***In Vitro* Antioxidant Potentials of Traditional Chinese Medicine, Shengmai San and Their Relation to *in Vivo* Protective Effect on Cerebral Oxidative Damage in Rats**

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The preventive effects of Shengmai San (SMS), a traditional Chinese herbal medicine (TCM), was studied on cerebral ischemia-reperfusion injury in rats as a model of antioxidant-based composite therapy. Two biochemical indicators of oxidative damage, thiobarbituric acid reactive substance (TBARS) formation and glutathione peroxidase (GPX) loss were measured in the brain after forebrain ischemia-reperfusion treatment and both were inhibited in all rats administered SMS (15 g original herbs/kg) 2 h before the ischemia-reperfusion. Histochemical study of the brain slice using TTC staining revealed that the SMS effectively reduced infarct area caused by the cerebral ischemia-reperfusion. The antioxidant potentials of SMS preparations were determined *in vitro* by five different assay methods and were related to the *in vivo* effectiveness of SMS in protection against brain damage. Inhibitory effect on TBARS formation *in vivo* showed better correlation with superoxide radical scavenging and DPPH quenching activity *in vitro* rather than with the other *in vitro* antioxidant indicators. On the other hand, the *in vivo* prevention of GPX activity loss showed better correlation with *in vitro* crocin bleaching inhibition than with the other *in vitro* antioxidant indicators. It was also suggested that the *in vitro* TBARS inhibitory activity of SMS is not a good indication to predict the *in vivo* effectiveness of SMS on inhibition of either TBARS formation or GPX activity loss.

Key words traditional Chinese medicine; Shengmai San; cerebral ischemia-reperfusion; antioxidant activity

Reactive oxygen species (ROS) are related to complex pathophysiological conditions such as lifestyle- and/or age-related diseases.^{1,2)} In particular, the brain is quite susceptible to oxidative insult due to the low levels of physiological antioxidants and antioxidant enzymes in the brain.³⁾ In fact, ROS have been implicated in brain neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.⁴⁾ Although ROS are instrumental in the initiation of lipid peroxidation, many other reactions such as cytoplasmic Ca^{2+} release,⁵⁾ also play crucial roles in the subsequent propagation of tissue injury. Thus, antioxidant protection of the brain against oxidative stress cannot be successfully achieved by the use of radical scavenging antioxidant alone. Indeed, several trials with small molecular antioxidants have been done but results were not enough satisfactorily.^{6–13)}

In this sense, antioxidative traditional Chinese medicine (TCM) is an attractive target for the study as a model of antioxidant-based composite therapy against cerebral oxidative damage, as TCM has been used as an alternative medicine for treating complex pathophysiological conditions, even for the diseases that Western medicine has failed to allocate a specific diagnostic name.^{14–17)}

However, the use of TCM is accompanied by difficulties in controlling the quantitative aspect of the treatment or setting up the therapeutic strategy, because the lack of sufficient knowledge about which component is ultimately active and how the component-component interaction affects the overall action. Therefore, some new overall criteria is necessary for evaluating therapeutic potential of mixed formulation such as TCM against complex pathophysiological conditions. Since, oxidative stress has been implicated in many complex conditions to which TCM can be applicable. It is of interest to study how the *in vitro* antioxidant potential of certain TCM formula relates to the *in vivo* activity of protecting oxidative tissue damage.

In the present study, we precisely examined the antioxidant potential of a traditional Chinese medicine, Shengmai San (SMS) *in vitro* and then discussed how the *in vitro* activity or which indication of the *in vitro* antioxidant assay relates to the *in vivo* effectiveness of SMS in preventing cerebral oxidative damage induced by ischemia-reperfusion in rats. SMS is a traditional formulation being used for treating symptoms related to cardiac disorder but was shown to have effective protection against oxidative damage in rat brain in our previous study.¹⁸⁾

MATERIALS AND METHODS

Reagents Constituent herbs of Shengmai San, *Panax ginseng* and *Ophiopogon japonicus* and *Schisandra chinensis* were the products of Korea, Sichuan, P.R. China, Hei Long Liang, P.R. China, respectively. Dimethylpyrrolone oxide (DMPO) was purchased from LABOTEC Co., Ltd., Tokyo. FeSO_4 was purchased from Kanto Chemical Co, Ltd., Japan. Xanthine oxidase (XOD, 20 U/ml, from cow milk) was purchased from Boehringer Mannheim Co., Germany. Crocin was extracted by diethyl ether from dried saffron purchased from a local supermarket. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chem. Co. All other reagents, including 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH), and diethylene triamine-*N,N,N',N'',N'''*-penta acetic acid (DTPA) were purchased from Wako Pure Chemical Industries Co., Ltd., Japan. BCA protein assay reagent was purchased from Pierce Chemical Co.

SMS Preparation The SMS preparation used in this study was kindly prepared by Kotaro Kampo Co., Ltd., Japan. Briefly, the three component herbs of Shengmai San, *Panax ginseng* (48 g), *Ophiopogon japonicus* (48 g), and *Schisandra chinensis* (24 g) were suspended in 1200 ml dis-

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tilled water, soaked for 1 h, and then decocted for 1 h. The supernatant was filtered through gauze, and the filtrate was then freeze-dried. The dried filtrate was mixed with Neusilin FL2 as an additive to produce a granulated SMS preparation. The SMS granules were stored at -80°C until use. For each experiment, the SMS granules were weighed precisely and solubilized in distilled water to make the final concentrations indicated.

Animal Treatment Male Wister rats (6 weeks old and 160–168 g body weight) were purchased from SLC Inc., Japan. They were allowed free access to pelleted diet and water before the experiments. Anesthesia was induced by diethyl ether and maintained by pentobarbital. In order to examine the dose-response effect of SMS against cerebral ischemia-reperfusion injury, 2 ml of each SMS solution at different concentrations (0.15, 0.3, 0.6, 1.2 g/ml) was administered directly into the lumen of duodenum exposed by abdominal incision 2 h before the ischemia operation. Cerebral ischemia was produced by occlusion of both the right and left common carotid arteries exposed through a middle skin incision, using aneurysm clips for 85 min. Ischemic condition was confirmed by the change in color of the eyeball from red to white. At the end of the ischemic period, the carotid arteries were declamped to allow blood reperfusion. All rats were decapitated under anesthesia after 45 min of reperfusion, and then the whole brain was removed for biochemical analysis.

For each experiment outlined below, biochemical measurements were taken for three groups of rats 1: Normal control; rats not given any treatment, 2: Vehicle control; rats administered Neusilin instead of SMS before ischemia followed by reperfusion, 3: SMS treated; rats treated with SMS before cerebral ischemia-reperfusion as outlined above.

Tissue Homogenate The whole brain was removed, weighed, and then chilled in ice-cold saline. After washing with 0.85% NaCl, the tissue was suspended in cold 0.05 M phosphate buffer containing 1.15% (w/v) KCl (9 ml per 1 g wet tissue), and then homogenized at 0°C using an ULTRA TRAX homogenizer.

Protein concentration of the homogenate was determined by BCA protein assay using bovine serum albumin as a standard (PIERCE).

TTC Staining of Brain Tissue TTC (2,3,5-triphenyltetrazolium chloride) staining of brain slices was performed as previously described.²⁰⁾ The brains removed were froze for 10 min at -20°C , and then six coronal brain sections, 6–8 mm thick, were sliced from the front pole using a razor. The sections were incubated in phosphate buffered saline containing 2% TTC at 37°C for 10 min, and then kept in 10% buffered formalin (pH 7.0) overnight.

Thiobarbituric Acid Reactive Substance (TBARS) in Brain Tissue Homogenate TBARS formation was determined as previously reported.²¹⁾ Briefly, an aliquot of brain homogenate (1 mg as protein) was mixed with 0.2 ml of 15% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA, and then the volume was adjusted to 4.0 ml with distilled water. After boiling at 95°C for 60 min, the reaction solution was extracted with *n*-buthanol/pyridine (15:1 v/v). Absorbance at 532 nm was determined for the organic layer after centrifugation.

Glutathione Peroxidase (GPX) Activity in Brain Tissue

Homogenate GPX activity was determined according to the method of Albrecht.²²⁾ Briefly, an aliquot of brain homogenate (0.4 mg as protein) in 0.05 M phosphate buffer containing 1.15% (w/v) KCl was mixed in a quartz cuvette with 935 μl of coupling solution (containing 33.6 mg disodium EDTA, 6.5 mg NaN_3 , 30.7 mg of GSH, 16.7 mg NADPH and 100 units of GSH reductase in 100 ml of 50 mM Tris-HCl pH 7.6) and the volume then adjusted to 975 μl with 0.05 M phosphate buffer. Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em. 465 nm) was measured after the addition of 25 μl of 1 mM H_2O_2 as the substrate using a Hitachi model 650-60 fluorescence spectrophotometer.

Hydroxyl Radical Scavenging Activity Measurement in Vitro Hydroxyl radical scavenging activity of SMS was determined as reported previously using the Fenton reaction as the hydroxyl radical source.²³⁾ The reaction mixture (300 μl total volume) containing defined amounts of SMS, 20 mM DMPO, 10 mM H_2O_2 , and 0.1 mM FeSO_4 was quickly transferred into a 250 μl quartz flat cell. Exactly 1 min after the addition of FeSO_4 , the electron spin resonance (ESR) spectra of DMPO-OH was determined using a JEOL JES-TE 200 ESR spectrometer (X-band Microwave Unit) with the following settings: microwave power; 8 mW, microwave frequency; 9.18 GHz, modulation amplitude; 0.1 m, time constant; 0.03 s, sweep time; 30 s, center fields; 331.6/321.6 mT.

Superoxide Scavenging Activity Measurement in Vitro Superoxide scavenging activity of SMS was measured according to the methods of Finkelstein *et al.*,²⁴⁾ using the hypoxanthine (HPX)/xanthine oxidase (XOD) system as the superoxide anion radical generator. The reaction mixture (total 300 μl) containing defined amounts of SMS, 1 mM DTPA, 0.5 mM hypoxanthine, DMPO and 0.1 U/ml xanthine oxidase in 0.2 M phosphate buffer (pH 7.8) was quickly transferred into a 250 μl quartz flat cell. The ESR spectra of DMPO-OOH was determined exactly 1 min after the addition of XOD. The ESR settings were the same as for DMPO-OH.

Crocin Bleaching Activity Measurement Crocin extraction and the bleaching test were carried out according to the method of Wolf *et al.*²⁵⁾ with minor modifications. To 3.0 ml of 0.1 M phosphate buffer (pH 7.0) containing defined amounts of SMS and 10 μM crocin, 75 μl of AAPH (0.5 M stock solution) was added to initiate crocin bleaching at 40°C . The decrease in the absorbance at 443 nm was kinetically recorded.

AAPH-Induced Lipid Peroxidation in Vitro AAPH initiated lipid peroxidation was determined in the presence or absence of SMS, using the TBARS method. Briefly, reaction mixture (300 μl) containing liver homogenate (1 mg as protein), 0.1 ml AAPH (1 mM aqueous solution) and defined amount of SMS was incubated aerobically at 37°C for 30 min. TBARS formation was determined as outlined above.²¹⁾

DPPH Radical Scavenging Assay in Vitro DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of SMS was determined according to the method of Yoshida *et al.*²⁶⁾ Defined amounts of SMS was incubated with 0.1 mM DPPH in 2 ml methanol/0.05 M phosphate (1:1 v/v) buffer (pH 7.6) for 10 min at room temperature. The kinetic decrease in DPPH absorbance at 520 nm was determined for the first 10 min after SMS addition.

RESULTS

Shengmai San consists of three herbal components, *Panax ginseng*, *Ophiopogon japonicus*, and *Schisandra chinensis*. Although several antioxidant ingredients have been reported in *ginseng* and *schisandra* such as Ginseoside Rb₁, Rg₁, and Ro from *ginseng*²⁷⁾ and schisanhenol and schizandrin B from *schisandra*,²⁸⁾ it is important for the practical purpose to evaluate the antioxidant potentials of SMS as a TCM formulation itself. We first determined *in vitro* antioxidant activity of SMS by five different assay methods.

The superoxide and hydroxyl radical scavenging activities were determined by electron spin resonance (ESR) spectrometry using DMPO as the spin trap. Both the formation of superoxide and hydroxyl radical spin adduct, DMPO-OOH and DMPO-OH, respectively, were inhibited by the addition of SMS in a dose-dependent manner (Figs. 1A, B). SMS was found to have stronger scavenging activity for hydroxyl radicals than superoxide radical. Since the dose responses for scavenging both radicals only showed linearity up to approx. 50% inhibition, the scavenging activity of the SMS (0.6 g original herbs/ml) used for the following experiments was compared to that of Trolox as a reference antioxidant at 20% inhibitory concentration (IC₂₀). The IC₂₀ of the SMS was calculated as 200 and 0.4 μ M Trolox equivalent, respectively, for superoxide and hydroxyl radical. The IC₂₀ values of the SMS solution as determined by other *in vitro* antioxidant assays are summarized in Table 1. From the results, SMS was found to be rather specific scavenger toward hydroxyl radicals than toward other radicals.

The protective effect of the SMS preparation on cerebral oxidative damage induced by ischemia-reperfusion in rats was studied using two biochemical indicators: inhibition of TBARS formation and GPX activity loss in Fig. 2. After ischemia for 85 min and reperfusion for 45 min at room temperature, TBARS of the brain homogenate increased to 189.9% and GPX activity was dropped to 64.2% of untreated control brain, respectively. However, both change were effectively prevented in the brain of rats treated with SMS (15 g original herb/kg) 2 h before the ischemia operation, such that the inhibitory activity of TBARS formation and the prevention of GPX activity loss were 98.8 and 94.9%, respectively (Fig. 2). TBARS formation and GPX activity loss were inhibited by SMS in a dose-dependent manner up to 7.5 g/kg, and the damage was almost completely inhibited above this dose (Fig. 3). The dose dependence of the SMS induced GPX activity loss was not completely identical to that of inhibition of TBARS formation. SMS had little effect on the GPX preservation at the low concentration of 1.88 g/kg. However, GPX activity loss was also prevented dose-dependently with higher dose of SMS (at 3.75, 7.5 g/kg), and the effect was saturated at 15 g/kg as same as TBARS formation.

The protective effect of SMS was also demonstrated histochemically using the TTC staining method. TTC is reduced by mitochondrial dehydrogenases to form red hormazan pigment. The results clearly showed that SMS (15 g/kg) administered 2 h before ischemia-reperfusion protected against oxidative damage in the brain (Fig. 4). A large infarcted area observed mainly in the gray matter and the brain stem remained unstained in the damaged brain of vehicle treated rats, whereas the infarction was markedly smaller in the brain

Fig. 1A. Effect of Shengmai San on DMPO-OH Formation *in Vitro*

Hydroxyl radicals were generated by the Fenton reaction and determined by ESR using DMPO as the spin trap. DMPO-OH signals were recorded exactly at 1 min after the addition of FeSO₄ to 10 mM H₂O₂ solution with presence or absence of SMS. Other experimental conditions are given in the Materials and Methods.

Fig. 1B. Effect of Shengmai San on DMPO-OOH Formation *in Vitro*

Superoxide radicals were generated by the hypoxanthine (HPX)/xanthine oxidase (XOD) system and determined by ESR using DMPO as the spin trap. DMPO-OOH signals were recorded exactly at 1 min after the addition of XOD with presence or absence of SMS. Other experimental conditions are given in the Materials and Methods.

Table 1. Antioxidant Activity of SMS Compared to That of Trolox as a Reference Antioxidant in Several Assay Systems *in Vitro*

Antioxidant assays	SMS IC ₂₀ as Trolox equivalent (μ M)
TBARS formation	5
DPPH Quenching	3.2
Crocin bleaching test	1.1
Superoxide radical scavenging	200
Hydroxy radical scavenging	0.4

The antioxidant potential of various concentrations of SMS and Trolox were determined *in vitro* by crocin bleaching, TBARS formation, DPPH quenching, and hydroxyl and superoxide radical scavenging activities, and then their IC₂₀ determined from the dose-response curves.

Fig. 2. Prevention of Ischemia-Reperfusion Damage by SMS in Rat Brain

Inhibitory effects of SMS are given on (a) TBARS formation and (b) GPX activity loss in rat brain after ischemia-reperfusion. SMS (15 g/kg) was administered into rat duodenum 2 h before ischemia induced by bilateral carotid artery occlusion for 85 min followed by 45 min reperfusion. Triplicate determinations were done on the brain homogenate prepared from each rat. Data represent the mean of 3 rats $\pm$ S.D. * $p < 0.005$, compared to Neusilin (vehicle) control.

of SMS treated rats.

To further examine how the *in vitro* antioxidant potential of SMS preparation relates to the *in vivo* effectiveness in preventing oxidative brain damage, the antioxidant activities of SMS preparations used for Fig. 3 experiments were measured *in vitro* using five distinct assay systems, namely TBARS formation, DPPH quenching, crocin bleaching, and hydroxyl and superoxide radical scavenging activities. Then the *in vitro* antioxidant potentials were correlated with the *in vivo* protective activity (Figs. 5, 6).

Essentially, the SMS solutions showing stronger net *in vitro* antioxidant activity more effectively inhibited TBARS formation in the brain in all the *in vitro* antioxidant indices except TBARS inhibitory activity (Fig. 5). Of the *in vitro* antioxidant indices, DPPH quenching, and superoxide radical scavenging activities showed rather good correlation with inhibition of TBARS formation *in vivo* ($r^2=0.998$, 0.974 , respectively.) although a linear dose-response was only established in the narrow range of both *in vitro* antioxidant indices. For example, the *in vitro* DPPH quenching activity showed linearity with the *in vivo* inhibition of TBARS formation only up to its 30% inhibition level, because the *in vivo* protection was almost completely attained with the SMS

giving approx. 30% inhibition of DPPH quenching *in vitro*. The same trends were observed for the *in vitro* hydroxyl radical and superoxide radical scavenging activities, *i.e.*, linear correlation was only observed up to approx. 20 and 40% inhibition levels of both activities *in vitro*, respectively. On the other hand, the *in vitro* crocin bleaching assay covered the *in vivo* TBARS inhibitory activity with its rather wide range of activity, although their relationship was not completely linear.

The same correlation patterns were observed between the *in vitro* antioxidant indices and the *in vivo* protective effect of SMS as defined by GPX activity loss (Fig. 6). Only limited activity ranges of the *in vitro* indices (for example, in DPPH quenching and superoxide radical scavenging, up to approx. 30 and 60% inhibition levels, respectively) covered the whole range of the *in vivo* effect (0–100%) on protecting against GPX activity loss in all the *in vitro* assays except the crocin bleaching assay.

DISCUSSION

In the present study, we examined the protective effect of SMS against cerebral oxidative injury as a model of antioxidant-based composite therapy for oxidative stress-related diseases. Not only biochemical indicators but also histochemical observation clearly showed that SMS, a hydroxyl radical scavenging TCM, effectively prevented the cerebral oxidative damage induced by forebrain ischemia followed by reperfusion. The dose-response profile of the SMS effect was almost the same as those obtained for single antioxidant drug, when a granulated SMS decoction was used.

For therapeutic purposes, TCM is usually administered orally, but oral administration was found not suitable for the present experiment that relatively acute effect of SMS was investigated. We found the SMS administered directly into the rat duodenum gave stable and reproducible *in vivo* results.

The dose dependence of the SMS effect was not completely identical for the inhibition of TBARS formation and GPX activity loss *in vivo*, especially at the low SMS concen-

Fig. 3. Prevention of Ischemia-Reperfusion Damage by SMS in Rat Brain

Inhibitory effects of SMS are given for (a) TBARS formation and (b) GPX activity loss in rat brain after ischemia-reperfusion. SMS (1.88, 3.75, 7.5, 15 g/kg) was administered into rat duodenum 2 h before ischemia induced by bilateral carotid artery occlusion for 85 min followed by 45 min reperfusion. Triplicate determinations were done for the brain homogenate prepared from each rat. Data represent the mean of 3 the rats $\pm$ S.D.

Fig. 4. Protective Effect of Shengmai San against Cerebral Ischemia-Reperfusion Damage in Rat Evaluated by TTC Staining

Brain coronary sections were prepared after 85 min ischemia followed by 45 min reperfusion, and then stained with 2,3,5-triphenyltetrazolium. Normal control; rats not given any treatment, Neusilin control; rats administered Neusilin instead of SMS before ischemia followed by reperfusion, SMS treated; rats treated with SMS before cerebral ischemia-reperfusion. Each group contained 3 rats, and the results for the other two rats in each group were almost identical to those represented here.

Fig. 5. Comparison of *in Vivo* and *in Vitro* Antioxidant Activities of SMS

Antioxidant potentials of SMS solutions of different concentration were determined by five different *in vitro* assays. The *in vitro* antioxidant potential was determined and then correlation to the inhibitory activities on TBARS formation *in vivo* obtained by the experiments in Fig. 3. Data represent the mean $\pm$ S.D. of 3 independent *in vivo* and *in vitro* experiments.

Fig. 6. Comparison of *in Vivo* and *in Vitro* Antioxidant Activities of SMS

The *in vitro* antioxidant potentials at different concentration of SMS solution as above were assessed for correlation with the preventive effect on GPX activity loss *in vivo* as in Fig. 3.

tration (see Figs. 3a, b). Since GPX activity change reflects more likely the cellular physiological response, this differential effect of SMS on these two biochemical indices suggests that the SMS not only functions as a simple antioxidant to inhibit lipid peroxidation but also as a modulator of the cellular antioxidant potential by that antioxidant enzymes such as GPX was enhanced. Indeed, we recently found that SMS enhanced cellular GPX levels in cultured cells (unpublished data). Therefore, TCM is a reliable model for studying antioxidant-based composite therapy of the oxidative stress-related

disorders, although further studies are required to identify the specific agent participating in each biochemical steps for the brain damage protection.

Since oxidative stress is implicated ubiquitously in age and life-style related pathophysiology such as diabetes mellitus, cancer and dementia to that TCM is applicable, it is important to know how the *in vitro* antioxidant potential of TCM itself as a mixed formula correlates to the *in vivo* therapeutic effectiveness. However, it is still argued that the *in vitro* antioxidant potential determined by certain assay method is not

always consistent with those determined by other assay methods, and also, the *in vitro* antioxidant potential is not sufficiently reflected in the *in vivo* effectiveness. Therefore, we here again examined precisely the *in vitro* antioxidant potential of different SMS preparations by five distinct typical assays of antioxidants *in vitro* and then each activity determined by different methods was correlated to the *in vivo* effectiveness of the same SMS preparation in preventing oxidative brain damage produced by ischemia reperfusion in rats.

Results showed that all of the *in vitro* antioxidant indices essentially reflect the *in vivo* activity in preventing cerebral oxidative damage, that is, both in the inhibition of TBARS formation and the prevention of GPX activity loss. However, each *in vitro* antioxidant index differently correlated to the *in vivo* activity as was expected from the observation in Table 1 that each antioxidant assay gives different IC₂₀ for SMS. Moreover, crocin bleaching test covered whole range of the *in vivo* effect although the relation was not sufficiently linear, but in other indices, the linearity between the *in vitro* and *in vivo* activity was established only for a limited region of the observed *in vitro* activities, especially for TBARS inhibition and hydroxyl radical scavenging activity. It was thus suggested that TBARS inhibition and hydroxyl radical scavenging activities *in vitro* are not adequate index for predicting the *in vivo* effectiveness of different SMS solutions. This might be due to the reason that the linear dose-response is hardly established even for the *in vitro* antioxidant assays. For example, apparent hydroxyl radical scavenging activity is usually saturated at around 50% inhibition level even with increasing concentrations of test samples when determined by ESR using Fenton system as a radical generator (unpublished observation). It was also noted that the *in vivo* prevention was almost completely attained even with the SMS preparations showing rather low antioxidant activity *in vitro* (see Fig. 3).

Present study suggested that inhibitory activity of crocin bleaching and superoxide scavenging activity are rather reasonable index for the evaluation of the *in vivo* antioxidant potential of mixed formula like TCM *in vivo*. Moreover, attention must be paid to the activity range of the *in vitro* antioxidant index that is applicable for the quantitative estimation of the *in vivo* antioxidant potential of certain mixed formula.

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