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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_2O_2/$FeSO_4$ 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.

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Introduction

Reactive oxygen species (ROS), including superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$), 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, artherosclerosis 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_2O_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 95ºC, 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_2O_2 (1 μM)/$FeSO_4$ (50 μM) was used to generate $OH^{\bullet}$. A fluorescent dye, DCFH/DA (2′, 7′-dichlorofluorescin diacetate), which is sensitive to $OH^{\bullet}$, was used to monitor changes in DCFH oxidation by $OH^{\bullet}$ (Vanden Hoek *et al*., 1997). There is a little change in DCF fluorescence by H_2O_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_2O_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_2O_2 (1 μM)/$FeSO_4$ (50 μM); and (4) a combination of both SbE (100 μg/ml) and GSPE (10 μg/ml) with H_2O_2 (1 μM)/$FeSO_4$ (50 μM) and DCFH/DA (10 μM). DCF

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 $O_2^{\bullet}$. A fluorescent dye, dihydroethidium (Eth) which is sensitive to $O_2^{\bullet}$, was used to measure the changes in $O_2^{\bullet}$ 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 $H_2O_2/FeSO_4$ over 15 minutes. Significant attenuation of the increase DCF fluorescence was observed with addition of SbE (100 µg/m, $n = 5$, $p < 0.01$) 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 $OH^{\bullet}$.

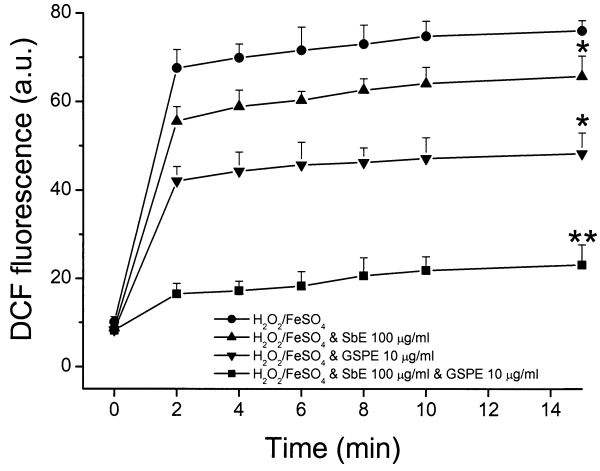

Figure 1a. Effects of SbE and GSPE on DCF fluorescence resulting from the Fenton reaction. After H_2O_2 (1 µM)/$FeSO_4$ (50 µM) was added to DCFH/DA (10 µM) balanced salt solution, a progressive increase in DCF fluorescence can be seen over 15 minutes. Addition of SbE (100 µg/ml) or GSPE (10 µg/ml) significantly attenuates DCF fluorescence (both $n = 5$, $^{*}p < 0.01$). Simultaneous addition of the two extracts further decreases the DCF fluorescence ($n = 5$, $^{**}p < 0.001$). ● = $H_2O_2/FeSO_4$. ▲ = $H_2O_2/FeSO_4$ with SbE (100 µg/ml). ▼ = $H_2O_2/FeSO_4$ with GSPE (10 µg/ml). ■ = $H_2O_2/FeSO_4$ and SbE (100 µg/ml) and GSPE (10 µg/ml).

Figure 1b shows, the synergistic effect of SbE and GSPE on $OH^{\bullet}$ 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 $OH^{\bullet}$.

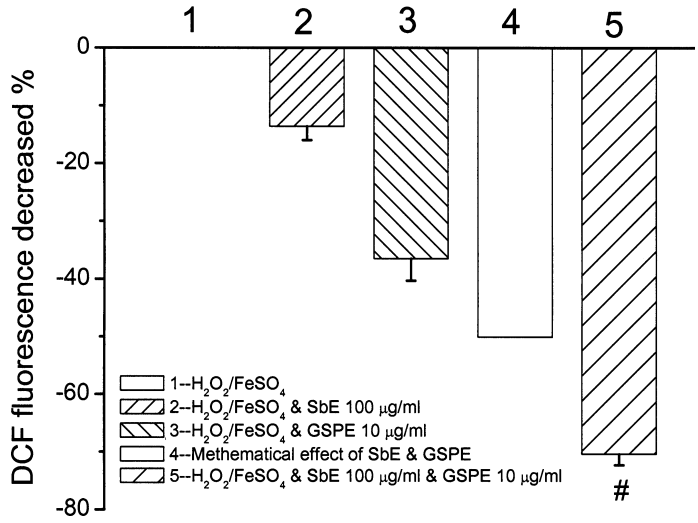

Figure 1b. SbE and GSPE act synergistically to attenuate DCF fluorescence resulting from reaction of H_2O_2/$FeSO_4$ at 15 minutes. Column 1: H_2O_2 (1 μM)/$FeSO_4$ (50 μM) produced $OH^{\bullet}$ at 15 minutes as a control (normalized to 0%, the column cannot be seen). Column 2: H_2O_2 (1 μM)/$FeSO_4$ (50 μM) plus SbE (100 μg/ml). DCF fluorescence was attenuated by 13.6% compared to control. Column 3: H_2O_2 (1 μM)/$FeSO_4$ (50 μM) plus GSPE (10 μg/ml). DCF fluorescence was attenuated by 36.5% compared to control. Column 4: sum of data from columns 2 and 3 (50.1%). Column 5: H_2O_2 (1 μM)/$FeSO_4$ (50 μM) and SbE (100 μg/ml) plus GSPE (10 μg/ml). DCF fluorescence was significantly attenuated by 70.1%, suggesting a synergistic effect. $^{\#}p < 0.05$ between Columns 4 and 5.

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 $O_2^{\bullet}$ (Fig. 2a). Figure 2b shows, the synergistic effect of SbE and GSPE on scavenging $O_2^{\bullet}$ 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 $O_2^{\bullet}$ scavenging.

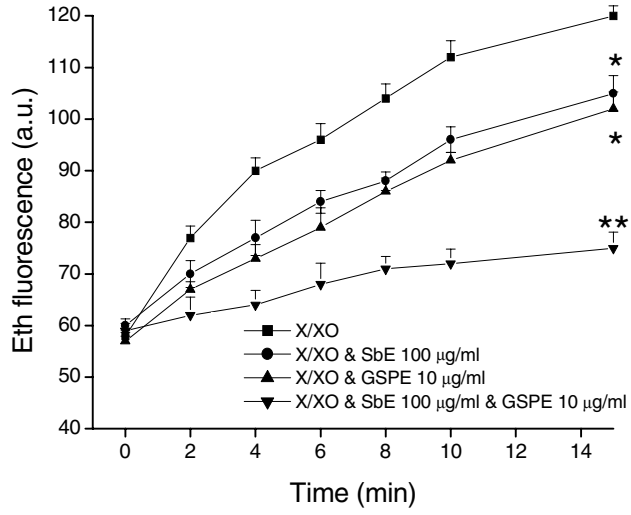

Figure 2a. Effect of SbE and GSPE on Eth fluorescence produced by xanthine (X)/xanthine oxidase (XO). After X (0.4 mM)/XO (0.02 U/ml) was added to Eth (100 μM) balanced salt solution, a progressive increase in Eth fluorescence was seen over 15 minutes. Addition of SbE (100 μg/ml) or GSPE (10 μg/ml) significantly 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. ● = X/XO with SbE (100 μg/ml). ▲ = X/XO with GSPE (10 μg/ml). ▼ = X/XO and SbE (100 μg/ml) and GSPE (10 μg/ml).

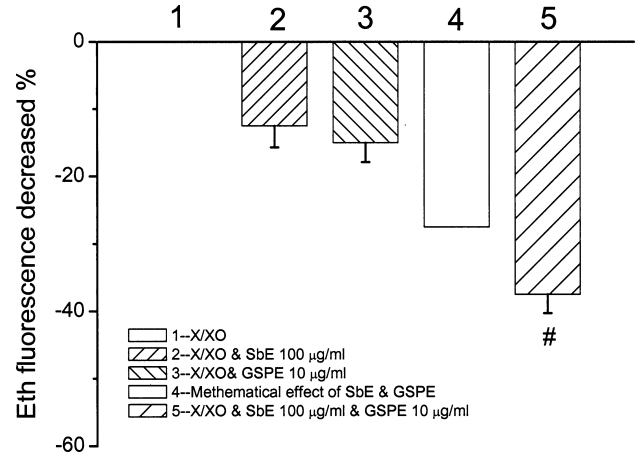

Figure 2b. Synergistic effect of SbE and GSPE on the attenuating Eth fluorescence produced by X/XO at 15 minutes. Column 1: X (0.4 mM)/XO (0.02 U/ml) produced $O_2^{\bullet}$ at 15 minutes as a control (normalized to 0%, the column cannot be seen). Column 2: X (0.4 mM)/XO (0.02 U/ml) plus SbE (100 μg/ml). 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) plus 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_2O_2, $OH^{\bullet}$ and $O_2^{\bullet}$ in a dose-dependent manner (Shao *et al*., 1999 and 2002). In addition, we have reported that GSPE dose-dependently scavenges exogenously added H_2O_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).

Acknowledgments

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