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Full Length Research Paper

Antioxidant ability of squid ink polysaccharides as well as their protective effects on deoxyribonucleic acid DNA damage *in vitro*

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Squid ink polysaccharides (SIPS) were isolated from squid ink, a plentiful and multifunctional marine material, and were demonstrated to have amelioratory effects on cyclophosphamide-induced damage in internal organs of model animals by our previous reports. To further investigate the protective effects of SIPS on chemotherapeutic damage caused by cyclophosphamide, this paper evaluated the bioactivities of the marine polysaccharides with a view to their antioxidant ability and their protective effects on deoxyribonucleic acid (DNA) damage using tests such as hydroxyl radicals, reducing power assay and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals, and pEGFP-N1 plasmid DNA treated with ultra violet (UV) plus H_2O_2. Data revealed that SIPS not only quenched hydroxyl radicals and 1,1-Diphenyl-2-picrylhydrazyl radicals, but showed its strong reducing power and also suppressed oxidative scission on plasmid DNA strand caused by hydroxyl radicals which originated from H_2O_2 shown by UV.

Key words: Squid ink polysaccharides (SIPS), antioxidant ability, deoxyribonucleic acid (DNA) damage

INTRODUCTION

It is well known that reactive oxygen species (ROS) produced in cells may induce oxidative damage to various biomacromolecules in cells, such as polysaccharide, protein, lipid and deoxyribonucleic acid, which results in metabolic and functional disturbance in cells and in turn leads to various pathological changes, for example aging and cancer, two major problems for human originated from ROS induced oxidative stress and deoxyribonucleic acid (DNA) damage (Cerutti, 1994; Wiseman and Halliwell, 1996). Thus quenching ROS in cells must be an effective way to prevent ROS-mediated oxidative damage induced by cellular metabolism and exogenous agents, especially some oxidative drugs such as cyclophosphamide, a most commonly used chemotherapeutic agent for cancer, which is also used in the treatment of some connective tissue and autoimmune diseases, minimal lesion glomerulonephritis, and for the control of organ rejection after transplatation (Emadi et al., 2009). Owing to the strong negative effects of cyclophosphamide-induced ROS on normal tissues except for its positive roles on tumor tissues (Emadi et al., 2009), resultant insufficient dose of cyclophosphamide results in an unfavourable therapeutic effect on cancer. So, development and application of natural antioxidants would be helpful to impair oxidative damage induced by cyclophosphamide and in turn improve the therapeutic effect of the chemotherapeutic agent on tumor.

It was reported that squid ink polysaccharides (SIPS) is a type of glycosaminoglycon with unique structure, $[3GlcA\beta1\text{-}4(GalNAc\alpha1\text{-}3)\text{-}Fuc\alpha1]_n$ (Takaya et al., 1996; Chen et al., 2008) and has been unveiled in our previous investigation (Liu et al., 2012) that the bioactive polysaccharides could increase antioxidant ability of some tissues including liver, heart, lung and kidney of model

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animals treated with cyclophosphamide, which implied that SIPS protected normal tissues of model animals from damage induced by chemotherapeutic drugs. Although the antagonism of SIPS on cyclophosphamide-induced oxidative stress has been discovered *in vivo*, the direct antioxidation of the natural marine polysaccharides is still unknown to us. In the present paper, *in vitro* investigation was carried out on the free radical scavenging ability of SIPS on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radicals and hydroxyl radicals, as well as its protective effects on DNA damage in a systematic way. The results should be beneficial to further elaborate the protective effects of SIPS on chemotherapeutic damage induced by cyclophosphamide, and improving the development of natural marine cytoprotectant as well as its clinical application for treatment on tumor.

MATERIALS AND METHODS

General

GeneJET™ polymerase chain reaction (PCR) Purification Kit was purchased from Fermentas, Ontario, Canada. Biowest Regular Agarose G-10 was produced in Spain. Papain and DPPH were purchased from Sigma. Red prussiate of potash was produced by Tianjin Hongyan Chemical Co. Trichloroacetic acid was from Shanghai Lingfeng Chemical Co. Ltd. $FeCl_3$ and EDTA-Na_2-Fe were from Guangzhou Chemical Co. Rromopyrogallol red was produced by Tokyo Chemical Industry Co. Ltd. ChemiDoc™ XRS+ System was purchased from Bio-Rad Lab. Inc. USA.

Preparation of SIPS

Fresh squid ink sacs collected from local market of aquatic products were stored at -28°C before use. SIPS was prepared via this procedure: ink harvested from dissected sacs thawed at 4°C was suspended into phosphate buffer (pH 6.7), and was then well grinded. After ultrasonic treatment, the ink solution was stored at 4°C for 24 h and was then centrifugated at speed of 14,000 g for 1 h at 4°C.

The supernatant was digested with 1% papain in phosphate buffer (pH 6.7) at 60°C for 24 h, mixed with 1/4 volume liquid mixture of chloroform and n-butanol (v/v, 4/1) followed by stirring for 30 min on a magnetic stirrer. After centrifugation at 5,000 g for 15 min, the supernatant was digested with papain again, the digestion process was performed twice. SIPS in the resultant supernatant was precipitated with four volumes of absolute alcohol and was dried at 50°C.

Reducing power assay

The mixture containing SIPS solution (scheduled concentrations, 2 ml), phosphate buffer saline (PBS) (pH 6.6, 2.5 mol/L, 2 ml) and $K_3Fe(CN)_6$ solution (1%, 2 ml) was heated in water bath at 50°C for 20 min, cooled with ice water and then mixed with trichloroacetic acid (10%, 2 ml) followed by centrifugation at 3,000 g for 10 min. Distilled water (2 ml) and $FeCl_3$ solution (0.1%, 0.4 ml) were in turn added into 2 ml of supernatant. After 10 min, optical density (OD) values at wavelength of 700 nm were measured (Shimada et al., 1992).

Detection of scavenging ability on hydroxyl radicals

SIPS (1 ml) and H_2O_2 (2%, 1 ml) were in turn added into Tris-HCl buffer (0.15 mol/L, pH 8.2, 3 ml) containing bromopyrogallol red (1 mmol/L, 1 ml) and EDTA-Na_2-Fe (1 mmol/L, 1 ml). Distilled water was used instead of SIPS in blank tube, and instead of SIPS and EDTA-Na_2-Fe in control tube. After 30 min, OD values at wavelength of 520 nm were measured. Scavenging ratio was calculated as follows:

$$\text{Scavenging ratio} = (OD_{BT} - OD_{ST}) / OD_{BT} \times 100\%$$

where BT = blank tubes, ST = sample tubes

Detection of quenching ability on DPPH radicals

The mixture containing DPPH (0.2 µmol/L, 2 ml) and SIPS (2 ml) was darkly incubated for 30 min. Optical density (OD) values at wavelength of 520 nm were measured. Scavenging ratio was calculated as the follows:

$$\text{Scavenging ratio} = (OD_C - OD_I) / OD_C \times 100\%$$

where C = absolute alcohol plus DPPH solution, I = SIPS solution plus DPPH solution.

Detection of DNA cleavage

The present DNA damage protection experiment was performed according to the reported methods that was modified slightly (Kumar and Chattopadhyay, 2007). Plasmid pEGFP-N1 DNA isolated with GeneJET™ PCR Purification Kit from *Escherichia coli* DH5α was used in this trial. A volume of 10 µl in a microfuge tube containing about 100 ng of plasmid DNA, H_2O_2 (100 mmol/L) and different final concentrations of SIPS were used. The reaction was initiated by UV irradiation for 2 min at room temperature. The reactive mixtures were subjected to electrophoresis in agarose gel and then stained with ethidium bromide followed by densitometric analysis.

Statistical analysis

Experimental data were analyzed by analysis of variance (ANOVA) using the JMP 7.0 statistical software. Results were expressed as the maen ± standard deviation (SD). Differences were separated by Duncan's multiple range test. Significance was considered at $p < 0.05$ or 0.01.

RESULTS

In present study, we investigated antioxidant ability of SIPS *in vitro*. Firstly, the total reducing power of the marine bioactive polysaccharides was analyzed in this paper. Data presented in Figure 1 showed a marked reducing power of SIPS on Fe^{3+} in a dose-dependent manner, which indicated that SIPS has an ability of maintaining systemic reduction state.DPPH is a stable organic free radicals, the stability originates from spatial obstacles of benzene ring that block the pairing process of the odd electron in central nitrogen atom. Based on a strong absorption of DPPH odd electron at wavelength of

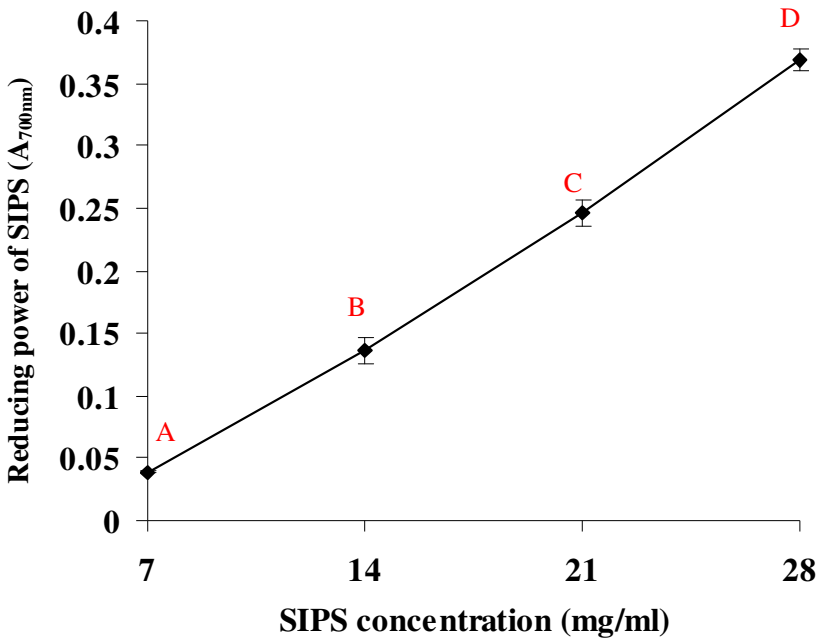

Figure 1. Reducing power of SIPS. The results are expressed as the optical density value in absorbance at 700 nm. Each value represents the mean ± SD of the four experiments.

517 nm and the purple characteristic of DPPH in ethanol solution, in the presence of free-redical scavengers, the odd electrons are paired off and the absorption decreases gradually, the resultant decolorization rate is stoichiometric in accordance to the number of electrons taken up. Because of the relatively short detection time of the assay method, we measured the scavenging ability of SIPS on DPPH radicals. Results showed an increasingly quenching activity on DPPH radicals in a positive dose dependent response to the scheduled doses of SIPS (Figure 2).

Hydroxyl radical is the strongest oxidant among all of free-radicals and can almost react with any macro-molecule, which leads to body damage and gene mutation resulting in aging and tumorigenesis. This experiment detected scavenging ability of SIPS on hydroxyl radical originated from H_2O_2 catalyzed by Fe^{2+} using Fenton reaction. Since hydroxyl radical can decolorize bromopyro-gallol red, hydroxyl radical content can be measured with Fenton reaction with respect to decolorization rate. The detection results are presented in Figure 3. The addition of SIPS produced a dose-dependent increment of scavenging activity on hydroxyl radicals.

This experiment is also designed to investigate the protective effects of SIPS on DNA damage induced by free radicals except for the aforementioned antioxidation. The protective roles of SIPS on the damage caused by H_2O_2 and UV together was performed on pEGFP-N1 plasmid DNA. Figure 4 shows the agarose gel electrophoretic pattern of DNA treated with different manner, in Figure 5 the results are expressed as percentage of optical density value of one DNA band with respect to total optical density value of three DNA bands in the plasmid lane. DNA prepared with kit from *E. coli* DH5α was observed with three bands in the agarose gel (lane 1), the fastest moving prominent band was supercoiled plasmid DNA (SC-DNA), the faster moving band was linear DNA (LIN-DNA) and the top faint band was the slowest moving open circular plasmid DNA (OC-DNA). Lanes 3 and 4 showed an unobvious changes compared with control DNA (lane 1), which indicated that UV irradiation or H_2O_2 did not obviously affect normal double helix structure of DNA. However both UV irradiation and H_2O_2 co-treatment resulted in marked decrease of SC-DNA and serious increase of the two abnormal DNA, LIN-DNA and OC-DNA (lane 2).

It is clear that normal SC-DNA was damaged by the combined action of the two physical and chemical factors, which showed hydroxyl radicals generated from H_2O_2 photolyzed by UV seriously cut single chain or double chains, especially double chains of SC-DNA. Also, the two figures showed that SIPS significantly impaired the damage on native SC-DNA induced by hydroxyl radicals generated from UV-photolyzed H_2O_2 in a dose-dependent manner. The low dose of SIPS (lane 5) hardly produced protective effects on the DNA damage, the percentage of densitometric units of SC-DNA in this lane were almost similar to that of the unprotected co-treated DNA in lane 2 but with the increase of SIPS concentrations, the percentage of SC-DNA increased markedly and the ratio of OC-DNA or LIN-DNA declined sharply.

Summarily, the results reveal that the marine bioactive

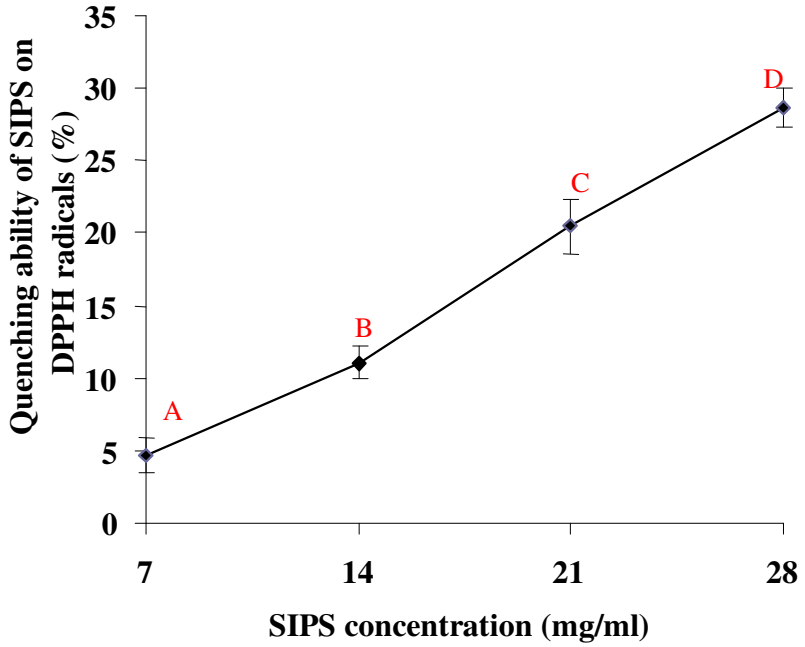

Figure 2. Scavenging ability of SIPS on DPPH radicals. The results are expressed as percentage of in absorbance at 520 nm with respect to control. Each value represents the mean ± SD of the four experiments.

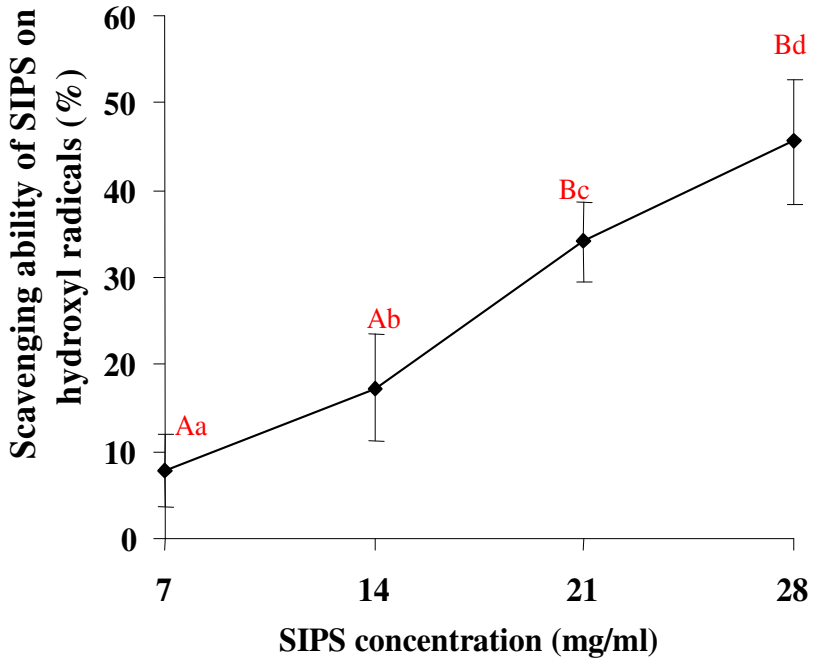

Figure 3. Scavenging ability of SIPS on hydroxyl radicals. The results are expressed as percentage of in absorbance at 520nm with respect to control. Each value represents the mean ± SD of the four experiments.

polysaccharides SIPS not only has strong scavenging activity on DPPH and hydroxyl radicals as well as total reducing power which are collectively called antioxidant ability, but also seriously protects DNA from oxidative damage induced by free-radicals originated from combined action of UV and H_2O_2.

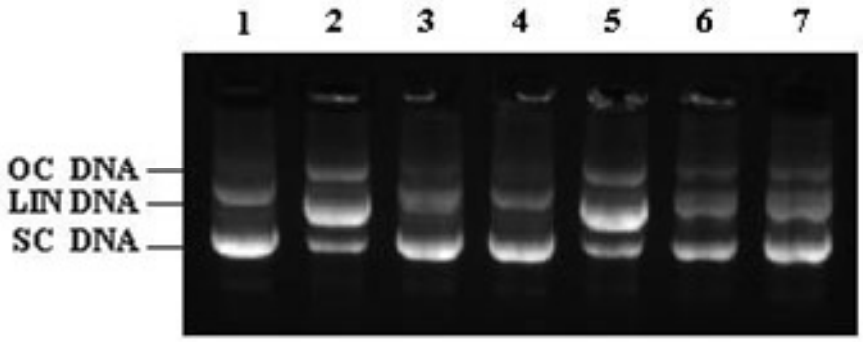

Figure 4. Protective effects of SIPS on DNA damage. DNA was treated by different treatment manners for 2 min, lane 1 to 7 represent treatment manners as follows. 1: control, 2: UV + H_2O_2, 3: UV only, 4: H_2O_2 only, 5: SIPS (1.75 mg/ml) + UV + H_2O_2, 6: SIPS (7.0 mg/ml) + UV + H_2O_2, 7: SIPS (28.0 mg/ml) + UV + H_2O_2. OC, LIN and SC represent open circular, linear and supercoiled DNA, respectively.

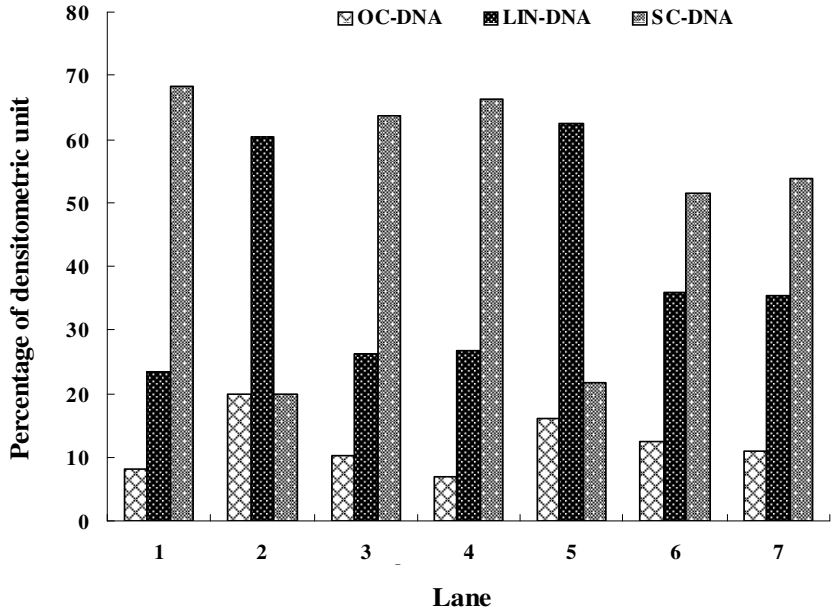

Figure 5. Densitometric analysis of supercoiled, linear and open circular plasmid DNA after different treatment. The value was represented with percentage between densitometric unit of each DNA and total densitometric unit of three DNAs. Lane 1 to 7 represent treatment manners as follows. 1: control, 2: UV + H_2O_2, 3: UV only, 4: H_2O_2 only, 5: SIPS (1.75 mg/ml) + UV + H_2O_2, 6: SIPS (7.0 mg/ml) + UV + H_2O_2, 7: SIPS (28.0 mg/ml) + UV + H_2O_2. OC, LIN and SC represent open circular, linear and supercoiled DNA, respectively.

DISCUSSION

Since 1950s, radiation biologists have discovered that ionizing radiation acting on organism can lead water to produce reactive oxygen species which in turn result in damage of biomacromolecules, but regretfully considered the phenomenon as an instantaneous reaction all the time. Until 1968 superoxide dismutase and its derivatives functioning on scavenging ROS was found, it was realized that ROS is constantly being created and scavenged in organism which mediates ROS to normal level of free radical homeostasis (Fang et al., 2004). However, under the circumstances such as aging, diseases or special physiology, the ROS can cause body injury. For instance, cancer patient inevitably accepts clinical chemotherapy, although cyclophosphamide is one of the most successful and widely utilized antineoplastic drugs, a well-known severe side effect of the drug is wide

oxidative damage in patient body caused by cyclophosphamide-induced oxidative stress (Emadi et al., 2009). Recently, in model animals we found that cyclophosphamide serviously reduced activities of anti-oxidative enzymes and elevated contents of 3,4-methylenedioxyamphetamine (MDA) and nitrogen oxide (NO) in blood/organs (Liu et al., 2012; some data unpublished). So eliminating ROS is an important pathway to improve chemotherapeutic effects.

Squid ink has been proved to be a multifunctional marine bioactive substance, such as antitumor (Sasaki et al., 1997; Chen et al., 2010), hemostasis (Xie et al., 1994), antioxidation (Liu et al., 2011; Lei et al., 2007), antiretroviral (Rajaganapathi et al., 2000) and anti-bacterial effect (Sadok et al., 2004) etc. In recent years, we discovered the alleviatory effects of squid ink on oxidative damage induced by cyclophosphamide (Wang et al., 2009, 2010) and polysaccharide in the dark ink was screened to be an active component. Our previous report (Liu et al., 2012) revealed that SIPS effectively suppressed cyclophosphamide-induced oxidative stress on partial internal organs and impaired chemotherapeutic damage, the antioxidation of SIPS was supported by another direct evidence *in vitro* in the present paper that showed SIPS with strong reducing power which effectively quenched hydroxyl and DPPH radicals.

It is documented that a cause of cyclophosphamide-induced DNA damage is put down to the two metabolites of the antineoplastic drug, nitrogen mustard and acrolein. The former binds covalently to DNA and induces DNA damage in the form of strand breaks through alkylation of DNA at the N_7 position of guanine, DNA-DNA cross-links, both interstrand and intrastrand, as well as DNA-protein cross-links (Emadi et al., 2009), and the latter inactivates O_6-methylguanine-DNA methyltransferase, a DNA repair protein. Another important reason of DNA damage mediated by cyclophosphamide is that although H_2O_2 and superoxide anion have potential cytotoxicity, hydroxyl radical generated from H_2O_2 and superoxide anion catalyzed by metal ions is the major oxidant causing oxidative damage in organism and is a cause of DNA strand scission in cellular damage (Wiseman et al., 1996; Guitteridge, 1984). To find natural material able to control oxidative DNA damage, the effetcs of SIPS on DNA cleavage were investigated. The results indicated that addition of SIPS suppressed scission of SC-DNA and formation of LIN-DNA and OC-DNA.

Summarily, our investigation unveils a direct evidence of antioxidation and protective effects on DNA damage of SIPS *in vitro*. The results may be helpful in development of SIPS as a cytoprotector, as well as its application in clinical chemotherapy on tumor. It is necessary to see that although this preliminary study described antioxidant ability and protection on DNA damage of SIPS *in vitro*, and our previous reports revealed antioxidant ability of the marine polysaccharides *in vivo*, regretfully, we cannot merge both of them into one conclusion, since we do not presently know what molecular forms SIPS employed to exert its antioxidative function *in vivo*. So it is important for us to further investigate mechanisms of SIPS-induced antioxidation *in vivo.*

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