Synergistic Effect of *Scutellaria baicalensis* and Grape Seed Proanthocyanidins on Scavenging Reactive Oxygen Species *in Vitro*

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Abstract: *Scutellaria baicalensis* (SbE) is a commonly used Chinese herb medicine and grape seed proanthocyanidins is a popular herbal supplement in the United States. Both herbs have been shown to possess potent antioxidant effects. Using an *in vitro* model to produce the reactive oxygen species (ROS) generation (H\textsubscript{2}O\textsubscript{2}/FeSO\textsubscript{4} for hydroxyl radicals, xanthine/xanthine oxidase for suproxide), we observed that *Scutellaria baicalensis* and grape seed proanthocyanidins acted synergistically to scavenge ROS. Our data suggest that a combination of these two herbs can potentially enhance their antioxidant efficacy, allowing lower dosages of each drug to be used. This has the advantage of avoiding possible side effects that may arise when higher doses of a single herb are used in an attempt to achieve a maximum degree of antioxidant activity.

**Keywords**: *Scutellaria baicalensis*; Grape Seed Proanthocyanidins; Herbal Medicine; Hydroxyl Radicals; Suproxide; Antioxidant; Synergistic Effect.
Introduction

Reactive oxygen species (ROS), including superoxide (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^•$), have been implicated in the pathogenesis of ischemia/reperfusion injury (Opie, 1993; Das and Maulik, 1994; Li and Jackson, 2002; Cicconi et al., 2003). Herbal antioxidants may confer significant cellular protection against such oxidant-mediated injury (Sato et al., 1999; Yamakoshi et al., 1999; Shieh et al., 2000; Bagchi et al., 2003).

Scutellaria baicalensis (SbE), also known as Chinese skullcap or Hung Qin, has been used clinically in Japan and China for treatment of allergies, inflammatory diseases, atherosclerosis and cancers (Kubo et al., 1984; Zhang et al., 2003). Several studies have reported finding antioxidative and cardioprotective effects of SbE (Hodnick et al., 1994; Gao et al., 1999). Grape seed proanthocyanidin extract (GSPE), which contains the major polyphenol compounds in red wine, is a popular herbal supplement that has been found to possess potent free radical scavenging capacity as compared with vitamins C, E and β-carotene, and to improve post-ischemic ventricular function and reduce myocardial infarction (Gao et al., 2001; Sato et al., 2001; Bagchi et al., 2002).

Many current commercially available dietary supplements contain several different herbal medicines. However, interaction between antioxidant herbs has not been investigated. This study was designed to evaluate potential synergistic effects from a combination of SbE and GSPE on ROS scavenging using an in vitro chemical ROS generation system (H$_2$O$_2$/FeSO$_4$ and xanthine/xanthine oxidase).

Materials and Methods

Scutellaria baicalensis (SbE) root was obtained from the Shanghai Chinese Herbal Medicine Company. The roots were cut into small pieces, and then soaked in cold water for 2 hours. The mixture was heated to 95°C, and stirred constantly for 1 hour. The hot water-soluble fraction was filtered, and then lyophilized. SbE constituents were identified with liquid chromatography/mass spectrometry (LC/MS; Hitachi M1000, Hitachi Denshi, Ltd., Tokyo, Japan) and atmospheric pressure chemical ionization interface. The flavones in the extract contained 36% baicalein (Shao et al., 1999). GSPE was kindly provided by InterHealth Nutraceuticals (Benicia, CA). SbE and GSPE concentrations used in this study were approximately at EC$_{50}$ identified in previous experiments for cell survival and ROS scavenging (Shao et al., 1999, 2002 and 2003).

In this in vitro study, Fenton reaction chemistry from a mixture of H$_2$O$_2$ (1 µM)/FeSO$_4$ (50 µM) was used to generate OH$^•$. A fluorescent dye, DCFH/DA (2′, 7′-dichlorofluorescein diacetate), which is sensitive to OH$^•$, was used to monitor changes in DCFH oxidation by OH$^•$ (Vanden Hoek et al., 1997). There is a little change in DCF fluorescence by H$_2$O$_2$ (1 µM) or FeSO$_4$ (50 µM) alone (data not shown). Four groups of cuvettes were prepared: these contained (1) DCFH/DA (10 µM) and H$_2$O$_2$ (1 µM)/FeSO$_4$ (50 µM) in balanced salt solution (as a control); (2) SbE (100 µg/ml); (3) GSPE (10 µg/ml), with DCFH/DA (10 µM) and H$_2$O$_2$ (1 µM)/FeSO$_4$ (50 µM); and (4) a combination of both SbE (100 µg/ml) and GSPE (10 µg/ml) with H$_2$O$_2$ (1 µM)/FeSO$_4$ (50 µM) and DCFH/DA (10 µM). DCF
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fluorescence was measured using fluorescence spectrophotometer (Molecular Devices, CA) at excitation of 488 nm/ emission of 529 nm at arbitrary unit (a.u.).

Reaction between xanthine (X, 0.4 mM) and xanthine oxidase (XO, 0.02 U/ml) was used to generate O$_2^\cdot$. A fluorescent dye, dihydroethidium (Eth) which is sensitive to O$_2^\cdot$, was used to measure the changes in O$_2^\cdot$ levels. Another four groups of cuvettes were prepared: (1) Eth (100 µM) and X (0.4 mM)/XO (0.02 U/ml) in balanced salt solution (as a control); (2) SbE (100 µg/ml); (3) GSPE (10 µg/ml) with Eth (100 µM) and X (0.4 mM)/XO (0.02 U/ml); and (4) a combination of SbE (100 µg/ml) and GSPE (10 µg/ml) with Eth (100 µM) and X (0.4 mM)/XO (0.02 U/ml). Eth fluorescence was measured at excitation 520 nm/emission 610 nm.

Data were expressed as mean ± SEM. Statistical significance (p < 0.05) was determined with Student t-test.

Results and Discussion

As seen in Fig. 1a, a progressive increase in DCF fluorescence was seen in the mixture of H$_2$O$_2$/FeSO$_4$ over 15 minutes. Significant attenuation of the increase DCF fluorescence was observed with addition of SbE (100 µg/ml, n = 5, p < 0.01) or GSPE (10 µg/ml, n = 5, p < 0.01). Simultaneous addition of these two extracts also caused significant attenuation of DCF fluorescence (n = 5, p < 0.001), suggesting SbE, GSPE and the combination of SbE and GSPE directly scavenge OH$^\cdot$.

Figure 1a. Effects of SbE and GSPE on DCF fluorescence resulting from the Fenton reaction. After H$_2$O$_2$ (1 µM)/FeSO$_4$ (50 µM) was added to DCFH/DA (10 µM) balanced salt solution, a progressive increase in DCF fluorescence can be seen over 15 minutes. Addition of SbE (100 µg/ml) or GSPE (10 µg/ml) significantly attenuates DCF fluorescence (both n = 5, *p < 0.01). Simultaneous addition of the two extracts further decreases the DCF fluorescence (n = 5, **p < 0.001), suggesting SbE, GSPE and the combination of SbE and GSPE directly scavenge OH$^\cdot$. 
Figure 1b shows, the synergistic effect of SbE and GSPE on OH• scavenging at 15 minutes. Addition of SbE (100 µg/ml) or GSPE (10 µg/ml) resulted in a significant decrease of DCF fluorescence to 13.6 ± 2.4% or 36.5 ± 3.8%, respectively. An even greater attenuation in DCF fluorescence was observed (70.4 ± 3.2%) when SbE and GSPE were simultaneously added, compared with the expected simple additive reduction of 50.1% (13.6% + 36.5%). This suggests that SbE and GSPE work synergistically to scavenge OH•.

A similarly significant increase in Eth fluorescence was seen with a mixture of X (0.4 mM)/XO (0.02U). After the addition of SbE (100 µg/ml) or GSPE (10 µg/ml), Eth fluorescence was attenuated to 12.5 ± 3.2% (n = 5, p < 0.01) or 15.0 ± 2.9% (n = 5, p < 0.01), respectively. An even more significant attenuation was observed with addition of a combination of SbE (100 µg/ml) and GSPE (10 µg/ml), suggesting SbE, GSPE and the combination of SbE and GSPE directly scavenge O2•* (Fig. 2a). Figure 2b shows, the synergistic effect of SbE and GSPE on scavenging O2•* at 15 minutes. When SbE and GSPE were simultaneously added, Eth fluorescence was significantly attenuated (37.5 ± 3.1%; n = 5, p < 0.001), compared with the expected simple additive reduction of 27.5% (12.5 + 15%), p < 0.05). This, also, indicates a synergistic effect of SbE and GSPE on O2•* scavenging.
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Figure 2a. Effect of SbE and GSPE on Eth fluorescence produced by xanthine (X)/xanthine oxidase (XO). After X (0.4 mM)/XO (0.02 U/ml) was added to Eth (100 µM) balanced salt solution, a progressive increase in Eth fluorescence was seen over 15 minutes. Addition of SbE (100 µg/ml) or GSPE (10 µg/ml) significantly attenuates Eth fluorescence (both n = 5, *p < 0.01). Simultaneous addition of the two extracts significantly decreases the Eth fluorescence (n = 5, **p < 0.001). ▲ = X/XO & SbE (100 µg/ml) and GSPE (10 µg/ml).

Figure 2b. Synergistic effect of SbE and GSPE on the attenuating Eth fluorescence produced by X/XO at 15 minutes. Column 1: X (0.4 mM)/XO (0.02 U/ml) produced O$_2^*$ at 15 minutes as a control (normalized to 0%, the column cannot be seen). Column 2: X (0.4 mM)/XO (0.02 U/ml) plus SbE (100 µg/ml). Eth fluorescence was attenuated by 12.5% compared to control. Column 3: X (0.4 mM)/XO (0.02 U/ml) plus GSPE (10 µg/ml). Eth fluorescence was attenuated by 15.0% compared to control. Column 4: sum of data from columns 2 and 3 (27.5%). Column 5: X (0.4 mM)/XO (0.02 U/ml) and SbE (100 µg/ml) and GSPE (10 µg/ml). Eth fluorescence was significantly attenuated by 37.5%, suggesting a synergistic effect. *p < 0.05 between Columns 4 and 5.
In our previous study, we demonstrated that SbE extract has potent antioxidant activity, attenuating DCFH oxidation and reducing the cell death in chick cardiomyocytes exposed to simulated ischemia/reperfusion. We also found that SbE directly scavenges H$_2$O$_2$, OH$^\cdot$ and O$_2^\cdot$ in a dose-dependent manner (Shao et al., 1999 and 2002). In addition, we have reported that GSPE dose-dependently scavenges exogenously added H$_2$O$_2$ and endogenous oxidants induced by antimycin A (a mitochondria electron transport chain complex III inhibitor) and confers cardioprotection in cardiomyocytes (Shao et al., 2003).

In contrast to Western medicine, several herbs are usually used in a single prescription of Chinese herbal medicine. There are often four components: monarch (principal), ministerial (subsidiary), adjuvant and conducting herbs. It is believed that usage of several herbs in a formula will enhance efficacy, and at the same time reduce the side effects. Data from this study demonstrate a synergistic effect of SbE and GSPE on scavenging ROS, above and beyond their additive effects. Co-administration of these extracts has the potential to increase antioxidant treatment efficacy while decreasing possible side effects that may result from high doses of single herb preparations (Vanden Hoek and Shao, 2003).

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References


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