ANTIOXIDANT AND β-GLUCURONIDASE INHIBITORY ACTIVITIES OF THE INTERNAL ORGAN EXTRACT FROM SEA CUCUMBER *STICHOPUS JAPONICUS*

HOẠT TÍNH CHỐNG OXY HÓA VÀ ỨC CHẾ ENZYME β-GLUCURONIDASE CỦA DỊCH CHIẾT TỪ NỘI TẠNG HẢI SÂM *STICHOPUS JAPONICUS*

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ABSTRACT

Antioxidants and β-glucuronidase inhibitors have been used for treatment and prevention of various human diseases. The study aimed to investigate the antioxidant and β-glucuronidase inhibitory activities of the aqueous methanolic extract (80% MeOH) and its solvent fractions (*n*-hexane, $CHCl_3$, EtOAc, BuOH, and water) prepared from the internal organs of sea cucumber *Stichopus japonicus*. The antioxidant and β-glucuronidase inhibitory properties varied between the solvent extract and fractions. The $CHCl_3$ fraction showed the most potent DPPH scavenging activity; whereas, the EtOAc fraction exhibited the highest hydroxyl peroxide scavenging and lipid peroxidation inhibitory activities. The $CHCl_3$ and EtOAc fractions significantly inhibited β-glucuronidase. The results obtained from the present study suggested that the *S. japonicus* internal organ extracts might be possible new sources of antioxidant and β-glucuronidase inhibitors suitably used for prevention of certain cardiovascular diseases and cancers.

Keywords: *Antioxidant, β-glucuronidase inhibitor, internal organs, Stichopus japonicus.*

TÓM TẮT

Các chất chống oxy hóa và chất ức chế enzyme β-glucuronidase đã được sử dụng để ngăn ngừa và điều trị nhiều loại bệnh của con người. Mục đích của nghiên cứu này là đánh giá hoạt tính chống oxy hóa và ức chế enzyme β-glucuronidase của dịch chiết (80% methanol) và các phân đoạn dịch chiết (*n*-hexane, $CHCl_3$, EtOAc, BuOH và nước) từ nội tạng của hải sâm *Stichopus japonicus*. Kết quả nghiên cứu cho thấy, có sự khác nhau về hoạt tính chống oxy hóa và ức chế enzyme β-glucuronidase giữa các phân đoạn dịch chiết. Phân đoạn $CHCl_3$ có hoạt tính khử gốc tự do DPPH mạnh nhất; trong khi đó, hoạt tính khử hydrogen peroxide và ức chế quá trình peroxy hóa lipid của phân đoạn EtOAc đạt cao nhất. Phân đoạn $CHCl_3$ cho hoạt tính ức chế mạnh enzyme β-glucuronidase và cao nhất trong các phân đoạn dung môi chiết. Từ kết quả nghiên cứu đạt được cho thấy dịch chiết từ nội tạng hải sâm *S. japonicus* có thể là nguồn nguyên liệu tiềm năng sử dụng để ngăn ngừa các bệnh về tim mạch và ung thư.

Từ khóa: *Chất chống oxy hóa, chất ức chế enzyme β-glucuronidase, nội tạng, Stichopus japonicus.*

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1. INTRODUCTION

The liver is an important organ that is involved in the maintenance of metabolic functions and helps in detoxification processes [1]. Live glucuronidation is the major pathway responsible for endogenous and xenobiotic metabolism of potentially damaging compounds such as pollutants, drugs, bile acids, steroids etc. leading to their elimination from the body [2].

β-Glucuronidase (E.C.3.2.1.31) is one of the most extensively studied enzymes, it is present in many organisms including plants, animals, humans, bacteria [3]. It is responsible for the elimination of potentially toxic compounds from the body as glucuronides [3]. However, excessive expression of this enzyme in the gut is capable of retoxifying compounds that have been detoxified by liver glucuronidation [4]. It has been demonstrated that increased activity of β-glucuronidase in the blood could result several diseases such as Crohn's disease [5], liver cancer and liver damage [6] and colon cancer [7]. Inhibition of bacterial β-glucuronidase in the intestine will promote excretion of xenobiotics and thus decrease their toxicity [8]. Therefore, it is important to find specific inhibitors of this enzyme that will serve hepatoprotective functions with little or no side effects.

Research has demonstrated that oxidation degeneration by free radicals is one of the leading causes of some chronic diseases including cancer and cardiovascular diseases in humans [9]. Antioxidant compounds can be used to control the

production of free radical, which may inhibit the lesions produced by these substances [10]. Natural antioxidants are generally more suitable for human consumption than synthetic ones such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), some undesirable side effects [11]. Thus, it is necessary to search for new natural antioxidants which are safe and inexpensive.

Sea cucumbers are marine invertebrates of the phylum Echinoderm and the class Holothuroidea, found on the sea worldwide [12]. They are one of the most important marine organisms, they have been used traditionally in the treatment of some diseases such as hypertension, eczema and cancer because of their high nutraceutical value [13]. *Stichopus japonicus*, a widespread sea cucumber species found in East Asia is one of the highest commercially valuable species as seafood [14, 15]. The body wall of this species is the major edible part and has been known to possess antifungal, antioxidant and anticoagulation activities [16, 17]. In Japan, a fermented delicacy, *Konowata*, is prepared from the visceral mass of *S. japonicus* [18]. There is limited literature available on investigations of the constituents of *S. japonicus* internal organs except for a report observed in *Konowata*, which analyzed the fatty acid composition of *Konowata*. So far, there is limited information concerning the health-beneficial effects of the internal organs of *S. japonicus*.

Therefore, considering that a considerable amount of bioactive compounds can be found in the sea cucumber waste materials, this study aimed at investigating the antioxidant potential and β-glucuronidase inhibitory activities of the aqueous methanolic extract and its solvent fractions prepared from internal organs of *S. japonicus*.

2. MATERIALS AND METHODS

2.1. Materials

Live specimens of sea cucumber *S. japonicus* were purchased from a local fishery market (Gangneung, Korea). The samples were authenticated by sea cucumber experts of the Department of Marine Food Science and Technology, Gangneung-Wonju National University Gangneung-Wonju National University [17]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, ascorbic acid, butylated hydroxytoluen (BHA), saccharic acid 1,4-lactone, β-glucuronidase, and *p*-nitrophenyl-β-D-glucuronide (*p*-NPG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

2.2. Sample preparation and extraction procedures

The internal organs of sea cucumber were collected and washed with tap water, followed by lyophilization. The lyophilized sample was ground to fine powder. The powder of sea cucumber internal organs (10g) was extracted with 80% MeOH (200ml) for 5h at room temperature with a magnetic stirrer. Extraction of the residue was repeated twice under the same conditions. The supernatant and the sediment were separated by filtration with an Advantec No. 5C filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The combined three filtrates were evaporated and concentrated under vacuum in a rotary evaporator at < 40°C (Buchi RE121 Rotavapor, Swizland). The concentrated extract was suspended in water and then partitioned with a series of solvent partitions. At first, the extract (200ml) was partitioned using *n*-hexane (200ml) by standing for 30 min at room temperature after vigorous shaking (*n*-hexane fraction), which was repeated 3 times until any color in the *n*-hexane layer was absent. The aqueous layer of the *n*-hexane fraction was then partitioned using $CHCl_3$, EtOAc, and BuOH in the same procedures as mentioned above. The combined organic layers for each solvent were concentrated to dryness in *vacuo* at < 40°C. The left-over aqueous layer was lyophilized to yield the aqueous fraction (water fraction). Dry extracts were stored in glass bottles at -20°C until analyses.

2.3. Determination of DPPH radical scavenging activity

Scavenging activity on DPPH radical was determined according to the method of Pendota et al. [19]. Briefly, 3ml the extract at different concentrations was mixed with 1ml of DPPH solution (0.1mM) in ethanol. The mixture was then vortexed vigorously and incubated at room temperature for 30 min in the dark. The absorbance of each sample solution was measured at 517nm using the spectrophotometer. Ascorbic acid and BHT were used as a positive control. A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as A_{blank}. The ability to scavenge the DPPH radical was calculated as percent DPPH scavenging using the following equation:

DPPH radical scavenging activity (%) = $[A_{blank} - A_{sample}]/A_{blank} \times 100$

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentrations (0 - 100µg/ml). Tests were carried out in triplicate.

2.4. Determination of antioxidant assay using β-carotene linoleate model system

The antioxidant activity of the sea cucumber internal organ extract and fraction was determined by the β-carotene-linoleate model system [19]. β-Carotene (0.2mg), 20mg of linoleic acid and 200mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed in 0.5ml of chloroform. Chloroform was removed at 40°C under vacuum using a rotary evaporator. The resulting mixture was immediately diluted with 10ml of triple-distilled water and was mixed well for 1 - 2min. The emulsion was further made up to 50ml with oxygenated water. Aliquots (4ml) of this emulsion were transferred into different test tubes containing 0.2ml of test samples (0.5mg/ml) in ethanol. A control, containing 0.2ml of ethanol and 4ml of the above emulsion, was prepared. The tubes were placed at 50°C in a water bath. Absorbances of all

the samples at 470nm were taken at zero time (t = 0). Measurement of absorbance was continued until the colour of β-carotene disappeared in the control reaction (t = 180 min) at 15 min intervals. A mixture prepared as above without β-carotene was used as the blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula:

AA= $[1-(A_o-A_t)/(A°_o-A°_t)]\times100$, where A_o and $A°_o$ are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and $A°_t$ are the absorbance measured in the test sample and control, respectively, after incubation for 180 min.

2.5. Determination of hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured according to the previous method [20]. The test sample (0.5mg/ml), dissolved in ethanol, was mixed with 100µL of phosphate buffer (0.1M, pH 5) and 20µl of hydrogen peroxide (10mM) in a 96-well microplate and then incubated at 37°C for 5 min. And then ABTS (30µl, 1.25mM) and peroxidase (30µl, 1U/ml) were added to the mixture, which was incubated at 37°C for 10 min. The absorbance was read with microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 405mn to determine the extent of hydrogen peroxide scavenging activity.

2.6. Determination of β-glucuronidase activity

β-Glucuronidase inhibitory activity was determined according to the modified method [21]. A 0.1ml of 10mM *p*-NPG as a substrate in 0.01M phosphate buffer (pH 7) was premixed with 2.2ml of sample solution and 0.1ml of 20U/ml β-glucuronidase in the same buffer was added to the mixture to start the reaction. The reaction was carried out at 37°C for 60 min and stopped by adding 1.5ml of 0.1M Na_2CO_3. Enzymatic activity was quantified by measuring the absorbance at 405nm. One unit of β-glucuronidase activity was defined as the amount of enzyme liberating 1.0µM of *p*-nitrophenol per min. One unit of β-glucuronidase inhibitory activity was defined as one unit decrease of β-glucuronidase activity.

2.7. Statistical analysis

Data were analyzed with Duncan's multiple comparison test ($p < 0.05$) using the SPSS software package version 10.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. DPPH radical scavenging activity

The DPPH method is described as a simple, rapid and convenient method due to independent of sample polarity for screening of many samples for radical scavenging activity [22]. The dose-response curve of DPPH scavenging activities of the extract/fractions is presented in Figure 1. At 500µg/ml, the scavenging abilities on DPPH radicals were 78.52, 46.81, 95.92, 65.24, 72.87 and 49.06% for the 80% MeOH extract, *n*-hexane, $CHCl_3$, EtOAc, BuOH and water fractions, respectively. At the same concentration, the scavenging abilities of ascorbic acid and BHA, the well-known antioxidants, were 96.6 and 83.5%, respectively. In order to compare the DPPH scavenging potencies among the extract/fractions, the concentration required to inhibit 50% radical-scavenging effect (IC_{50}) was determined from the results of a series of concentrations tested. The scavenging activity of the extracts on DPPH increased in the order of the $CHCl_3$ fraction (63.18µg/ml) > 80% MeOH extract (85.79µg/ml) > EtOAc (184.73µg/ml) > BuOH (197.16µg/ml) > water (465.32µg/ml) > *n*-hexane (531.90µg/ml). In order to investigate the contribution of phenolic constituents to the DPPH scavenging ability of the extract and fractions, $1/IC_{50}$ for DPPH radical scavenging was plotted to total phenolic contents (data not shown). According to this result, the DPPH radical scavenging activity does not correlate with the total phenolic contents determined in the extract/fractions (R^2 = 0.012, $p < 0.05$). This result also agrees well with the report of Zhong et al. [23] who found that no correlation existed between radical scavenging capacity of fresh and processed sea cucumber (*Cucumaria frondosa*) extracts and total phenolic contents. However, Shetty and Sibi [24] mentioned the strongest positive correlation found between total phenolics and DPPH activity in *C. vulgaris* (r = 0.997). Husni et al. [25] also showed that total phenolic contents of the *S. japonicus* body wall extracts had significantly correlation (R^2 = 0.73) with their DPPH radical scavenging activities.

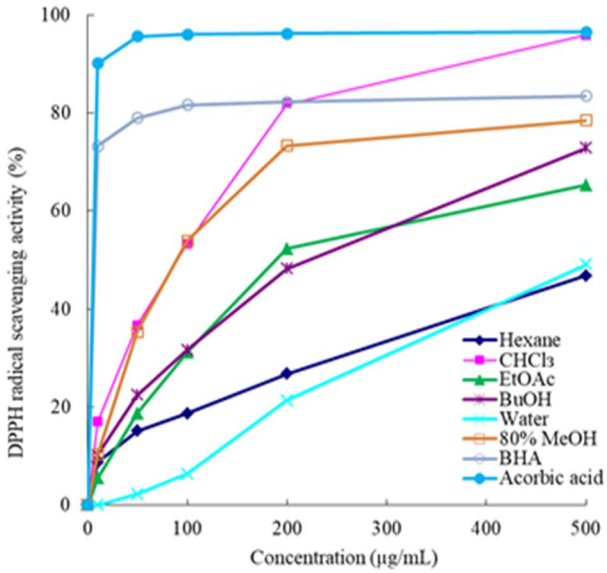

Figure 1. DPPH radical scavenging activities of the solvent-partitioned fractions from sea cucumber internal organ at different concentrations

3.2. Hydroxyl peroxide scavenging activity

The reactive oxygen species (ROS) such as superoxide anion ($O2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) are known to cause oxidative damage, contributing to the development of chronic

diseases such as cancer, heart disease, and cerebrovascular disease [25]. The ability of crude extract and its solvent partitioned fractions from *S. japonicus* internal organs to scavenge hydroxyl peroxide is presented in Figure 2. Hydroxyl peroxide scavenging activities of 80% MeOH extract, *n*-hexane fraction and EtOAc fraction were 100% at 0.5mg/ml, which were similar to that of BHA. These extracts and fractions showed significantly ($p < 0.05$) higher hydroxyl peroxide scavenging activities than those of BuOH, $CHCl_3$, and water fractions. *n*-Hexane and EtOAc fractions showed stronger hydroxyl radical scavenging activity than other fractions. This indicated that not only phenolic compounds but other compounds such as fatty acids, chlorophylls, etc. also contributed to antioxidant activity on the term of hydroxyl peroxide scavenging activity [26, 27].

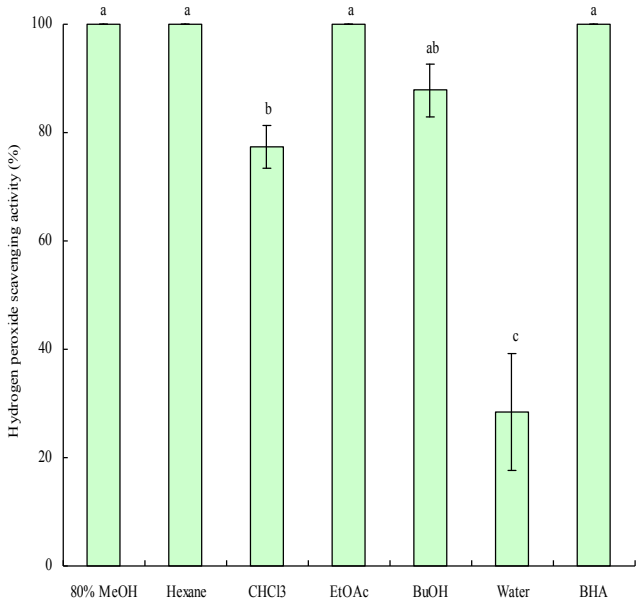

Figure 2. Hydroxyl radical scavenging activity of the solvent-partitioned fractions of sea cucumber internal organ. [a-d]Different superscripts on the bars indicate significant difference ($p < 0.05$)

3.3. Antioxidant activity in β-carotene/linoleic acid emulsion system

Cell membranes are composed of phospholipid bilayers, with intrinsic/extrinsic proteins, and are direct targets for lipid oxidation by reactive oxygen species. In β-carotene/linoleic acid model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant [28]. Figure 3 shows the antioxidant activities of *S. japonicus* internal organs extract/fractions in comparison with BHA at the concentration of 0.5mg/ml. Their antioxidant powers were ranked in the order: BHA > EtOAc fraction > *n*-hexane fraction > 80% MeOH extract ≈ BuOH fraction > water ≈ $CHCl_3$ fraction. The inhibition ratio of EtOAc fraction (90.63%) was found to be the greatest, and almost equal to the inhibition capacity of the positive control BHA (98.33%). In contrast, $CHCl_3$ and water fractions showed the weakest antioxidant activity potential in this system with the inhibition rate of 20.49 and 28.55%, respectively. This result was different from radical and peroxide scavenging activity tests (DPPH and hydrogen peroxide), recommending that antioxidant activities of sea cucumber internal organs should be evaluated by different methods than depending on the results of a single method [29]. These results suggest that the antioxidant activity of the extract/fractions might be attributed to the presence of non-phenolic compounds. On the other hand, their antioxidant activities may depend on the basis of high antioxidant activity of some individual phenolic units, which may act as efficient antioxidants rather than contributing to high total phenolic contents [30].

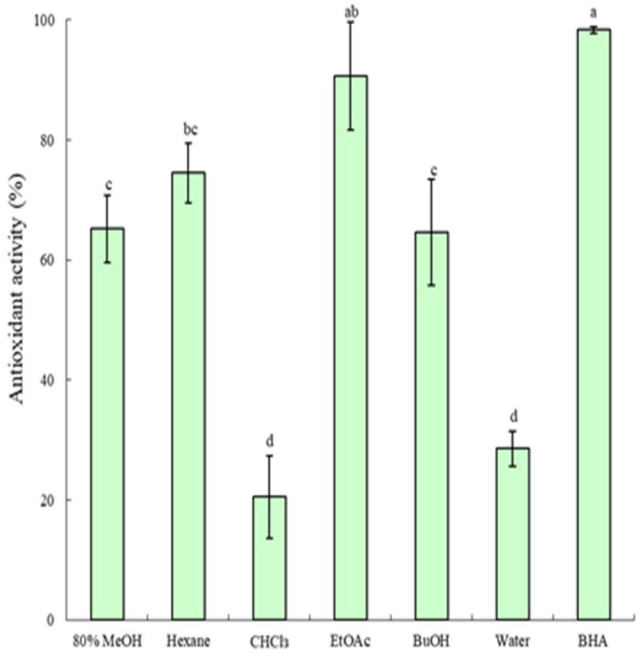

Figure 3. Antioxidant activity of the solvent-partitioned fractions from sea cucumber internal organ in the β-carotene/linoleic acid system. [a-d]Different superscripts on the bars indicate significant difference ($p < 0.05$)

3.4. β-Glucuronidase inhibitory activity

β-Glucuronidase of intestinal bacteria is capable of retoxifying compounds that have been detoxified by liver glucuronidation, which is one of the most important detoxication processes in the liver. Therefore, this enzyme is known to accelerate colon cancer invasion and metastasis. Inhibition of bacterial β-glucuronidase in the intestine will promote excretion of xenobiotics and thus decrease their toxicity. β-glucuronidase inhibitors are mainly purified from terrestrial plants [31]. In previous studies, two β-glucuronidase inhibitors were purified and identified as the bromophenol from *G. elliptica* [21]. In present study, the β-glucuronidase inhibitory activity sea cucumber internal organs extract and its solvent fractions were investigated (Table 1). The inhibitory activity of aqueous methanol extract of *S. japonicus* internal organs against *E. coli* β-glucuronidase was 50.1% at 0.4mg/ml. In

the consecutive solvent fractionations of inhibitors, β-glucuronidase inhibitory activities of $CHCl_3$ fraction at the concentration of 0.01, 0.1 and 0.4mg/ml were 9.1, 49.4 and 79.7%, respectively, whereas those of EtOAc fraction were 9.5, 16.1 and 45.6%, respectively. At 0.4mg/ml, BuOH fraction inhibited β-glucuronidase by 22.8%. However, there were no β-glucuronidase inhibitory activities in *n*-hexane and water fractions. The β-glucuronidase inhibitory activity (79.7% at 0.4mg/ml) of $CHCl_3$ fraction of *S. japonicus* internal organs extract was much higher than that of EtOAc fraction (45.6% at 4mg/ml) of red alga, *G. elliptica* [21]. The β-glucuronidase inhibitory activities of various solvent fractions of sea cucumber internal organs extract were also compared with that of saccharic acid 1,4-lactone, a well-known β-glucuronidase inhibitor, with 50.1 and 80.2% inhibitory activity at 0.1 and 0.4mg/ml, respectively. This result suggested that the $CHCl_3$ fraction had inhibitory potency similar to that of saccharic acid 1,4-lactone.

The strong β-glucuronidase inhibitory activity exhibited by the $CHCl_3$ fraction from the extract of sea cucumber internal organs, may contain hepatoprotective effects as well as being a prodrug that is transformed to tectorigenin [32]. In order to understand the mechanisms of action, the identification and characterization of active compounds are currently underway in our laboratory. Furthermore, we are also attempting to find other biofunctional activities of sea cucumber *S. japnicus* internal organs.

Table 1. β-Glucuronidase inhibitory activities of the solvent-partitioned fractions from sea cucumber internal organs

Fractions	β-Glucuronidase inhibitory activity (%)		
	0.01mg/ml	0.1mg/ml	0.4mg/ml
n-Hexane	NI*	NI	NI
Chloroform	9.1 ± 0.6^a	49.4 ± 2.3^a	79.7 ± 0.5^a
Ethyl acetate	9.5 ± 2.0^a	16.1 ± 0.2^b	45.6 ± 0.1^b
Buthanol	NI	3.5 ± 1.7^c	22.8 ± 0.5^c
Water	NI	NI	NI
Saccharic acid 1,4-lactone	ND**	50.1 ± 2.0^a	80.2 ± 3.2^a

^{a,b,c}Means in the same column with different superscripts are significantly different ($p<0.05$).

**No inhibition.*

***Not determined.*

4. CONCLUSION

Free radicals and reactive oxygen species (ROS) play an important physiological role in many diseases, such as cancer, gastric ulcers, Alzheimer's disease, arthritis, renal injury, and ischemic reperfusion injury. All the extract/fractions of sea cucumber internal organs showed remarkable antioxidant activity, and the extent of which depends on the type of solvent used for the extraction as well as on the antioxidant test. The $CHCl_3$ fraction had the β-glucuronidase inhibitory potency comparable to that of saccharic acid 1,4-lactone, a well-known β-glucuronidase inhibitor. Therefore, *S. japonicus* internal organs could be used as a functional food ingredient and pharmaceutical to prevent oxidative stress related diseases and colon cancer.

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