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Inhibition effect of procyanidins from lotus seedpod on mouse B16 melanoma *in vivo* and *in vitro*

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ABSTRACT

Significant growth inhibition effects of procyanidins from lotus (*Nelumbo nucifera Gaertn.*) seedpod (LSPCs) on mouse melanoma B16 were found both *in vivo* and *in vitro*. *In vivo* treatment with LSPCs inhibited tumour growth in C_{57}BL/6 J mice by 55.3% in terms of average tumour weight. LSPCs can significantly ($P < 0.05$) decrease lipid peroxidation (LPO) levels and increase the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in liver tissue. *In vitro* assay of anti-cancer activities suggested that LSPCs (25–100 μg/ml) possessed cytotoxicities against mouse melanoma B16 in a dose-dependent mode. Furthermore, LSPCs had significant ($P < 0.05$) stimulatory effects on mouse splenocyte proliferation. The prevention of tumour growth was exerted through diverse mechanisms, including cell-cycle arrest, induction of tumour cell death by apoptosis and increase of Ca^{2+} ions, together with stimulation of antioxidant enzyme activities and immunomodulatory activities.

1. Introduction

Procyanidins are naturally occurring polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics, and are widely available in fruits, vegetables, nuts, seeds, flowers and bark (Ye et al., 1999). As natural antioxidants, procyanidins are known to possess a broad spectrum of biological, pharmacological and chemoprotective properties against free radicals and oxidative stress, such as antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory functions (Pinent et al., 2006; Yusuf & Toledo, 2004), as well as inhibiting lipid peroxidation, platelet aggregation and capillary permeability and fragility, and modulating the activity of enzyme systems, including cyclo-oxygenase and lipo-oxygenase (Kolodziej, Haberland, Woerdenbag, & Konings, 1995). Furthermore, procyanidins have been reported to have anti-tumour activities (Ye et al., 1999). Reviews of recent literature indicate that several laboratories have explored the safety, efficacy and bioavailability of procyanidins and their potential application in interfering with cancer cell growth (Ray, Parikh, & Bagchi, 2005). It is extremely appealing that procyanidins can be lethal to a variety of cancer cells, including breast, lung, prostate, gastric, liver and oral cancers, while enhancing the growth and viability of normal cells (Ye et al., 1999).

Malignant melanoma is one of the most aggressive forms of skin cancer, with high metastatic potential and extraordinary resistance to cytotoxic agents (Hoang & Eichenfield, 2000). Currently, between 2 and 3 millions of non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally each year (Formento et al., 2009). If detected early and surgically excised, the 5-year survival rate is satisfactory. However, later stages of the disease are difficult to treat and long-term survival is low. Despite extensive research and partial successes gained by use of platinum analogues, nitrosoureas, taxanes, *Vinca* alkaloids and cytokines (Legha et al., 1998; Sun & Schuchter, 2001), currently there is no effective chemotherapy against invasive melanoma. Among the drugs exerting anti-cancer activity against malignant melanoma, dacarbazine has been extensively used, though it could achieve only low response rates (11–25%) and short survival time (Harhaji et al., 2008). Therefore, it is necessary to develop new drugs with potent activity and weak side-effects against melanoma.

Nelumbo nucifera Gaertn., commonly known as lotus, is a perennial aquatic plant, grown and consumed throughout Asia. Almost all parts of lotus are eaten as vegetables and are also used for various medicinal purposes in oriental medicine. Lotus seedpod is usually discarded, except when sometimes used as a traditional medicine with hemostasis function and for eliminating bruise. We have demonstrated that a procyanidin-rich fraction could be isolated from the lotus seedpod (LSPCs) (Ling, Xie, & Yang, 2005), which exhibited some powerful biological effects, including

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antioxidation (Duan & Xie, 2003), improving learning and memory abilities (Gong, Liu, Xie, Liao, Yang, & Sun, 2008), protective effects against experimental myocardial injury and ischemia (Zhang, Zhang, Gong, & Zeng, 2004). It might have good potential for use as functional food material. Many natural antioxidant substances have anti-cancer or anti-carcinogenic properties (Ju, Lee, Hwang, & Kim, 2004). However, little information is available on LSPCs anti-tumour effect, particularly on the role of anti-melanoma. The objective of this work was to study the anti-melanoma effect and mechanism of LSPCs on B16 mouse melanoma cells, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

Fetal calf serum (FCS), penicillin G, streptomycin, and RPMI-1640 (culture medium) were obtained from GIBCO BRL. Dimethyl sulphoxide (DMSO), ribonuclease (RNase), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, acridine orange (AO), ethidium bromide (EB), methythiazoletetrazolium (MTT), sodium dodecyl sulfate, pyridine, pyrogallol, (hydroxymethyl-) aminomethane (Tris), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO, USA). Sodium azide and glutathione were purchased from Guoyao Group Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals used were of analytical purity.

2.2. Preparation of LSPCs

We extracted procyanidins from mature lotus seedpod, which was collected from Honghu Lantian (Hubei, China). This variety of *Nelumbo nucifera Gaertn* was named No. 2 Wuhan plant and authenticated by the Department of Botany, Wuhan Plant Institute of the Chinese Academy of Science.

The procyanidin extract of lotus seedpod (LSPCs) is a light red-brown amorphous powder extracted with Me_2CO/H_2O and purified by Sephadex LH-20 column chromatography, with a purity of >98%. As part of our ongoing research, we have confirmed the main molecular weight distribution of LSPCs (range 291.1–1155.3) by electrospray ionisation-mass (ESI-MS) analysis, and the LSPCs degree of polymerisation, $\leqslant 4$, containing monomers, dimers, and tetramers of procyanidins, in which the amounts of dimers are the greatest, and catechin and epicatechin are the major components (Ling et al., 2005). For all experiments, final concentrations of the tested compounds were prepared by diluting the stock solution with RPMI-1640; control cultures received the same volume of RPMI-1640 for anti-cancer activity assay *in vitro*. *In vivo* trials were conducted by diluting the stock solution with normal saline (NS).

2.3. Animals and experimental design

Healthy female $C_{57}BL/6$ J mice (weighing 18–22 g and 6 weeks old) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences & Peking Medical College, China. All animals were housed in a temperature-controlled room under a continuous 12 h light/12 h dark cycle with food and water *ad libitum* for 7 days. After 7 days of acclimatisation, all mice were randomly divided into six experimental groups of 12 mice each. Groups I (control) and II (B16 melanoma-bearing (BMB)) orally received NS but no LSPCs, and served as negative and positive controls, respectively. Group III (LSPCs120) was only given LSPCs at 120 mg/kg bwt (body weight), orally by intra gastric (*i.g.*) feeding without bearing B16 melanoma. Groups IV (LSPCs60 + BMB), V (LSPCs90 + BMB), and VI (LSPCs120 + BMB) were not only inoculated with B16 melanoma cells but also administered with LSPCs (60, 90,120 mg/kg bwt, respectively). Animals were treated humanely, in compliance with the guidelines of the National Institutes of Health (NIH), and the protocol conforming to the Institutional Animal Ethical Committee.

2.4. Cell line and culture

Mouse melanoma cell line, B16, was supplied by the Institute of Biochemistry and Cell Biology, SIBS, CAS. It was maintained in monolayer culture at 37 °C, 5% CO_2/95% air humidified atmosphere in RPMI-1640 supplemented with 10% heat-inactivated FCS, 10 U/ml of penicillin, 10 mg/ml of streptomycin (Sigma, USA). Cells were grown until they were 80% confluent, and were then harvested by trypsinization (0.5% trypsin/2.6 mM EDTA), and washed with phosphate buffered solution (PBS).

Mice were killed by cervical dislocation and spleens were removed aseptically. Single cells were prepared by mincing spleen fragments and pressing them through a stainless 200-mesh screen in the RPMI-1640 complete medium. The RPMI-1640 complete medium was supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Yu, Zhao, Yang, & Bai, 2009). An amount of 1×10^7 cells was placed in a 16 mm well and incubated for 3 h in humidified 5% CO_2 at 37 °C. The supernatants, together with the non-adherent cells, were collected by centrifugation at $630 \times g$ for 10 min. The cell pellets were re-suspended in the RPMI-1640 complete medium and then adjusted to 1×10^6 cells/ml.

2.5. In vitro study

2.5.1. Cytotoxicity assay of B16 melanoma cell

Inhibition of B16 cell proliferation by LSPCs was measured by MTT assay (Mosmann, 1983). Briefly, B16 cells were plated in 96 well culture plates (2.5×10^3 cells per well). After incubation for 24 h, the cells were treated with LSPCs (25, 50, and 100 μg/ml) for 5 days. The medium was replaced every other day. Dye solution (40 μl), specific for the MTT (5.0 g/l) assay, was added to each well for an additional 4 h incubation at 37 °C. After adding DMSO (100 μl/well), the absorbance was read with a microplate reader (Multiskan MK3, USA) at 570 nm. The inhibition rate (%) of samples against the proliferation of B16 was calculated using the following formula: (A570 of control cells − A570 of treated cells)/A570 of control cells × 100%.

2.5.2. Proliferation assay of splenocytes

The proliferation stimulatory effects of LSPCs on splenocytes were also evaluated by the MTT assay method described previously (Sun, Qin, & Pan, 2005). Briefly, 20 μl of cell suspension (1×10^6 cells/ml) and 40 μl of RPMI-1640 in the wells were added to 20 μl of LSPCs at 6.25, 12.5, 25, 50 and 100 μg/ml, which were dissolved in RPMI-1640 in a 96 well flat-bottom plate. Control cells were incubated in a medium containing an equivalent solvent amount without the test materials. The plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 44 h of incubation, MTT solution (5 g/l, 40 μl) was added to each well and then incubated for 4 h. The other steps were the same as described in Section 2.5.1. The absorbance at 570 nm was measured with a microplate reader, using wells without cells as control. The stimulatory effect (%) of samples on the proliferation of splenocytes was calculated as (A570 of treated cells − A570 of control cells)/A570 of control cells × 100% (Yu et al., 2009).