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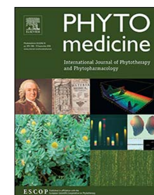

Original Article

Mechanisms underlying the wound healing potential of propolis based on its *in vitro* antioxidant activity

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A R T I C L E I N F O

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A B S T R A C T

Background: Propolis is a resinous substance collected by honeybees, *Apis mellifera*, from various plant sources. Having various pharmacological and biological activities, it has been used in folk medicine and complementary therapies since ancient times.
Purpose: To evaluate the effects and underlying mechanism of the protective effects of the ethanol extract of Chinese propolis (EECP) on L929 cells injured by hydrogen peroxide (H_2O_2).
Study design: The wound healing activities of EECP in L929 cells with H_2O_2-induced damage were investigated.
Methods: The main components of EECP were analyzed by RP-HPLC, and the free radical scavenging capacity and reducing power were also measured. The effects of EECP on the expression of antioxidant-related genes in fibroblast L929 cells were determined using qRT-PCR and western blotting.
Results: EECP had significant protective effects against cell death induced by H_2O_2 and significantly inhibited the decline of collagen mRNA expression caused by H_2O_2 in L929 cells.
Conclusion: EECP induced the expression of antioxidant-related genes, such as HO-1, GCLM, and GCLC, which has great implications for the potential of propolis to alleviate oxidative stress in wound tissues. The protective effects of propolis have great implications for using propolis as a wound healing regent.

Introduction

Skin is the largest organ of the body and acts as a wall to protect from attacks from various external factors, such as ultraviolet radiation (UVR), chemical toxics, microorganisms, and so on (Proksch et al., 2008). Therefore, the skin itself is directly impacted by toxic injuries, which cause adverse effects, such as erythema, edema, wrinkling, photoaging, inflammation and wound healing impairment (Nachbar and Korting, 1995; Parihar et al., 2008). Numerous reports have indicated that reactive oxygen species (ROS) appear to play a vital role in the pathogenesis of cutaneous wound healing (Bryan et al., 2012; Huo et al., 2009). ROS are generally produced during normal skin tissue metabolism and are kept at very low level by the strong oxidation–reduction system; therefore, they have few damaging effects (Kohen, 1999). However, defense mechanisms, though highly efficient (Kohen and Gati, 2000), have their limitations and may be overwhelmed when exposed to excessive levels of oxidative species. Uncontrolled release and inefficient removal of ROS may cause biomolecular oxidative damage and induce aberrant signal transduction, which contributes to an array of physiological manifestations in cells and tissues (Ichihashi et al., 2003). Thus, antioxidant therapy is believed to have significant benefits for improving oxidative stress-related cutaneous wound healing (Kant et al., 2014; Pinnell, 2003).

Propolis, a resinous substance, is collected by *Apis mellifera* from plant shoots or trunk lesions. The resin is mixed with mandibular gland secretions and beeswax. It has extensive plant sources and complex chemical compositions, including flavonoids, phenolic acids, terpenes, sugars, hydrocarbons and mineral elements (Bankova et al., 2000), which enable it to perform a wide range of pharmacological activities. Among these biological activities, the antioxidant activity of propolis has been widely studied. The chemical structure of the constituent polyphenols enable propolis to effectively eliminate free radicals.

Abbreviations: EECP, Ethanol extract of Chinese propolis; HPLC, High performance liquid chromatography; H_2O_2, Hydrogen peroxide; HO-1, Heme oxygenase-1; GCLC, Glutathione-cysteine ligase catalytic subunits; GCLM, Glutathione-cysteine ligase modify subunits; ROS, Reactive oxygen species; COL1A2, Alpha2 chain of type I collagen; COL3A1, Alpha1 chain of type III collagen

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Propolis promotes skin wound healing by stimulating epithelial regeneration (Pessolato et al., 2011), modulating extracellular matrix (collagen etc.) deposition (Olczyk et al., 2012; Olczyk et al., 2013b), and facilitating the formation of granulation tissue (Han et al., 2005). Hence, the antioxidant activity of propolis may contribute to its protective effects in cutaneous diseases. Burn wounds treated with propolis were found to have lower concentrations of free radicals (Olczyk et al., 2013a). In addition, it was reported that propolis could alleviate cell damage in fibroblast cells by suppressing intracellular ROS production induced by excessive light (Murase et al., 2013). Propolis has been used as a folk medicine to treat burns, ulcers and wounds for a long time (Kuropatnicki et al., 2013). It has been recorded that propolis was used to treat wounds during the Boer war in the early 1900 s (Ghisalberti, 1979).

However, few studies have been carried out to explore the mechanisms underlying the wound healing potential of propolis. In consideration of the crucial role that oxidative stress plays in skin damage, an *in vitro* study was conducted to gather additional evidence to support the clinical application of propolis in wound healing.

Composition variations exist between propolis from different plant origins, allowing it to exhibit different pharmacological activities. Our previous study demonstrated that poplar type propolis possesses strong anti-oxidant activity due to the abundant active polyphenols that it contained (Wang et al., 2013; Zhang et al., 2016). Thus, in this study, we investigated the antioxidant activity of Chinese propolis (poplar type) and its protective effects on hydrogen peroxide-induced changes (cell viability and collagen genes expression) in mouse L929 fibroblasts, and further examined the molecular mechanisms behind it. To our knowledge, this is the first report using Chinese propolis to evaluate the *in-vitro* protective effects of propolis against oxidative injury in fibroblasts.

Materials and methods

Materials

An alkaline phosphatase-conjugated secondary antibody (anti-rabbit IgG), DPPH, ABTS, α-tocopherol (Vitamin E), Trolox and the standards used in the HPLC analysis were purchased from Sigma (St. Louis, MO). Primary rabbit antibodies against HO-1 (lot #: YJ071709CS, monoclonal), GCLM (lot #: 5529–1, monoclonal), β-tubulin (lot #: YH082302D, monoclonal), and other analytical grade chemicals were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Sample collection and extraction

Poplar type propolis (*Populus* spp.) was collected from *Apis mellifera* colonies in Shandong province of China during the summer of 2010. A voucher specimen of the samples (no. 130520) was deposited at the College of Animal Sciences, Zhejiang University. Samples were stored at −20 °C until used. Briefly, propolis samples were weighed and broken into powder form using a grinder, extracted with 95% (v/v) ethanol three times, and sonicated at 40 so for 3 h. Subsequently, the supernatants were filtered using Whatman No. 4 filter papers. The residues were extracted with 95% ethanol, the supernatants were collected and evaporated in a rotary evaporator under reduced pressure at 50 °C. After drying, the residues were collected and weighed. All samples were stored at −20 °C and redissolved in ethanol and filtered with a 0.22-μm syringe filter before use. During the cell experiments, the final concentration of ethanol in the medium did not exceed 0.1% (v/v).

HPLC analysis of propolis

To separate and determine the 24 main constituents of EECP, including flavonoids and phenolic acids, HPLC was conducted according to previously described methods (Cui-ping et al., 2014; Kumazawa et al., 2003). A Sepax HP-C18 column (150 mm × 4.6 mm, 5 μm; Sepax Technologies, Inc., Newark, DE) was used. The mobile phase was composed of 1.0% aqueous acetic acid (v/v) (A) and methanol (B) in gradient mode at 33 °C as follows: 15–40% (B) at 0–30 min, 40–55% (B) at 30–65 min, 55–62% (B) at 65–70 min, and 62–100% (B) at 70–85 min at a flow rate of 1.0 ml/min. The injection volume was 5 μl, and the results were detected at 280 nm.

Determination of the free radical scavenging activities and reducing power

DPPH radical scavenging activity (DPPH)

The hydrogen donating activity was measured by direct hydrogen donation to the DPPH radical as described in a previous report with minor modifications (Yang et al., 2011). The reaction solution consisted of 100 μL of sample and 100 μl of DPPH solution; 100 μl of the mixture per well was incubated in a 96-well plate at room temperature for 30 min in the dark. All experiments were performed in triplicate. The absorbance was detected at 517 nm, and the results are expressed as IC50 values (μg/ml).

ABTS cation radical scavenging activity (ABTS)

The ABTS radical-scavenging activity assay was performed according to the modified method (Yang et al., 2011). The ABTS working reagent was diluted with methanol to reach an absorbance of 0.7 at 734 nm. 50 μl of sample and 100 μl of the ABTS working solution were aliquoted into the 96-well plate away from light for 16 h before use, and the absorbance was measured at 734 nm after being incubated for 10 min in the dark. All experiments were performed in triplicate. The scavenging ability results were expressed as IC50 values (μg/ml).

Measurement of the reducing power (RP)

The power of reducing ferric ions was measured by a modified method (Moreira et al., 2008). 125 ml of the propolis sample was mixed with 312.5 μL of phosphate buffer (0.2 M, pH 6.6) and 312.5 ml of 1% potassium ferrocyanate. The mixture was preheated at 50 °C for 20 min, followed by the addition of 312.5 μl of 10% trichloroacetic acid, and then centrifuged at 2000 rpm for 10 min. The reaction solution consisted of 1 ml supernatant, 312.5 μl of distilled water and 62.5 μl of 0.1% ferric chloride. Four-hundred microliters of the reaction solution was pipetted into a 96-well plate (200 μL/well). The absorbance was detected at 700 nm, and trolox was used as the reference sample. The results were expressed as the Trolox equivalent (mmol) per gram of propolis.

Cell culture and cell viability assay

Fibroblasts L929 cells were incubated in DMEM (Keyi Biotechnology Company, Hangzhou, China) containing 10% fetal bovine serum (Gibco, Grand Island, NY) at 37 °C and 5% CO_2 in a humidified incubator. The toxicity of EECP and H_2O_2 was determined by using a CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 550, CA).

ROS levels in L929 cells

Fibroblast L929 cells were cultured with the indicated concentrations of propolis for 3 h, followed by stimulation with 600 μM H_2O_2 for 12 h. Next, the cells were washed twice with PBS to remove extracellular reactive oxygen species (ROS) and incubated with a new culture coupled with 200 μM DCHF-DA for 30 min. The cells were removed from the culture medium and washed with PBS, collected using trypsin and centrifuged at 2500 rpm for 5 min to remove extracellular compounds. Next, the ROS levels were determined by a BD FACS Calibur (Franklin, NJ).

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