

Original Article

Effects of sea buckthorn procyanidins on healing of acetic acid-induced lesions in the rat stomach

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The aim of this study was to investigate the effects of sea buckthorn procyanidins (SBPC) on healing of acetic acid-induced lesions in the rat stomach and its possible mechanism. The sea buckthorn procyanidins (SBPC) were extracted with 60% alcohol/H₂O from sea buckthorn bark and purified by macropore adsorption resin column, with a purity of >96%. The chemical character of SBPC was analyzed by reverse phase high-performance liquid chromatography/mass spectrometry (HPLC/MS). Chronic gastric ulceration was induced by injecting acetic acid into the subserosa of stomach. Different concentrations of SBPC were orally administrated to gastric ulcers rats. After treatment 7d and 14d, rats were sacrificed respectively. The healing of the acetic acid induced ulcerations was measured by ulcer index (UI). The level of epidermal growth factor (EGF) in plasma was determined; the expression of epidermal growth factor receptor (EGFR) and proliferating cell nuclear antigen (PCNA) around ulcer was detected by immunohistochemical method. SBPC was found to reduce the size of the ulcers at day 7 and 14 in a dose-dependent manner. Compared with the control, the UI of SBPC group was significantly lower ($p < 0.01$) and the level of EGF in the plasma of SBPC group increased significantly ($p < 0.01$), meanwhile the expression of EGFR and PCNA around ulcer in high-dose SBPC stomach were enhanced ($p < 0.05$). The results implied that SBPC plays an important role in healing of acetic acid-induced gastric lesions possibly by the acceleration of the mucosal repair.

Key Words: sea buckthorn, procyanidins, acetic acid, gastric ulcer

Introduction

Sea buckthorn (*Hippophaë rhamnoides* L.) is a Euro-Asian wild, newly cultivated, edible berry with exceptionally high contents of nutrients and phytochemicals such as lipids, water and fat soluble vitamins, and flavonoids.¹ Sea buckthorn has a long history of application (more than 1000 years) in Tibetan and Mongolian medicines in the treatment of various diseases. The bark of Sea buckthorn is widely used in Mongolian folk medicine for the treatment of a wide range of gastrointestinal symptoms.

Proanthocyanidins are major phenolic constituents in many fruits and mainly procyanidins with (-)-epicatechin and (+)-catechin as constitutive units. They have been reported to possess a variety of physiological activities, e.g. antioxidant,²⁻⁵ anti-atherosclerotic, anti-allergenic and anticarcinogenic effects, and to inhibit the activities of some physiological enzymes and receptors.⁶ Recently, some polyphenolics have been found to have a preventive action on gastric injury in rats. Some research has focused on the antiulcer activity of polyphenol from grape seed or cacao liquor.⁷⁻⁸ Many researchers have utilized acute gastric lesions induced by water-immersion⁹ and HCl/ethanol¹⁰⁻¹¹ to study the effects of procyanidins extracts on gastric ulcer, while the gastro-protective activity was mainly explained by its strong antioxidant power because reactive oxygen and free radicals are related to the occurrence of ulcers.

Previously, EGF, a protect factor to heal process of chronic gastric and duodenal ulcers,¹²⁻¹³ has been observed in the efficiency of on ulcer recovery.¹⁴ Some drugs (eubrotidine, a H₂-receptor antagonist with gastro-protective, and sucralfate, a potent cytoprotective agent) accelerated ulcer healing associated with a marked transient increase in the expression of PCNA.¹⁵⁻¹⁶ Therefore, investigation the change of EGFR and PCNA expression with ulcer healing treatment with SBPC is a source of insight into the effect of SBPC taking place during mucosal repair. In present study, procyanidins- rich fraction has been isolated from the sea buckthorn bark, partially characterized by chromatogram and chemical methods.¹⁷ The effects of procyanidins extract from Sea buckthorn on chronic gastric ulcer were investigated for the first time.

Materials and methods

Plant material

The plant was collected in November from Northwest Sci-Tech University of Agriculture and Forestry (Yanglin, China).

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The bark was chopped into pieces about 3cm in size, Freeze-dried and milled. All samples were stored under vacuum in the dark at -20°C .

Animals

Male Wistar rats, SPF (190-210g each), were purchased from the Central Animal House of Southern Medical University where all experiment were performed.

Drugs and chemicals

D3520 Microporous adsorption resin was purchased from the Chemical Plant of Nankai University (Tianjin, China), and detailed information can be obtained from the following to web sites: <http://www.nksg.com/chemical/English/index.htm>. Goat anti-EGFR(1005): sc-03 against a peptide mapping at the carboxy terminus of the EGF receptor of human origin (identical to corresponding mouse sequence); Santa Cruz Biotechnology, Inc. Mouse anti-PCNA, DAB kit (Boster Biotechnical co., Ltd., Wuhan, China), Kit 125I-mEGF reagent pack for RIA (Biotinge Biomedicine co., Ltd., Beijing, China).

Preparation of the sea buckthorn procyanidins

Sea buckthorn bark were immersed in 65% (v/v) aqueous ethanol at 20°C , the combined extracts were concentrated by low-pressure evaporation at 42°C to eliminate ethanol, and the concentrated aqueous solution was extracted with petroleum ether and the remaining aqueous solution was filtrated before loading to macroporous adsorption resin column ($3.5\times 50\text{cm}$). The column was eluted with five column volumes of distilled deionised water and then eluted with 30% ethanol. The fraction of 30% ethanol was concentrated by low-pressure evaporation at 42°C to eliminate ethanol, and the concentrated aqueous solution was lyophilized.¹⁷

Determination of sea buckthorn procyanidins

The determination of procyanidins were performed by n-butanol/HCl/Fe(III),¹⁸ the content of procyanidins was 96.5% and further characterized by reverse phase high-performance liquid chromatography/mass spectrometry (HPLC/MS) and normal phase HPLC. HPLC/MS analyses were performed using an Agilent (Agilent Technologies, Palo Alto, Ca, USA) 1100 series LC/MSD trap equipped with an auto-injector, binary HPLC pump, column heater and diode array detectors. The sample was loaded on Zorbax (Agilent Technologies) SB-C18 column ($150\text{mm}\times 2.1\text{mm}$, $5\mu\text{m}$). The column was equilibrated in solvent A (0.2%(v/v)acetic acid), and proanthocyanidins ($5\mu\text{L}$ injected) were eluted with a gradient of solvent B (5%(v/v) acetonitrile) from 0 to 5% B between 0 and 10min, from 5 to 20% B between 10 to 20min, from 20 to 40% B between 20 to 40min, from 40 to 50% B between 40 and 45min, from 50 to 5% B between 45 to 50min and held isocratic at 5% B between 50 and 60min. The mass spectrometer was operated in negative mode for LC/ESI-MS and scanned from m/z 100 to 1200. A nebulising pressure of 20 psig and a gas drying temperature of 325°C were used. Data were collected on an HP Chemstation (Agilent Technologies). Peaks were detected at 280nm and identified by comparison with retention times of standards.

Acetic acid-induced gastric ulcers

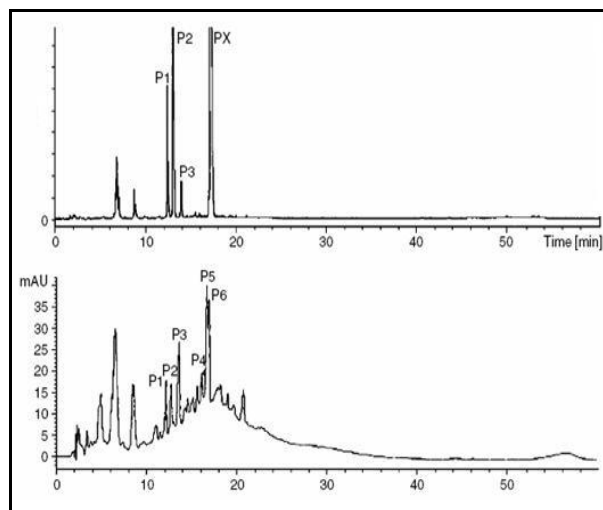


Figure 1. Top: reverse phase HPLC/MS trace from negative ion TIC of SBPC (1mg mL^{-1} , $5\mu\text{L}$ injection) using acetic acid to assist ionization. Bottom: UV trace at 280nm for SBPC. P₁, dimmers; P₂, dimmers; P₃, (+)-catechin; P₄, trimers; P₅, (-)-epicatechin; P₆, dimmers.

Table 1. Ions identified by HPLC/MS analysis of SBPC

Oligomer	Peaks	Molecular ion (M-H) ⁻
Monomers	P ₃ , P ₅	289
Dimers	P ₁ , P ₂ , P ₆	577
Trimers	P ₄	865

The experiments were performed according to the method Takagi et al with some modifications.¹⁹ Male Wistar rats were fasted for 16h and the experiments performed between 9:00 and 11:00 a.m. Under pentobarbital sodium anesthetization, a laparotomy was done through a midline epigastric incision. After exposing the stomach, 0.04mL (v/v) 50% acetic acid solution was injected into the subserosal layer in the glandular part of the anterior wall and 16 rats of control group were injected with saline. Stomachs were bathed with saline to avoided adherence to the external surface of the ulcerated region. The abdomens were then closed and the animals were allowed to recover with free access to food and water. Three days after laparotomy, 80 rats of experimental group were assigned into 5 at random. SBPC were suspended in distilled water and Oral administered at the daily doses of 50mg/kg, 100mg/kg and 150mg/kg, respectively, Control groups received distilled water. Ranitidine (30mg/kg) was given orally, as the reference drug.

Radioimmunoassay of EGF in plasma of rats

Radioimmunoassay of EGF was performed using commercial kit-mEGF reagent pack for RIA. Each sample was assayed triplicate exactly as described in the kit protocols.

Measurement of ulcer index

After treatment 7d and 14d, rats were sacrificed respectively. The rats were anesthetized with pentobarbital sodium and underwent a laparotomy. The stomach was excised and opened along the greater curvature. The glandular portion was examined under a dissecting microscope ($\times 10$) for the presence of ulcers. The ulcer was calculated

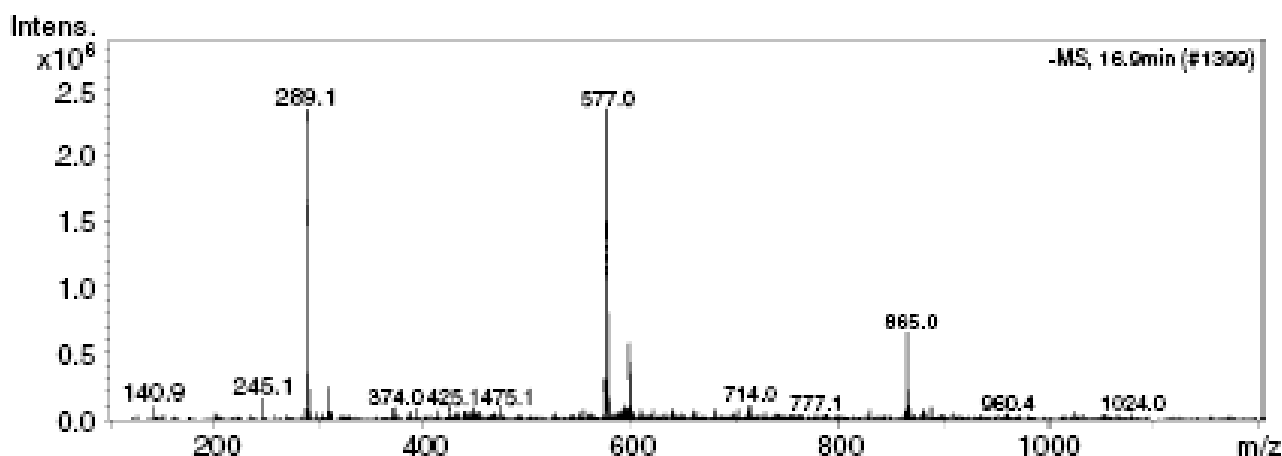


Figure 2. Mass spectrum of Px retention time 16.9min) from sea buckthorn bark extract determined by LC/ESI-MS analysis.

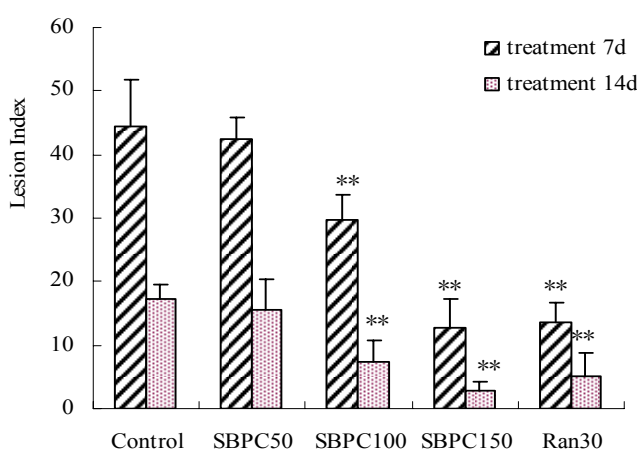


Figure 3. Effect of SBPC on Gastric Mucosal Lesion Index (LI) in Gastric Ulcer Rats Induced by Acetic Acid Cautery; $\bar{x} \pm s$, $n=8$ ** $p < 0.01$ when compared with the corresponding controls.

as follows: $\pi/4 \times a \times b$ mm², where a was the long axis and b was the short axis. The ulcer area is expressed as an ulcer index. Gastric tissues with the ulcers were also sampled and fixed in 10% buffered formalin for histological or immunohistochemical analysis.

Immunohistochemical analyses

For immunohistochemical analyses of EGFR and PCNA expression, gastric tissues were fixed with 10% buffered formalin overnight. Paraffin-embedded tissues were cut into 4 μ m sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Sections were rinsed in PBS after 3% H₂O₂ treatment for 10 min, the deparaffinized sections were preincubated with normal goat serum to prevent nonspecific binding and then incubated with a primary polyclonal antibody EGFR at an optimal dilution of 1:100 in a wet chamber overnight at 4°C. After rinsing with PBS, a secondary goat anti-mouse IgG peroxidase-conjugated antibody was applied and the sections were again placed in a wet chamber at room temperature 15min. Next, the sections were incubated in a 3'-diaminobenzidine (DAB) solution, EGFR positive cytoplasm were brown coloured. For the immunohistochemical study of PCNA, a primary monoclonal antibody PCNA was used. The slides were incubated with an anti-mouse

immunoglobulin antibody. The slides were examined with a Leica M420 Macroviewer with an Apozoom lens under the $\times 200$ field. Positive area percent was determined utilizing a Leica Q500MC Image Analysis System, values were expressed as the percentage of total mucosal sections area occupied by cell positively stained for EGFR or PCNA. All slides were read blind, and analysis was performed by a single operator to minimize operator bias.

Statistical analysis

All data are expressed as means \pm SD. Statistical analysis of data was performed using analysis of variance (ANOVA) and where appropriate by Student's t test, a value of 0.05 being considered significant.

Results and discussion

Identification of SBPC by reverse phase HPLC/MS: The total ion current (TIC) for SBPC is shown in Fig 1, and compared with the UV trace at 280nm. Mass spectral data were used to identify the peaks labeled P1-P6. P3 and P5 were also identified via reference standards.

The ions observed for each procyanidins oligomer are listed in Table 1. Px includes ions at m/z 289, 577 and 865, which may indicate that the eluted peaks P4-P6 were not separated completely.

The major m/z signals of peak Px (retention time 16.9min) are shown in Fig 2. HPLC/MS analysis shows that Procyanidin dimmers and trimers predominated among oligomer procyanidins of the SBPC.

Effect of SBPC on gastric ulcer healing: Rats can renew injured mucous membrane of stomach by instinct. As shown in Fig.3, without given any medicine, mucosal lesion index (LI) could sharply decrease in 14 days. SBPC could accelerate the gastric ulcer healing up. LI in different SBPC dose group is significant decreased comparing with the corresponding control. On the other hand, LI in high dose group (150mg/kg) are similar to which treated with Ranitidine (30mg/kg).

Assessment the content of EGF in plasma of rats during the process of healing: The content of EGF in plasma of normal group was very steady; therefore, abdomen surgery couldn't cause the content of EGF fluctuation (Tab 2). After treated with SBPC or Ranitidine 7 days, the EGF level in plasma could raise comparing with the control group ($p < 0.05$, $p < 0.01$). After 14 days treatment,

Table 3. Effects of SBPC on area percent of EGFR and PCNA Expression in Gastric Mucosal of gastric ulcer rats induced by acetic acid cautery (after 7d treatment); ($\bar{x} \pm s$, n=8)

Group	EGFR Expression (%)	PCNA Expression (%)
Normal	11.0±1.66	2.24±0.37
Control (0mg·kg ⁻¹)	11.0±1.22	2.36±0.17
SBPC(50mg·kg ⁻¹)	12.2±0.85	2.48±0.42
SBPC(100mg·kg ⁻¹)	12.7±1.43	2.60±0.26
SBPC(150mg·kg ⁻¹)	12.9±0.56*	2.92±0.14*
Ranitidine Hydrochloride (30mg·kg ⁻¹)	12.6±0.65	3.14±0.16**

vs control: * $p < 0.05$, ** $p < 0.01$

there was no difference between all groups.

Effect of SBPC on EGFR and PCNA expression: Tab.3 shows that up-regulated expression of EGFR at the gastric ulcer tissues accompanied by a significant elevation in mucosal PCNA expression after 7 days treatment with SBPC (150mg/kg).

The present study demonstrated that the major component of the fractions of 60% alcohol/H₂O extract of Sea buckthorn bark, oligomer procyanidins, were shown to accelerated the healing of gastric ulcers induced by acetic acid. SBPC treatment was found to dose-dependently increase the content of EGF in plasma which could facilitate epithelial proliferation during ulcer healing stage.

The processes of gastric mucosal repair-characterized by massive cell migration, proliferation, differentiation and remodeling are regulated by growth factors and the extent of cellular expression of their receptors. An orderly synchronized induction of signaling cascades that trigger these events is of paramount importance to the reconstruction of the mucosal architecture as well as to the quality of mucosal structure restoration.²⁰ Acetic acid-induced chronic gastric ulcer healing is accompanied by a marked increase in mucosal expression of the growth factor receptors and a dynamic variation in the receptor bound growth factors, such as epidermal growth factor.²¹⁻²² We observed that accelerated ulcer healing was accompanied by a significant elevation in mucosal EGFR expression treatment with SBPC (150mg, 7d). Simultaneously, the content of EGF in plasma of rat was raised. As EGF and EGFR are crucial for cell proliferation, migration, re-epithelialization and gland reconstruction within the scar, their changes was consistent with the up-regulate expression of PCNA. The finding above implicated that SBPC might exert effect on the expression of cell factors that accelerate the ulcer healing.

In addition to the finding obtained in this research, other beneficial effects of SBPC maybe also contributed to their healing mechanisms. Oxygen-derived free radicals have been shown to participate in reperfusion damage in the intestine and in the stomach.²³⁻²⁴ Free radicals are involved in several pathological processes, such as inflammation, an experiment revealed that procyanidins have potent inhibitory effect on inflammation,²⁵ procyanidins may stimulate the production of antioxidants in wound site and provides a favorable environment of tissue healing which may also attributes to their mechanisms.

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