Investigation of Antioxidant Interactions between Radix Astragali and Cimicifuga foetida and Identification of Synergistic Antioxidant Compounds

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Abstract

The medicinal plants of Huang qi (Radix Astragali) and Sheng ma (Cimicifuga foetida) demonstrate significantly better antioxidant effects when used in combination than when used alone. However, the bioactive components and interactional mechanism underlying this synergistic action are still not well understood. In the present study, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was employed to investigate the antioxidant capacity of single herbs and their combination with the purpose of screening synergistic antioxidant compounds from them. Chromatographic isolation was performed on silica gel, Sephadex LH-20 columns and HPLC, and consequently to yield formononetin, calycosin, ferulic acid and isoforulic acid, which were identified by their retention time, UV λmax MS and MS/MS data. The combination of isoforulic acid and calycosin at a dose ratio of 1:1 resulted in significant synergy in scavenging DPPH radicals and ferric reducing antioxidant power (FRAP) assay. Furthermore, the protective effects of these four potential synergistic compounds were examined using H2O2-induced HepG2 Cells bioassay. Results revealed that the similar synergy was observed in the combination of isoforulic acid and calycosin. These findings might provide some theoretical basis for the purported synergistic efficiency of Huang qi and Sheng ma as functional foods, dietary supplements and medicinal drugs.

Introduction

Traditional herbs in Asia are widely applied to medical treatments and as dietary supplements. Many of these edible and medicinal plants are excellent source of phytochemicals, such as phenolic acids and flavonoids, which have more potent antioxidant activity than common dietary [1,2]. However, accumulated evidence show that an individual isolated ingredient commonly exhibited poorer antioxidant activity than a whole or partially purified extract of a plant [3,4], suggesting that the isolation and purification of phytochemicals might lead to the reduction of their antioxidant ability. Thus, synergistic interactions between phytochemicals are of vital importance for the whole antioxidant efficacy of a plant.

Free radical scavenging effect is one kind of the most important antioxidant abilities of phytochemicals. Various free radicals that occurred in body can cause damage to cells, injure the immune system and lead to a series of chronic degenerative diseases such as heart disease, Alzheimer’s disease and cancer [5]. Thus, the study on synergistic interactions between phytochemicals which possess free radical scavenging ability tends to get more and more attention. Alpha-tocopherol was found to show potent synergistic action in scavenging radicals when used in combination with proanthocyanidins [6] and quercetin [7]. The study on the scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and β-carotene against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals revealed that the combination of lycopene, vitamin E and vitamin C resulted in significant synergism in the three-component system [8]. Hugo et al. found a synergistic effect in the majority of the all combinations of phenolics (chlorogenic, gallic acid, protocatechuic and vanillic acid) using the DPPH assay [9]. However, most of these present researches commonly confined to some specific compounds, especially the vitamin C and vitamin E, the synergistic interactions between phenolics were rarely reported.

Traditional herbs are commonly used in the form of combination. Herb pair is a basic unit of multi-herb recipes [10], which consists of two single herbs and presents significantly better pharmacological efficacy than individual herbs [11]. Practitioners always believed that synergistic interactions between the components of herbs greatly contribute to the enhancement of their therapeutic efficacy. And they also have made great efforts in screening of active compounds from various herbs. However, the target object of these researches was generally one kind of plant, few studies kept a watchful eye on the screening and identification of synergistic compounds from two or more herbs. Huang qi (Radix Astragali) is the root of Astragalus membranaceus (Fisch.) Bunge and/or A. membranaceus var. mongolicus (Bunge) Hsiao. It is commonly used as food supplement on the western market and as diuretics in East Asia [12]. Sheng ma (rhizome of Cimicifuga

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*foetida* L.) has been widely applied as a cooling and detoxifying agent and for alleviation of fever, pain, and inflammation [13]. The herb pair of Huang-qi and Sheng-ma is one of the most widely used combinations in traditional Chinese medicine. Our previous study (unpublished data) has found that the ethanol extract of Huang-qi and Sheng-ma exhibited synergistic antioxidant activity when used in combination. In the present study, the synergistic antioxidant compounds from these two edible and medicinal plants were investigated, which may provide some theoretical basis for the purported synergistic efficiency of Huang-qi and Sheng-ma as functional foods, dietary supplements and medicinal drugs.

Figure 1. The antioxidant abilities of AME and CFO and different solvent-extracted fractions. (A): DPPH free radical scavenging ability of AME and four solvent-extracted fractions; (B): DPPH free radical scavenging ability of CFO and four solvent-extracted fractions; (C): ABTS free radical scavenging ability of AME and four solvent-extracted fractions; (D): ABTS free radical scavenging ability of CFO and four solvent-extracted fractions; (E): FRAP value of AME and four solvent-extracted fractions; (F): FRAP value of CFO and four solvent-extracted fractions.

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Materials and Methods

Materials

The herb plant materials of Huang-qi (Astragalus membranaceus var. mongholicus (Bunge) Hsiao) (AME) and Sheng-ma (rhizome of Cimicifuga foetida L.) (CFO) were purchased from the Shijiazhuang Pharmaceutical Group of China and authenticated by Professor Lingchuan Xu, Shandong University of Traditional Chinese Medicine, P. R. China.

Chemicals

Folin-Ciocalteu’s phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ) and 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonicacid) (ABTS) were purchased from Sigma (St. Louis, MO); Calycosin (purity, ≥98%), formononetin (purity, ≥98%) were purchased from Mairier company (Shanghai, China); Ferulic acid (purity, ≥98%) and isoforulic acid (purity, ≥98%) were purchased from Yuanye Company (Shanghai, China); Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s minimum essential medium (DMEM) and Trypsin-EDTA (0.25% trypsin with EDTA-4Na) were purchased from Gibco (Grand Island, NY); The mixture of penicillin and streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Solarbio (Beijing, China); HPLC-grade acetonitrile was obtained from Fisher Scientific (Walham, MA); Water was purified using a Milli-Q system from Millipore (Bedford, MA).

Determination of Antioxidant Activity

**DPPH free radical scavenging assay.** The DPPH radical scavenging assay used in this study was slightly modified on the basis of previous reports [14,15]. Briefly, 0.1 mL of samples of different concentrations was added to 3.9 mL of DPPH solution in ethanol (0.1 mmol/L) and mixed immediately. After reacting at 37°C for 60 min, the absorbance was measured at 517 nm, and the antioxidant capability (AA) was expressed as the percentage of DPPH+ reduced, which was calculated with the following formula:

\[
AA_{DPPH} = \left(1 - \frac{A_S}{A_B}\right) \times 100
\]

Where \( A_S \) was the absorbance of the DPPH solution after reacting with the sample at a given concentration and \( A_B \) was the absorbance of the DPPH+ solution with an ethanol blank instead of the sample. The percentage of DPPH+ reduced was plotted against the concentration of each sample, and an IC50 value, which is defined as the concentration of the sample needed to scavenge 50% of the DPPH+, was calculated from the graph.

**ABTS free radical scavenging assay.** The ABTS free radical scavenging assay was based on previous method with a few of modifications [16]. Potassium persulfate was added into 7 mmol/L of ABTS+ and kept for 12–16 h at room temperature in the dark environment. Then the ABTS+ solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm before analysis. 0.1 mL of samples of different concentrations was added to 3.9 mL of ABTS+ solution in ethanol and mixed immediately. The absorbance of the mixture was measured at 734 nm after reaction for 15 min at room temperature, and the antioxidant capability (AA) was expressed as the percentage of the reduced ABTS+. The calculation method is similar to DPPH free radical scavenging assay.

**Ferric Reducing Antioxidant Power (FRAP) assay.** The FRAP working solution was prepared freshly as previously described with slight modifications [17]. Briefly, it was the mixture of acetate buffer (300 mmol/L, pH 3.6), TPTZ solution (10 mmol/L) in 40 mmol/L HCl) and FeCl3·6H2O solution (20 mmol/L) in a proportions of 10:1:1. 0.1 mL of samples of ethanol was added directly to 3.9 mL of FRAP working solution. The absorbance of the mixture was measured at 593 nm after 10 min of reaction. The calibration curve was constructed with aqueous solutions of FeSO4·7H2O (100–1000 μmol/mL), and the results were expressed as mmol Fe(II)/g dry weight of herb extract.

**Determination of Total Phenolics and Flavonoids Content.** Total phenolics content was determined using the Folin-Ciocalteu method with some modifications [18]. Briefly, 0.1 mL of sample was mixed with 1 mL of the Folin-Ciocalteu working solution (diluted ten-fold) and incubated at room temperature for 5 min, then 1 mL of Na2CO3 solution(0.1 g/mL) was added to the mixture. After incubation for 90 min at room temperature, the absorbance of sample was measured at 765 nm, and the results were expressed as gallic acid equivalents per gram sample (mg GAE/g).

Total flavonoids content of the samples was measured according to previous colorimetric assay with some modifications [19]. 0.1 mL sample was mixed with 0.3 mL of NaNO2 solution (0.05 g/mL) and incubated for 5 min, then 0.3 mL of AlCl3 solution (0.1 g/mL) was added and incubated for another 6 min. The reaction was terminated by adding 2 mL of NaOH solution (1 mol/L). Then absorbance of the mixture was measured at 510 nm immediately. The results were expressed as rutin equivalents per gram sample (mg RE/g).

**Determination of Protective Effect against H2O2-induced Oxidative Damage in HepG2 Cells.** HepG2 cell was obtained from American Type Culture Collection (ATCC, Rockville, MD) and incubated in DMEM supplemented with heat-inactivated 10% FBS, 100 U/mL penicillin, 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO2 at 37°C, and the medium was changed every other day.
Figure 2. The correlation between the increments of phenolics/flavonoids and antioxidant activities. (A): ● The correlation between the increment of total phenolics and DPPH scavenging IC₅₀ in AME; ○ The correlation between the increment of total phenolics and FRAP value in AME. (B): The correlation between the increment of total phenolics and ABTS scavenging IC₅₀ in AME. (C): ● The correlation between the increment of total phenolics and DPPH scavenging IC₅₀ in CFO; ○ The correlation between the increment of total phenolics and ABTS scavenging IC₅₀ in CFO. (D):
The correlation between the increment of total phenolics and FRAP value in CFO. (E): The correlation between increment of flavonoids and DPPH scavenging IC_{50} in AME. (F): The correlation between the increment of flavonoids and FRAP value in AME. (G): The correlation between the increment of flavonoids and ABTS scavenging IC_{50} in CFO. (H): The correlation between the increment of flavonoids and FRAP value in CFO. PC means Spearman Correlation Coefficient.
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**Cell viability assay.** Cells were seeded in 96-well plates at a density of 1×10^{4} cells/well and incubated for 24 h. Thereafter, the medium was replaced with fresh medium containing various concentrations of tested compounds for another 24 h incubation prior to exposure to 1.4 mmol/L H_{2}O_{2} for 4 h. At the end of the incubation period, the medium was decanted, and 100 μL of MTT dye solution (0.5 mg/mL in PBS) were added into each well, and the plates were incubated for 4 h at 37°C. Then, 150 μL of DMSO was added to dissolve/extract tetrazolium dye. Relative cell viability was calculated by determining the absorbance at 570 nm and untreated control cells were assigned a relative

**Evaluation of Synergistic Effect**

In chemical-based assays, the herb pair was consisted of two single herbs at a dose ratio of 1:1. Its scavenging ability against free radicals was defined as Herb pair IC_{50}. In cell-based assays, the two drugs in the combination were not at a constant ratio. The synergistic effect of the herb pair was evaluated using CalcuSyn software, which was developed for dose-effect analysis in drug-combination studies. The combination index (CI) was expressed as CI_{25}, CI_{50} and CI_{75} when the fraction affected (Fa) by the dose was 0.25, 0.50, 0.75, respectively. CI ≤ 0.90, 0.90< CI < 1.10 or CI ≥ 1.10 represent synergistic, additive, and antagonistic effects, respectively.

In the FRAP assay, the theoretical value was calculated as the sum of two single herbs' values multiplying 0.5 for their proportion in herb pair was 0.5:0.5. When the actual value of herb pair was significantly larger than their theoretical value (P<0.05), the synergistic effect was sure to exist in the combination.

**Preparation of Crude Extracts**

The powder of single herbs (AME, CFO, 500 g) was extracted twice with 2.5 L of 95% of ethanol by soxhlet extractor and each time lasted 2.5 h. The extracts then were merged, concentrated by rotary evaporator to obtain the residues. The residues were re-suspended in appropriate volume of deionized water and successively extracted with equivalent volume of petroleum ether (PE), chloroform (CF), ethyl acetate (EA), and n-butyl alcohol (NB), giving four fractions for each herb extract (AME-PE, AME-CF, AME-EA, AME-NB) and (CFO-PE, CFO-CF, CFO-EA, CFO-NB). The crude extracts were stored at −20°C until use.

| Samples          | Herb pair IC_{50} (mg/mL) | CI_{25} | CI_{50} | CI_{75} |
|------------------|---------------------------|---------|---------|---------|
| AME-CF+CFO-CF    | 1.706                     | 0.543   | 0.494   | 0.449   |
| AME-CF+CFO-EA    | 1.727                     | 1.956   | 1.917   | 1.881   |
| AME-EA+CFO-CF    | 1.715                     | 0.581   | 0.526   | 0.477   |
| AME-EA+CFO-EA    | 1.526                     | 0.355   | 2.144   | 12.986  |

**Separation and Purification of Crude Extracts**

The samples of AME-CF (9.0 g) and CFO-CF (3.5 g) were respectively subjected to silica gel column chromatography to isolate the active ingredients. AME-CF was eluted in the following order by petroleum ether-acetone (16:4, 15:5, 14:6, 11:9, v/v), chloroform-methanol (18:2, 17:3, 16:4, 15:5, 13:7, 0:20, v/v). Ten fractions were collected according to thin-layer chromatography (TLC). After being stored at 4°C for some time, precipitation appeared in fraction 2 (0.407 g) and fraction 4 (0.258 g), then needle-like white crystalization (3.5 mg) was obtained by re-crystalization from fraction 4, while the yellow precipitation of fraction 2 did not change after re-crystallization. The fraction 3 (0.208 g) and fraction 5 (0.209 g) were purified by Sephadex LH-20 chromatography using chloroform-methanol solution as the eluent. The eluent for separating CFO-CF was chloroform-methanol (19:1-18:2-17:3-16:4-10:10-0:20, v/v). After TLC analysis, four fractions were obtained. Fraction 1 (0.793 g) was repeatedly subjected to Sephadex LH-20 chromatography for purification.

**Preparation of Standard Solutions**

3.1 mg of ferulic acid and 4.1 mg of isoferulic acid were weighed, and dissolved in 1 mL of methanol respectively to yield the stock solutions. 200 μL of ferulic acid stock solution and 200 μL of isoferulic acid stock solution were mixed with 600 μL of methanol to obtain the mixed standard solution (ferulic acid 0.62 mg/mL, isoferulic acid 0.82 mg/mL). 5.0 mg of calycosin and 2.4 mg of formononetin were weighed, and dissolved in 1.5 mL of methanol respectively. 60 μL of calycosin solution and 60 μL of formononetin solution were mixed with 480 μL of methanol to obtain the mixed standard solution (calycosin 0.33 mg/mL, formononetin 0.16 mg/mL).

**HPLC Conditions**

The Waters Acquity Ultra Performance LC system (Waters Corporation, Milford, MA, USA) consisting of binary solvent manager, an auto-sampler, column manager, and a diode-array detector (DAD) was used for setting the reverse-phase liquid chromatographic conditions (data analysis software Empower). Chromatographic separations were carried out using an Atlantis ®dC18 column (250 mm × 4.6 mm, 5 μm, Waters). HPLC separation was performed using the linear gradient at 30°C and the flow rate of 0.8 mL/min. The injection volume was 10 μL. Detection wavelength was set at 254 nm and UV spectra from 200 to 500 nm were also recorded for peak identification.

For the samples of AME, the mobile phase consisted of water (A) and acetonitrile (B) using the elution gradient. The gradient program was adopted as follows: Isocratic elution of 10% B over the first 5 min, linear from 10 to 20% B (5–10 min), linear from 20 to 35% B (10–20 min), linear from 35 to 45% B (20–30 min), linear from 45 to 55% B (30–40 min), linear from 55 to 65% B (40–50 min), linear from 65 to 10% B (50–55 min), held for 3 min until the separation finished. For the samples of CFO, the mobile phase was 0.1% of phosphoric acid in water (A) and acetonitrile (B) using the elution gradient. The gradient program was adopted as follows: Isocratic elution of 20% B over the first 10 min, linear from 20 to 30% B (10–20 min), linear from 30 to 35% B
(20–30 min), linear from 35 to 40% B (30–40 min), linear from 40
to 20% B (40–45 min), held for 5 min until the separation
finished.

Mass Spectrometry
The Waters Micromass Quattro Ultima PT system (Waters
Corporation, Milford, MA, USA) equipped with the electrospay
ionization (ESI) source was used for mass spectrometric mea-
surement (data analysis software MassLynx 4.0 SP4). The column,
mobile phase and elution program were transferred directly from
the system described in HPLC method to this system excepted for
the mobile phase for samples of CFO. Since phosphoric acid
would seriously damage the mass spectrometric, the 0.1% of
phosphoric acid in water was replaced by 0.1% of formic acid in
water. The injection volume was 5 μL. In order to adapt to the
requirement of ESI-MS/MS, a post-column flow splitter (split
ratio 1:1) was connected to the C18 column, and the flow rate of
0.8 mL/min was reduced to 0.4 mL/min before the elution buffer
entered the ESI interface. The mass spectrometry detector (MSD)
parameters were as follows: positive ion mode; capillary voltage,
3.5 KV; cone voltage, 45.0 V; collision energy, 10.0–25.0 eV;

Figure 3. The DPPH radical scavenging ability of different fractions isolated from AME-CF and CFO-CF. A: The DPPH radical scavenging
ability of 10 fractions isolated from AME-CF. B: The DPPH radical scavenging ability of 4 fractions isolated from CFO-CF.
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source temperature, 110°C; desolvation temperature, 400°C; cone gas (argon, 99.9999% purity) flow rate, 80 L·h⁻¹; desolvation gas (nitrogen, 99.9% purity) flow rate, 562 L/h. Mass analyzer scanned from 100 to 1000 u. The MS/MS spectra were recorded.

**Statistical Analysis**

All experiments were carried out in triplicate and the data were expressed as mean ± standard deviation. Statistical analysis was performed with the one-way analysis of variance (ANOVA), the Duncan test and the Bivariate Correlations using the SPSS 16.0 software. Values of P<0.05 were considered to be statistically significant.

**Results**

**Antioxidant Activity of AME, CFO and Different Solvent–extracted Fractions**

As shown in Fig. 1, compared with other solvent-extracted fractions of AME, the chloroform extracts and ethyl acetate extracts showed higher scavenging efficacy against DPPH free radicals. In addition, AME-CF owned the strongest ABTS free radical scavenging ability and ferric reducing antioxidant power, which was basically in consistent with the previous study [20]. Among the different solvent-extracted fractions of CFO, the ethyl acetate extracts presented the strongest scavenging ability against DPPH radicals, and the results were shown in Fig. 3. Among the ten fractions isolated from AME-CF, fraction 2, 3, 4 and 5 showed higher free radical scavenging efficacy than other fractions and the original CFO-CF sample. However, these fractions exhibited higher potency in scavenging DPPH radicals than the original AME-CF sample (P<0.05). Among the four fractions isolated from CFO-CF, fraction 1 showed the strongest free radical scavenging ability compared with other fractions and the original CFO-CF sample. Thus, AME-CF, AME-EA, CFO-CF and CFO-EA were selected for further analysis.

**Total Phenolics and Flavonoids Content of AME, CFO and Different Solvent-extracted Fractions**

Phenolic and flavonoid compounds are important antioxidants in many plants. Thus, their contents in AME, CFO and different solvent-extracted fractions were determined. From Table 1, it was found that the phenolics and flavonoids content of CFO was significantly higher than that of AME, which might be responsible for the stronger antioxidant activity of CFO. Among the solvent-extracted fractions of AME, AME-CF owned the highest phenolics and flavonoids content. In the case of CFO, the highest phenolics and flavonoids content was observed in CFO-EA.

**The Correlation between the Phenolic/Flavonoid Increments and Antioxidant Activity**

Phenolic substances play a very important role in antioxidant effects [21]. Fig. 2 showed the Spearman correlation coefficients (PC) between the total phenolic/flavonoid increments and antioxidant activity increments determined via three anti-oxidative assays. AME-PC<sub>DPPH</sub>: 0.6/0.6, AME-PC<sub>ABTS</sub>: 0.7/0.8, CFO-PC<sub>DPPH</sub>: 0.7/0.7 and CFO-PC<sub>ABTS</sub>: 0.7/0.7 were ≥0.3, suggesting that the phenolic/flavonoid increments of different extracts were closely related to the enhancement of free radical scavenging abilities. Besides, there was significant linear correlation between the phenolic/flavonoid increments and FRAP values increments as indicated by the correlation coefficient of 0.98 in various extracts of AME, suggesting that the phenolic/flavonoid substances in AME played an important role in the ferric reducing antioxidant activity. However, CFO-PC<sub>FRAP</sub> was 0.8/0.3, mean that the active substances that greatly contributed to the ferric reducing antioxidant capacity of CFO were phenolics, not flavonoid compounds.

**Screening of Synergistic Antioxidant Fractions from Active Solvent-extracted Fractions**

Ten fractions isolated from AME-CF and four fractions isolated from CFO-CF were examined for their scavenging abilities against DPPH free radicals, and the results were shown in Fig. 3. Among the ten fractions isolated from AME-CF, fraction 2, 3, 4 and 5 showed higher free radical scavenging efficacy than other fractions. Moreover, these fractions exhibited higher potency in scavenging DPPH radicals than the original AME-CF sample (P<0.05). Among the four fractions isolated from CFO-CF, fraction 1 showed the strongest free radical scavenging ability compared with other fractions and the original CFO-CF sample. Thus, AME-CF<sub>2</sub>, AME-CF<sub>3</sub>, AME-CF<sub>4</sub>, AME-CF<sub>5</sub>, CFO-CF<sub>1</sub> were selected for the further examination of synergistic effects after purified.

**Screening and Identification of Potential Synergistic Antioxidant Compounds**

AME-CF<sub>2</sub>, AME-CF<sub>3</sub>, AME-CF<sub>4</sub> and AME-CF<sub>5</sub> were paired with CFO-CF<sub>1</sub> to examine the possible synergistic antioxidant efficacy, respectively. As shown in Table 3, (AME-CF<sub>2</sub>+CFO-CF<sub>1</sub>) pair and (AME-CF<sub>3</sub>+CFO-CF<sub>1</sub>) pair resulted in synergistic effects. However, comparing with (AME-CF<sub>2</sub>+CFO-CF<sub>1</sub>) pair (CI<sub>50</sub>, 0.49), the synergy weakened to some extent along with the isolation and purification.

These components were further characterized by HPLC-ESI-MS/MS, and their MS/MS spectra, retention time and UV<sub>λ</sub>max were shown in Table 4. Based on the comparison with standard solutions, the antioxidant compounds were unambiguously identified as calycosin (1), formononetin (2) in AME-CF<sub>2</sub>, calycosin (3) in AME-CF<sub>3</sub>, and ferulic acid (4), isoeugenol acid (5) in CFO-CF<sub>1</sub>. Their structures were listed in Fig. 4.

**Verification of the Synergistic Combination using DPPH Radical Scavenging Assay**

In DPPH radical scavenging assay, the poor scavenging activity was observed in formononetin with a percent inhibition of 0.72%
Figure 4. HPLC spectrogram and structures of synergistic antioxidants. A: Spectrogram of purified sample from AME-CF2. B: Spectrogram of purified sample from AME-CF4. C: Spectrogram of purified sample from CFO-CF1. D: Spectrogram of mix standard solution of calycosin and formononetin, peak 6 was calycosin, peak 7 was formononetin. E: Spectrogram of mix standard solution of ferulic acid and isoferulic acid, peak 8 was ferulic acid, peak 9 was isoferulic acid. doi:10.1371/journal.pone.0087221.g004
at the concentration of 8.05 mg/mL. The IC50 of calycosin was 0.64 mg/mL. These results might suggest that the potent free radical scavenging abilities of AME-CF2 and AME-CF4 were greatly attributed to calycosin. Therefore, calycosin was selected to combine with ferulic acid (IC50, 0.26 mg/mL) and isoferulic acid (IC50, 1.50 mg/mL) respectively to examine their synergistic effects. The results from Fig. 5 showed that the combination of calycosin and ferulic acid exhibited slight antagonism (CI50, 1.16), while the combination of calycosin and isoferulic acid showed synergistic effect (CI50, 0.77). In order to observe whether the dose ratio could affect the synergistic effect between calycosin and isoferulic acid, different dose ratios were tested and the strongest synergy was observed at a dose ratio of 1:1. Furthermore, with the reduction of the dosage, the radical scavenging ability decreased, while the synergistic effect of this combination was improved.

ABTS Radical Scavenging Ability and FRAP Value of the Potential Synergistic Compounds

ABTS radical scavenging ability and ferric reducing antioxidant capacity of these four potential synergistic antioxidant compounds were also measured in order to evaluate their synergistic effects from different aspects. The results in Table 5 indicated that these compounds exhibited strong radical scavenging ability and ferric reducing antioxidant capacity with an exception of formononetin. In FRAP assay, the synergistic effect was observed in the combination of isoferulic acid and calycosin. While, as for ABTS radical scavenging assay, the combination of isoferulic acid and calycosin only showed additive effect (CI50, 1.062), and the other combinations even exhibited slight antagonism.

Protective Effect of Potential Synergistic Compounds against H2O2-induced Oxidative Damage in HepG2 Cells

Many natural antioxidants that provide an antioxidant effect in vitro may have a pro-oxidant effect in vivo or in cultured cells, so it is necessary to proceed to in vivo assays after evaluating antioxidant activities in vitro using chemical-based methods. The animal models and clinical studies are advanced but expensive and time-consuming, making the cell-based assays exceedingly attractive [22]. Three concentration gradients were screened from each drug according to the results in Fig. 6, i.e. 2 μg/mL, 1 μg/mL, 0.5 μg/mL for Ferulic acid, isoferulic acid and calycosin, and 1 μg/mL, 0.5 μg/mL, 0.25 μg/mL for formononetin. Therefore,
Table 5. ABTS radical scavenging ability, ferric reducing antioxidant activity and synergistic effect of different samples.

| Samples     | IC_{50}(ABTS) (mg/mL) | CI_{50} (mg/mL) | FRAP value (mmol/g) | Theoretical value (mmol/g) |
|-------------|------------------------|-----------------|----------------------|-----------------------------|
| FA^a        | 0.061                  | 8.400           |                      |                             |
| IFA^b       | 0.109                  | 11.424          |                      |                             |
| FOR^c       | 7.468                  | –               |                      |                             |
| CAL^d       | 0.159                  | 4.905           |                      |                             |
| FA+ FOR     | 0.133                  | 1.263           |                      |                             |
| FA+ CAL     | 0.111                  | 1.230           |                      |                             |
| IFA+ FOR    | 0.278                  | 1.148           | 6.699                | 6.652                       |
| IFA+ CAL    | 0.117                  | 1.062           | 8.706^*             | 8.164                       |

^aFerulic acid,
^bIsoferulic acid,
^cFormononetin,
^dCalycosin.

^*There was a significant difference between the actual FRAP value and the theoretical value (P<0.05).

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Figure 6. The cell viability of potential synergistic antioxidant compounds. A: The cell viability of ferulic acid in different concentrations. B: The cell viability of isoferulic acid in different concentrations. C: The cell viability of formononetin in different concentrations. D: The cell viability of calycosin in different concentrations.

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Discussion

Free radicals are highly unstable and tend to induce oxidative damage to other molecules by extracting electron, then cause various diseases such as inflammation, atherosclerosis, and aging [23]. Therefore, antioxidants with free radical scavenging activities may play a significant role in the prevention and therapeutics of these diseases. As we all know, phenolic acid and flavonoid compounds are important radical scavengers. In this study, the correlation analysis between the total phenolic/flavonoid increments and the enhancement of antioxidant activities demonstrated the significant contribution of the phenolic/flavonoid compounds to the antioxidant efficacy of AME and CFO. Extraction with the significant contribution of the phenolic/flavonoid compounds may regenerate the latter, thus improving the overall free radical scavenging ability than ononin from n-butyral alcohol extracted fraction, while saponins were generally present in the petroleum ether extracted fraction.

Up to date, a variety of phenolic compounds have been extensively investigated for their antioxidant activity. However, to our knowledge, these studies mainly focused on the single phenolic compound, the informations regarding the interactive actions among them are still limited. Peng et al. isolated ten flavonoid compounds from Polygonum hydropiper L., and found that there was not synergistic anti-oxidative capacity when the flavonoid compounds were used in combination [27]. Other recent study on phenolic compounds (chlorogenic acid, hesperidin, luteolin, myricetin, naringenin, p-coumaric acid, and quercetin) of navel oranges showed that three combinations of 2 compounds (e.g. hesperidin/myricetin) and five combinations of 3 compounds (e.g. hesperidin/chlorogenic acid/naringenin) represented synergistic effects [4]. In our study, the combination of calycosin and isoferulic acid at a dose ratio of 1:1 resulted in a significant synergy in DPPH radical scavenging assay. Both isoferulic acid and calycosin possess hydroxyl groups, after being combined, the number of free hydroxyl groups may be increased and promote access of the free radical scavengers to the radical center [28]. Therefore, the concentration of the potential antioxidant components may be elevated and then appeared synergistic effect. Although isoferulic acid owned poorer DPPH free radical scavenging ability than ferulic acid, it showed antioxidant synergism when used in the combination with calycosin. This fact demonstrated that the combination of two strong antioxidants may not necessarily engender stronger efficacy, it may even produce antagonistic interaction. In contrast, when a weaker antioxidant was combined with a stronger one, the former may regenerate the latter, thus improving the overall free radical quenching capacity of the combination [29].

### Table 6. Cell protective ability and the synergistic effect for the combination of Ferulic acid and Formononetin, Calycosin.

| Samples (μg/mL) | Fraction affected (Fa)a | CI value |
|----------------|-------------------------|----------|
| FAa (1)        | 0.131                   |          |
| FORb (0.25)    | 0.103                   |          |
| FA+FOR (FA: 0.5, FOR: 0.125) | 0.424 | 0.424 |
| FA (0.5)       | 0.103                   |          |
| FA (2)         | 0.226                   |          |
| CALa (2)       | 0.343                   |          |
| CAL (1)        | 0.152                   |          |
| FA+CAL (FA: 0.25, CAL: 1) | 0.335 | 0.526 |
| FA+CAL (FA: 0.25, CAL: 0.5) | 0.182 | 0.656 |
| FA+CAL (FA: 1, CAL: 0.5) | 0.220 | 0.877 |

Fa = \frac{\text{cell viability of (drug + H}_2\text{O}_2 \) - \text{cell viability of H}_2\text{O}_2}{(100 - \text{cell viability of H}_2\text{O}_2) \times 100}

### Table 7. Cell protective ability and the synergistic effect for the combination of Isoferulic acid and Formononetin, Calycosin.

| Samples (μg/mL) | Fraction affected (Fa)a | CI value |
|----------------|-------------------------|----------|
| IFAa (0.5)     | 0.103                   |          |
| FORb (0.5)     | 0.143                   |          |
| FOR (0.25)     | 0.100                   |          |
| IFA+FOR (IFA: 0.25, FOR: 0.25) | 0.252 | 0.314 |
| IFA+FOR (IFA: 0.25, FOR: 0.125) | 0.273 | 0.193 |
| IFA (0.5)      | 0.070                   |          |
| IFA (1)        | 0.172                   |          |
| CAL (0.5)      | 0.078                   |          |
| IFA+CAL (IFA: 0.25, CAL: 0.25) | 0.168 | 0.442 |
| IFA+CAL (IFA: 0.5, CAL: 0.25) | 0.172 | 0.636 |

Fa = \frac{\text{cell viability of (drug + H}_2\text{O}_2 \) - \text{cell viability of H}_2\text{O}_2}{(100 - \text{cell viability of H}_2\text{O}_2) \times 100}

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9. Li et al. [26] proved the significant contribution of phenolics to the antioxidant ability of Sheng-ma, and indicated that phenolic compounds were mainly obtained from ethyl acetate extracted fraction, while saponins were generally present in the petroleum ether extracted fraction.
that polyphenols in green tea could regenerate V_E, and L-ascorbic acid could accelerate the regeneration of oxidized polyphenols in green tea, then formed an antioxidant cycle system [30]. Furthermore, ingredients which possess different antioxidant mechanisms also could exhibit synergistic effect. For example, quercetin as a metal chelating agent could support a tocopherol to improve the antioxidant ability of oil [31]. Likewise, isoflavone has been found to be a metal ion chelating agent [32], which might help calycosin to strengthen the antioxidant activities of the combination. The combination of isoferulic acid and calycosin at low dose showed better synergistic effect than at a high dose. This fact indicated that the synergistic interactions may be weakened and even can be transformed into antagonistic interactions under a higher dose, then to bring about adverse effects on body.

In addition, although these obtained compounds showed potent synergistic effect, this effect was weakened to some extent when compared with the original samples prior to purification. These findings may indicate that some components which contributed to the synergistic effect might be separated into other fractions. It also demonstrated the complexity of synergistic antioxidants when the edible and medicinal plants were utilized in the combination. The fact that the synergistic combination screened based on DPPH radical scavenging assay did not exhibit similar synergy in scavenging ABTS radicals radicals demonstrated the difference between various antioxidant methods. Not a single method can give a comprehensive prediction of antioxidant efficacy, so more than one in vitro antioxidant method should be applied to screening the synergistic compounds from plants. Since most of the chemical assays are done in non-physiological pH values, the results of chemical assays and in vivo models may be inconsistent. In this paper, more than one synergistic combination was observed in cell-based assays, suggesting that the in vitro chemical methods are convenient but clearly limited. Thus, in order to better elucidate the mechanism behind the antioxidant synergism of natural product, further cells bioassay and in vivo research should be done to study the bioavailability of the potential antioxidant like metabolism, uptake and portioning in membranes.

Author Contributions
Conceived and designed the experiments: DL, FW. Performed the experiments: FW SZ FL BZ YQ TS TL. Analyzed the data: FW SZ. Contributed reagents/materials/analysis tools: DL SZ. Wrote the paper: FW FL DL BZ.

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