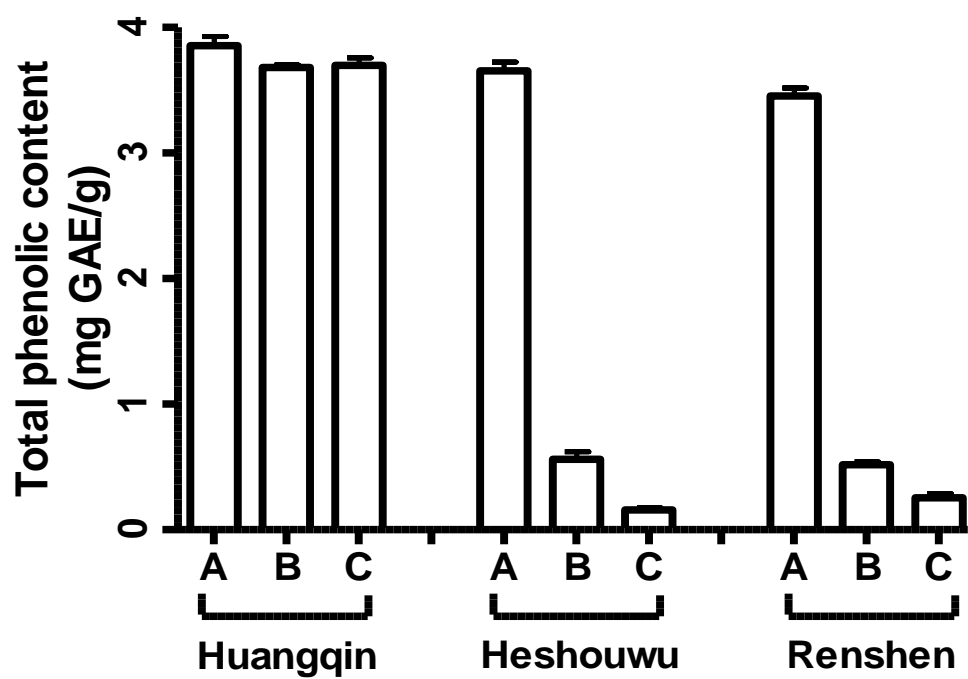


Figure 2.

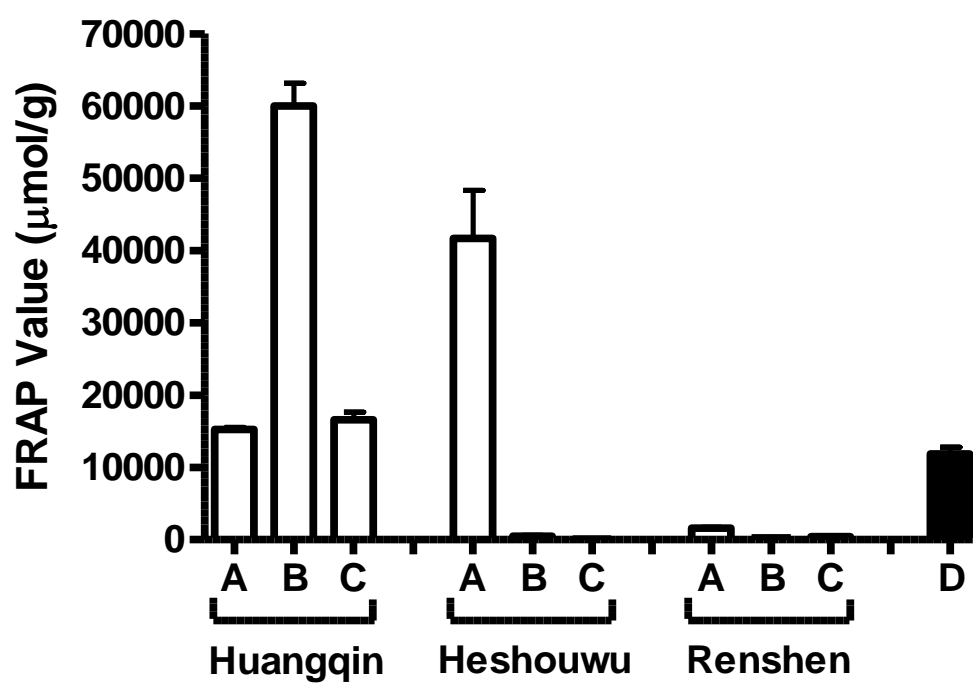


Figure 3.

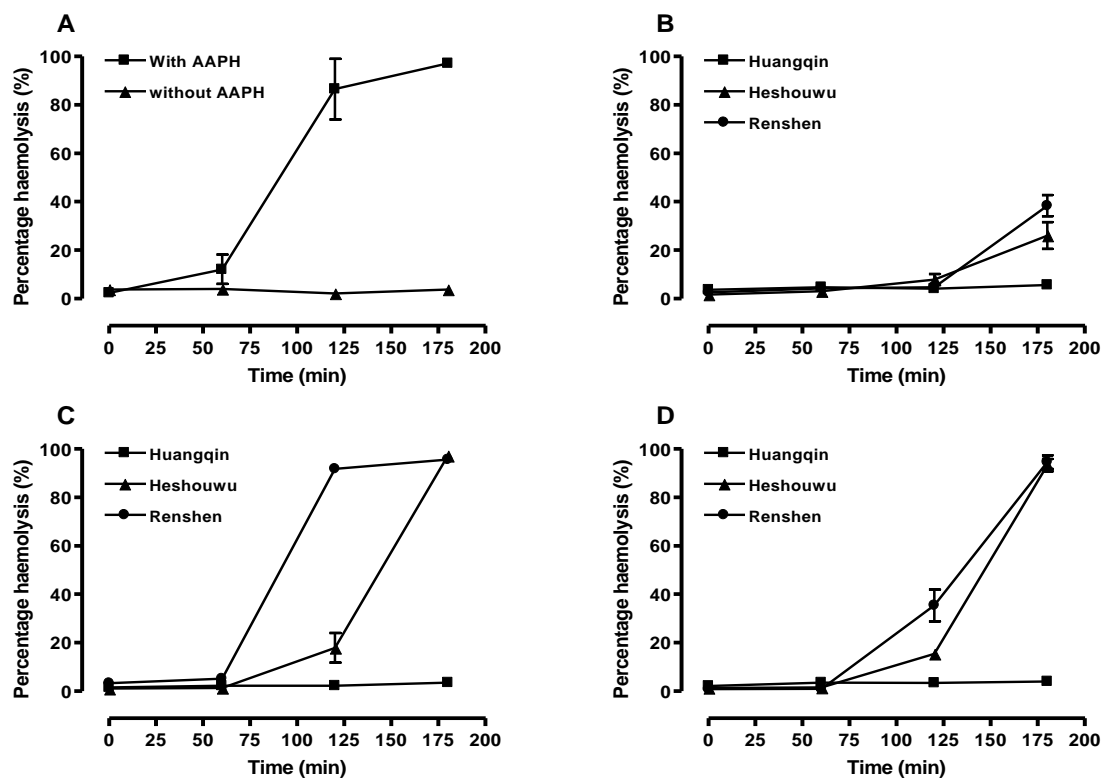


Figure 4.

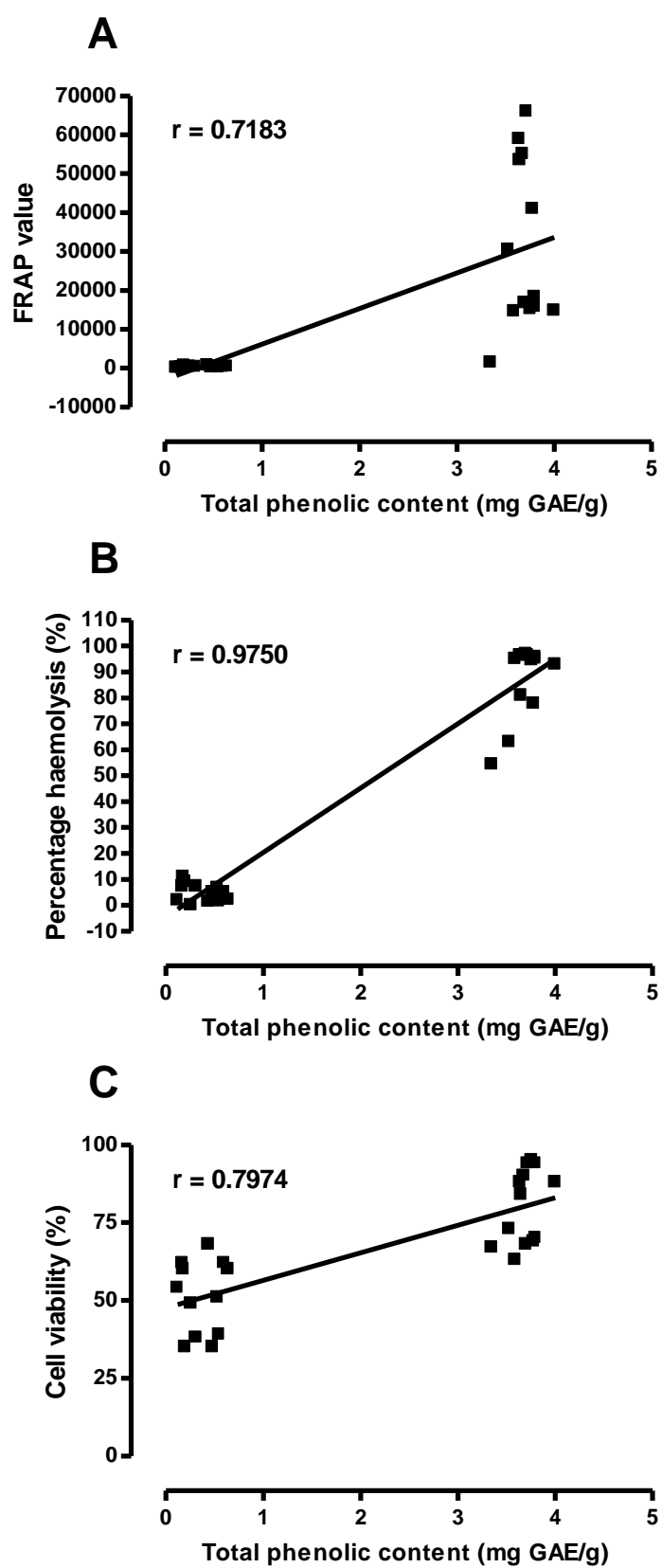


Figure 5.