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# Water-soluble carbohydrate compound from the bodies of Herba Cistanches: Isolation and its scavenging effect on free radical in skin

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ABSTRACT

A polysaccharide was isolated from the Herba Cistanches. This polysaccharides contained glucose, galactose, rhamnose, arabinose and fructose. The native polysaccharide could be fractionated by size exclusion chromatography into one major fraction. The apparent average molecular weight of the purified polysaccharide was estimated to be approximately 385 kDa by size-exclusion HPLC. Furthermore, this polysaccharides still exhibited strong antioxidant activities in aged rats' skin. Namely, Herba Cistanches polysaccharides inhibited the oxidative modification of lipids, thus protecting cells from injury by the oxidized molecules in aged rats' skin. The present results were in accordance with previous studies on the mode of the protective effect of the plant extract which showed positively that the protection may possibly be attributed to the combination of more than one biological activities and that the use of antioxidants might be an useful approach in the treatment of some skin aging diseases.



## 1. Introduction

The stems of Herba Cistanches, an important traditional Chinese medicine (TCM), has the function of strengthening the kidney and moistening dryness by nourishing blood in the accepted terminology of TCM. It is used primarily for kidney deficiency characterized by impotence, cold sensation in the loins and knees, female infertility and constipation due to dryness of the bowel among the aged population (The State of Pharmacopoeia Commission of the People's Republic of China, 2005). Two species, i.e. *Cistanche deserticola* Y.C. Ma and *Cistanche tubulosa* (Schenk) R. Wight, have been collected in the Chinese Pharmacopoeia (ChP, 2005; Sun et al., 2009; Tao, Zhang, & Zhang, 2009). However, another two *Cistanche* species, *Cistanche salsa* (C.A. Mey.) G. Beck and *Cistanche sinensis* G. Beck, are also used in some regions of China due to the resource shortage (Tu, He, & Lou, 1994). Herba Cistanches has various pharmacological activities, including antinociceptive, anti-inflammatory, and immuno-enhancing effects (Lin, Hsieh, Tsai, Wang, & Wu, 2002; Wu, Gao, Tsim, & Tu, 2005).

It is now accepted that free radical-mediated oxidation of biological molecules such as lipids, proteins, and DNA is involved in a variety of disorders and diseases (Halliwell & Gutteridge, 2007). Above all, lipids are very susceptible to free radical attack and lipid peroxidation induces alterations in integrity, disturbances in fine structure, and functional loss of biomembranes (Niki, 2009). Furthermore, lipid peroxidation mediated by free radicals proceeds by a chain mechanism, amplifying the damaging effect of free radicals (Niki, 2009; Yin & Porter, 2005). Lipid peroxidation products are potentially cytotoxic and modify proteins and DNA (Marnett, Riggins, & West, 2003). It is now accepted that lipid peroxidation is involved in the pathogenesis of various diseases (Yin, 2008), and consequently the role of free radical-scavenging antioxidants has received much attention. The aging process of the skin is a complex biological phenomenon and it can be divided into intrinsic and extrinsic aging. Intrinsic aging, which is largely genetically determined, affects the skin in a manner similar to most internal organs (Uitto, Matsuoka, & Kornberg, 1986; Amrani, Rhallab, Alaoui, Badaoui, & Chakir, 2009). Oxidized lipids and proteins induces alterations in skin conditions. Topical application of oxidized squalene (squalene monohydroperoxide) on the skin disrupts the skin barrier function as an acute response and induces skin roughness as a chronic response (Chiba, Kawakami, Sone, & Onoue, 2003). Alkyl aldehydes further oxidize lipid hydroperoxides and proteins to produce carbonylated proteins in the stratum corneum (SCCP). The SCCP levels increase following UV-exposure (Fujita, Hirao & Takahashi, 2007) and during the winter season (Kobayashi, Iwai, Akutsu, & Hirao, 2008). In addition, patients suffering from atopic

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dermatitis have higher levels of SCCP compared with normal subjects (Niwa et al., 2003). SCCP levels appear to reflect the degree of oxidative stress in the skin induced by the environment. Thus, oxidative stress initiated by ROS alters skin conditions.

Plants contain a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, cartenoids, dietary glutathionine, vitamins and endogenous metabolites and such natural products are rich in antioxidant activities (Cao, Sofic, & Prior, 1996; Hertog, Hollman, & Katan, 1992; Kivits, Vam der Sman, & Tijburg, 1997; Larson, 1988; Naim & Pasaribu, 2009; Magadula, Innocent, & Otieno, 2009). These plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists (Larson, 1988). Electron acceptors, such as molecular oxygen, react easily with free radicals to become radicals themselves, also referred to as reactive oxygen species (ROS). The ROS include superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^-$) (Grisham & McCord, 1986).

Therefore, the aim of the present research was to isolate and determine the structural features and antioxidant activity of the Herba Cistanches polysaccharides. The present paper is concerned with the isolation, chemical characterisation and evaluation of the inhibitory effect of Herba Cistanches polysaccharides on age-induced skin oxidative injury.

## 2. Materials and methods

### 2.1. Extraction and purification of polysaccharides

The Herba Cistanches (300 g) were extracted with 1 l of water at 100 °C for 2.5 h, repeated three times, combined the solution, filtrated and concentrated. The extract was submitted to graded precipitation with three volumes of ethanol. The precipitate was collected by centrifugation, washed successively with ethanol and acetone, and then dried at 45 °C under reduced pressure. The sample (32 g) was dissolved in $H_2O$ (400 ml), and then frozen, thawed gently, centrifugated to remove the precipitate. Freezing dry of the supernatant provided crude polysaccharide (Wagner et al., 2004).

The crude polysaccharide was deproteined according to the Sevage method (Staub, 1965). Briefly, the saturated solution of the crude polysaccharide was added to 20 mg proteinase (enzyme:protein = 1:50), dialyzed in distilled water at 37 °C for 16 h (changing water every 4 h), and then the sugar solution was treated with Sevage reagent (1-butanol:chloroform = 1:4) fully oscillated and centrifuged. The process was repeated until the supernatant was no free protein. The crude polysaccharide was further purified by column chromatography. Fifty milligrams of crude polysaccharide dissolved in 10 ml of $dH_2O$ was applied to a DEAE-cellulose column (3 cm × 45 cm) pre-equilibrated with water and eluted in NaCl gradient (0–3 M) until no carbohydrate was detected. Each fraction was assayed for carbohydrates content by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The carbohydrate-positive fractions were pooled together and dialyzed (MWCO 14,000) for 24 h against $dH_2O$ and lyophilized.

### 2.2. Capillary electrophoresis analysis

Polysaccharides samples (amounts between 0.2 and 4 mg) were taken up in 2 M trifluoroacetic acid (TFA) ($100^{-1}$ TFA/0.2 mg sample) and hydrolysed in capped glass vials for 2 h at 110 °C (Kharbade & Joshi, 1995). Optimum hydrolysis time is dependent on a balance between the rate of release of hydrolysable polysaccharides and the degradation of monosaccharides that occurs during prolonged treatment under the experimental conditions (Kennedy & Marsden, 1976).

After hydrolysis, the samples were frozen and then lyophilised over night at 200 mT and −56 °C. Then, 0.3 ml of the separation electrolyte was added to the dried samples, ultrasonicated for 1 min, the solution centrifuged for 2 min at 13,000 × *g* in a tabletop centrifuge (Eppendorf, model 5415D, Hamburg, Germany), and capillary electrophoresis analysis was carried out with the supernatant. The oils samples were saponified prior to CE as described (Surowiec, Kaml, & Kenndler, 2004).

### 2.3. Spectral analysis

The sample resolved with distilled water (0.5 mg/ml) was scanned on an Ultraviolet spectrophotometer from 190 nm to 400 nm.

Dried HCP and KBr were mixed, ground and squashed. IR spectroscopy was recorded on a Nicolet Nexus 5DXC FT-IR spectrometer (USA) within the range of 500–4000 $cm^{-1}$.

### 2.4. DPPH radical scavenging method

The method of Brand-Williams, Cuvelier, and Berset (1995) was used for measuring the DPPH radical scavenging ability of the compounds. DPPH was dissolved in methanol at a final concentration of about $6 \times 10^{-5}$ M. The exact concentration of DPPH was calculated from a calibration curve, $\varepsilon_{calc} = 11{,}870\ M^{-1}\ cm^{-1}$ at 515 nm ($\varepsilon$ = 12,509, Brand-Williams et al., 1995; $\varepsilon$ = 11,240, Goupy, Dufour, Loonis, & Dangles, 2003). Different aliquots of HCP and vitamin C were added to 2450 μl of DPPH solution and the volume adjusted to a final value of 2500 μl with methanol. Seven different concentrations were used for each assay. The decrease of the DPPH radical was followed at 515 nm, until the reaction reached a steady state. The thermally controlled multicell block was set at 25 °C. The final concentrations of DPPH at the steady state, corrected for the natural disappearance of the DPPH under the same conditions and after the same time intervals, were plotted as a function of the molar concentration ratio [AH]/[DPPH] to determine the Effective Concentration ($EC_{50}$).

### 2.5. Animal and polysaccharides treatments

Thirty-two male SD rats (age 15 months) were used. The animals were allowed free access to rat chow and water before polysaccharides treatment. After a 1-week period of adaptation, rats were randomly divided into untreated model group (group 2) and three polysaccharides-fed group. Each group contained eight rats. Rats were fed with standard diet containing 0.3, 0.5 and 0.7% polysaccharides, respectively. Another eight rats (age 3 months) were served as control group (group 1). The control and untreated model control groups were continuously administered by standard diet without polysaccharides. The experiment lasted for 7 weeks.

Rats were killed 6 and 24 h after polysaccharides treatment under ether anesthesia. Immediately after being killed, skin was isolated, washed well in ice-cold 0.15 M KCl, blotted on a filter paper, and weighed as soon as possible. The skins were stored at −80 °C until use. All animals received humane care in compliance with the guidelines of the Animal and Use Committee of China.

### 2.6. Statistical analysis

All experimental values were presented as mean ± standard error of mean (S.E.M.). Statistical analysis was performed with analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparison among the groups. Difference was considered

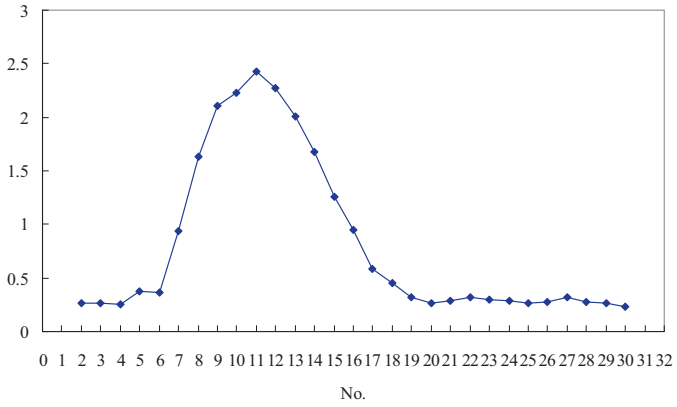


**Fig. 1.** Elution curve of Herba Cistanches polysaccharides.

to be statistically significant if the probability value was less than 0.05 ($p < 0.05$).

## 3. Result and discussion

### 3.1. Chemical analysis

The solution extracted with hot water was fractionated by ultrafiltration. Here, the membranes with different pore size were selected to find the main fractions of the polysaccharides, and the results are shown in Fig. 1. One peak was obtained. This indicated that HCP was homogeneous.

Chemical composition of the purified polysaccharide was determined. The monosaccharide composition of the purified HCP was determined to be glucose, galactose, rhamnose, arabinose and fructose, based on the capillary electrophoresis analysis. These results suggest that the HCP is a heteropolysaccharide. The apparent average molecular weight of the purified polysaccharide was estimated to be approximately 385 kDa by size-exclusion HPLC.

**Table 1**
Herba Cistanches polysaccharides reducing MDA level in the skin.

| Group | MDA |
|---|---|
| Normal control (1) | 8.43 ± 0.35 |
| Model control (2) | 14.28 ± 0.94[b] |
| HCP-treatment (3) | 13.21 ± 1.10 |
| HCP-treatment (4) | 10.32 ± 0.82[d] |
| HCP-treatment (5) | 8.82 ± 0.63[d] |

[b] $p < 0.01$, group 2 vs group 1.
[d] $p < 0.01$, group 3, 4, 5 vs group 2.

**Table 2**
Herba Cistanches polysaccharides reducing lipofuscin level in the skin.

| Group | Lipofuscin (μg/g) |
|---|---|
| Normal control (1) | 0.31 ± 0.02 |
| Model control (2) | 0.95 ± 0.05[b] |
| HCP-treatment (3) | 0.73 ± 0.06[a] |
| HCP-treatment (4) | 0.54 ± 0.04[d] |
| HCP-treatment (5) | 0.40 ± 0.03[d] |

[a] $p < 0.01$, group 2 vs group 1.
[b] $p < 0.01$, group 2 vs group 1.
[d] $p < 0.01$, groups 3, 4, 5 vs group 2.

Fig. 2 showed the infrared spectra of Herba Cistanches polysaccharides. The IR spectra of the Herba Cistanches polysaccharides displayed a broad stretching intense characteristic peak at around 3383 $cm^{-1}$ for the hydroxyl group. The difference spectrum showed some important spectral modifications in bands: a band at 1,378 $cm^{-1}$, three bands in the window dominated by carbohydrates vibrations (1279, 1158, and 1069 $cm^{-1}$) (Samuelsen et al., 1999; Sun, Lawther, & Banks, 1997), and two other bands in the region 900–800 $cm^{-1}$ (883 and 812 $cm^{-1}$) which was sensitive to anomeric configuration of carbohydrates (Fig. 3). The absorbance of polysaccharides in the range 1023–1279 $cm^{-1}$ were where the C–O–C and C–O–H link band positions were found.

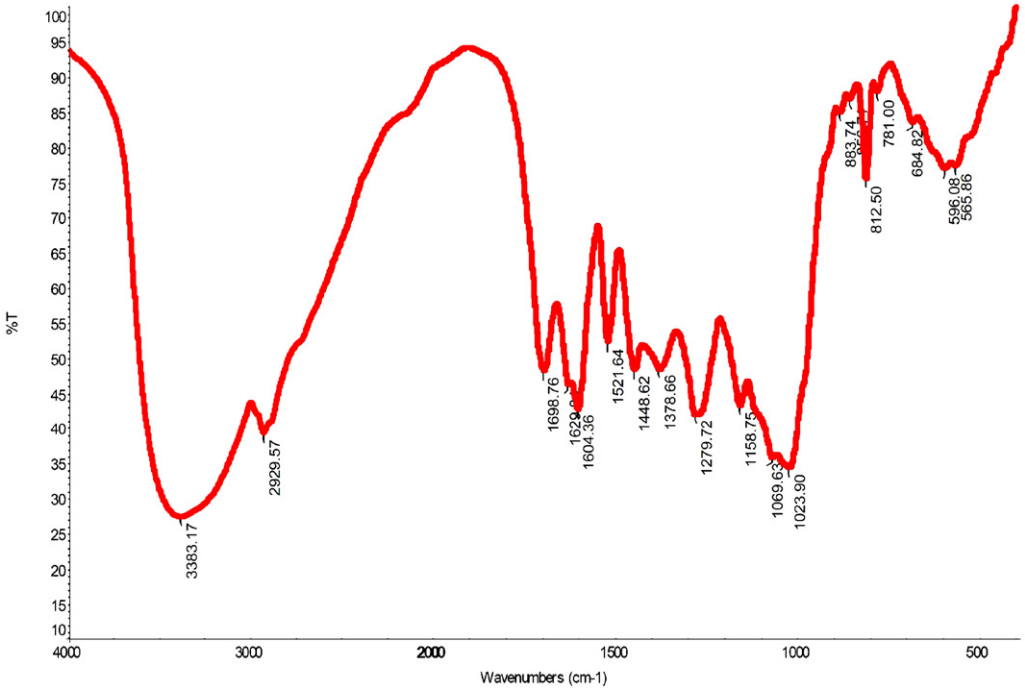


**Fig. 2.** FT-IR spectrum of Herba Cistanches polysaccharides.

**Table 3**
Herba Cistanches polysaccharides increasing SOD, CAT and GSH-Px activities in the blood and skin.

| Group | SOD | | CAT | | GSH-Px | |
|---|---|---|---|---|---|---|
| | Blood | Skin | Blood | Skin | Blood | Skin |
| Normal control (1) | 80.34 ± 3.87 | 141.32 ± 11.22 | 19.43 ± 1.07 | 22.09 ± 1.63 | 15.28 ± 1.22 | 17.39 ± 1.39 |
| Model control (2) | 57.32 ± 2.57[b] | 99.43 ± 3.88[b] | 9.74 ± 1.03[b] | 10.53 ± 0.75[b] | 8.09 ± 0.63[b] | 11.43 ± 0.92[b] |
| HCP-treatment (3) | 66.29 ± 3.98[d] | 123.11 ± 11.36[d] | 13.28 ± 1.12[d] | 16.38 ± 1.32[d] | 12.17 ± 0.93[d] | 13.27 ± 1.11[d] |
| HCP-treatment (4) | 75.21 ± 5.28[d] | 140.02 ± 11.04[d] | 16.32 ± 1.42[d] | 19.83 ± 1.52[d] | 14.08 ± 1.62[d] | 14.09 ± 0.98[d] |
| HCP-treatment (5) | 82.14 ± 6.09[d] | 145.21 ± 13.28[d] | 18.84 ± 1.66[d] | 22.09 ± 1.96[d] | 15.92 ± 1.06[d] | 16.82 ± 1.03[d] |

[b] $p < 0.01$, group 2 vs group 1.
[d] $p < 0.01$, group 3, 4, 5 vs group 2.

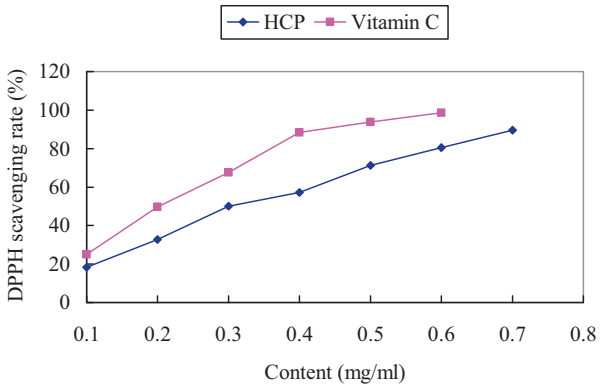


**Fig. 3.** DPPH radical scavenging activity.

### 3.2. DPPH radical scavenging capacity of HCP

Fig. 3 showed the scavenging capacity of HCP extract on DPPH (ascorbic acid as the positive control). A dose–response relationship was found in DPPH scavenging activity of HCP extract and an increase in concentration was synonymous of an increase in scavenging capacity. Ascorbic acid showed high scavenging activity ($EC_{50}$ = 0.2 mg/ml). The HCP extract also had strong DPPH scavenging effect, with an $EC_{50}$ value of 0.3 mg/ml. At 0.6 mg/ml, the scavenging ability of HCP extract on DPPH was significantly different from ascorbic acid. Among the levels used in the experiment, 0.7 mg/ml variety was the strongest one with a scavenging rate of 89.5%. The strong scavenging capacity of HCP extract on DPPH was possibly due to the phenolic compounds which could act as a hydrogen donor antioxidant.

### 3.3. In vivo antioxidant activities of HCP

Skin is a soft outer covering of an animal, in particular a vertebrate. Other animal coverings such as the arthropod exoskeleton or the seashell have different developmental origin, structure and chemical composition. The adjective cutaneous literally means "of the skin" (from Latin cutis, skin). In mammals, the skin is the largest organ of the integumentary system made up of multiple layers of ectodermal tissue, and guards the underlying muscles, bones, ligaments and internal organs ("Skin care" (analysis), 2007). Skin of a different nature exists in amphibians, reptiles, and birds (Alibardi, 2003). All mammals have some hair on their skin, even marine mammals which appear to be hairless.

Oxidation is a normal chemical reaction within the body. Oxidation occurs when a substance combines with oxygen and since our bodies need oxygen to sustain life this type of reaction occurs regularly. Although part of our normal metabolism oxidation can create free radicals. A free radical is an atom or group of atoms (molecule) that is missing an electron. Free radicals can be detrimental to not only the skin and the aging process, but it can cause diseases and illnesses, which can result in death (Tahara, Matsuo & Kaneko, 2001). Free radicals damage cells by stealing electrons from healthy atoms. This can damage the cell membrane and cell DNA causing the cell to function abnormally, which can trigger mutations. In skin cells, free radicals destroy elastin and collagen that keep the skin firm and tone. This speeds up the aging process causing wrinkles, sagging and drooping skin (Pomarede & Chandramouli, 2009).

Even though, it is part of our body's natural metabolic chemistry, nature created a substance that can counteract free radicals. Antioxidants are reducing agents that remove free radicals and inhibit oxidative reactions. Therefore antioxidants are powerful substances that reduce the risk for several diseases while keeping our skin healthy and youthful (Lau, Bagchi, Zafra-Stone, & Bagchi, 2009; Gauger, Rodríguez-Cortés, Hartwich, & Schneider, 2010). Antioxidants are able to counteract free radicals because they are oxidized themselves, therefore they are able to give free radicals an electron, to prevent them from damaging any cells in the body (Kaneko, Tahara, Taguchi, & Kondo, 2001).

The concentrations of MDA in the skin were significantly higher in the model control animals than in the normal control animals (Table 1). The supplementation of HCP suppressed the increase in the concentrations of MDA in the skin of the animals.

The concentrations of lipofuscin in the skin were significantly higher in the model control animals than in the normal control animals (Table 2). The supplementation of HCP suppressed the increase in the concentrations of lipofuscin in the skin of the animals.

The activities of serum SOD, CAT and GSH-Px activities were significantly lower in the model animals than in the normal animals (group 1). Compared with model group (group 2), treatment with HCP significantly (groups 3 and 4) prevented the decrease in serum SOD, CAT and GSH-Px activities of HCP-treated animals (Table 3).

A significant decrease in skin SOD, CAT and GSH-Px activities was noted in model control animals as compared with normal control animals (Table 3). Administration of HCP to animals for 30 days, resulted in a significant increase of skin SOD, CAT and GSH-Px activities when compared to model animals (Table 3).

## 4. Conclusion

The monosaccharide composition of the purified HCP was determined to be glucose, galactose, rhamnose, arabinose and fructose, based on the capillary electrophoresis analysis. Pharmacological analysis showed that Herba Cistanches polysaccharides could reduced oxidative injury in aged rats' skin. Therefore, Herba Cistanches polysaccharides could delay skin aging.

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