Biomedicine & Pharmacotherapy 103 (2018) 406–414

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Protective effects of radish (*Raphanus sativus L.*) leaves extract against hydrogen peroxide-induced oxidative damage in human fetal lung fibroblast (MRC-5) cells

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ARTICLE INFO

Keywords:
Protective effect
Oxidative damage
Radish leaves
Antioxidant
MRC-5 cell
Fractions

ABSTRACT

Natural antioxidants play a critical role in the promotion of good health for its prevention of oxidative stress. The main purpose of this study is to investigate the protective effects of radish leaves extract on the oxidative damage in human fetal lung fibroblast (MRC-5) cells. F2, a fraction of radish leaves extracts, which was fractionated by different polarity solvents and AB-8 macroporous resins column shows the best free radical scavenging ability, the highest total polyphenol contents (TPC), and the most potent protective effects on H_2O_2-induced oxidative damage in MRC-5 cells. The results indicated that pretreatment with F2 before the exposure of cells to H_2O_2 led to a significant increase in cell viability and internal antioxidant enzyme activities, and a decrease in the content of malondialdehyde (MDA). Furthermore, F2 attenuated the increase in intracellular reactive oxygen species (ROS) level and restored the loss of mitochondria membrane potential (MMP) caused by H_2O_2. In addition, pretreatment of F2 down-regulated the pro-apoptosis protein (Bax) and up-regulated the anti-apoptosis protein (Bcl-2) suggested its preliminary mechanism of protective effect. In summary, F2 from radish leaves might be used as a source of antioxidant for protecting the oxidative damage of lung.

1. Introduction

Raphanus sativus L., belonging to a large family of Cruciferae, is commonly known as radish which is widely consumed throughout the world as a vegetable or condiment in human diets. The most popular part for consumption is the napiform taproot, although the entire plant is edible and the aerial part can be used as a leaf vegetable. Different parts of radish, such as roots, seeds, flowers, sprouts, and leaves, have been known to show various medicinal properties. Recently, radish has been reported to possess a wide range of pharmacological activities, such as gut stimulatory effect [1], hepatoprotective activity [2], cardioprotective effect [3], antioxidant activity [4], antitumor and anti-inflammatory activity [5]. Meanwhile, pharmacological studies have also shown that radish leaves possess a wide range of biological activities, such as ameliorate hepatotoxicity through anti-oxidant and cytoprotective activity [6], spasmogenicity in guinea-pig ileum and colon [1], healing ulcers in patients suffering from peptic ulcer disease [7], and antihypertensive effect in spontaneously hypertensive rats [8]. Apart from this, it has been reported that the ethanol extracts from radish leaves may be a clinically useful antitumor agent for breast cancer because it directly inhibits the growth of tumor cells and induces apoptosis [9]. Several studies demonstrated that radish, radish leaves, and radish leaves extract have a preventative effect on excessive reactive oxygen species (ROS) generation.

Oxidative stress reflecting an imbalance between the production of free radicals and reactive metabolites is also known as oxidants or ROS in organisms. Meanwhile, oxidative stress causes profound alterations in various biological structures, including cellular membranes, lipids, proteins and nucleic acids [10]. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, aging, etc. Antioxidants are vital substances which possess the ability to protect the body of damage caused by free radical induced oxidative stress. Recently, interested in plant-derived natural products (natural antioxidants) has increasingly growing [11], mainly because synthetic antioxidants suffer from several drawbacks. Many plants bio-resources (e.g., fruits and vegetables) were screened for their

Abbreviations: MRC-5, human fetal lung fibroblast; TPC, total polyphenol contents; ROS, reactive oxygen species; MMP, mitochondria membrane potential; FBS, fetal bovine serum; GAE, gallic acid equivalents; PTFE, polytetrafluoroethylene; CLF, chloroform fraction; EAF, ethyl acetate fraction; WBF, water saturated *n*-butanol fraction; AQF, aqueous fraction; DCFH-DA, 2',7'-dihydrofluorescein-diacetate

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https://doi.org/10.1016/j.biopha.2018.04.049
Received 24 February 2018; Received in revised form 4 April 2018; Accepted 6 April 2018

antioxidant capacities, and some of them had strong activity as related to polyphenols and flavonoids [12]. Up to date, the search for new natural antioxidant and anti-aging agents from plants resources remain a potential area to investigate.

Antioxidant-guided separation is a method of fractionating a sample extracts, following which the most antioxidant-active fractions are chosen, and then the major contributors to the measured antioxidant capacity of the sample are identified. Hence, the present study is mainly aimed at fractionating the main antioxidant ingredients with protective effects on H_2O_2-induced oxidative damage in human fetal lung fibroblast (MRC-5) cells.

2. Materials and methods

2.1. Chemicals and reagents

2, 4, 6-tri (2-pyridyl)-*S*-triazine (TPTZ), Folin–Ciocalteau's reagent (FCR), and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Shanghai, China). The assay kits were purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China). Gallic acid, ferulic acid, *p*-coumaric acid, caffeic acid, and kaempferitrin were obtained from Nanjing Spring & Autumn Biological Engineering Co., Ltd (China). Trypsinization was purchased from HyClone (Logan, USA), and heat-inactivated fetal bovine serum (FBS) was purchased from Gibco (USA). Antibodies were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). HPLC grade methanol was purchased from Tedia Company, Inc. (USA). All other reagents were of analytical reagent (AR) grade. Double distilled water was used throughout.

2.2. Determination of total polyphenol contents (TPC)

TPC in the fractions was determined according to the Folin-Ciocalteau method [13] with a little modification, using gallic acid as a standard. In general, the properly diluted extracts (1 mL) were mixed with ten-fold diluted FCR (1 mL) and incubated at room temperature for 4 min. Then, 0.8 mL of 75 g/L Na_2CO_3 solution was added. Subsequently, it was added 2.2 mL distilled water. The solution mixture was incubated in the dark at room temperature for 1 h. The absorbance of the solution mixture at 760 nm was measured with UV-1601 Spectrophotometer (Chengshi). The estimation of TPC in the extracts was calculated by a calibration curve obtained with gallic acid. Results were expressed as gallic acid equivalents (mg GAE/g dw). Each test was repeated three times, and the results were averaged.

2.3. Antioxidative capacity in vitro

2.3.1. DPPH radical scavenging assay

The ability of the extracts to scavenge DPPH radical was measured as previously described [14] method with some modifications. DPPH was dissolved in 80% methanol to make a 0.2 mM DPPH solution. Extracts with a various concentration were dissolved in methanol (1 mL) and mixed with DPPH solution (2 mL), and the mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Methanol (1 mL) was used as the control for this experiment. The DPPH radical scavenging rate was estimated by measuring absorbance at 517 nm with a UV-1601 spectrophotometer. The radical scavenging activity of the extracts was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100\%$$

Where A_0 is the absorbance of the control (using methanol instead of extract's solution) and A_1 is the absorbance in the presence of the extract or standard sample. All determinations were performed in triplicate. Scavenging activity of the extracts was also estimated based on the percentage of the DPPH radical reduction by calculating the IC_{50} values (concentration in μg/mL that caused 50% inhibition of DPPH radical) using a non-linear regression analysis.

2.3.2. ABTS radical-scavenging activity

The ABTS radical scavenging activity was assessed according to the method described previously [15] with a little modification. In brief, 5.0 mL of a 7.0 mM ABTS solution was reacted in the dark for 12–16 h with 88.0 μL of a 140 mM potassium persulfate solution to yield the $ABTS^+$ radical cation. Prior to use in the assay, the $ABTS^+$ radical cation was diluted with methanol to an initial absorbance of about 0.700 ± 0.02 at 734 nm. ABTS solution (1.9 mL) was mixed with 100 μL of sample extracts and the absorbance was taken at 734 nm after incubated for 6 min at room temperature in the dark with an UV-1601 spectrophotometer.

2.4. Cell culture

MRC-5 cells, purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's modification of Eagle's medium (DMEM), which was supplemented with 10% fetal bovine serum, penicillin (100 UI/mL) and streptomycin (100 μg/mL). Cells were maintained at 37 °C under an atmosphere of 5% CO_2 with 90% relative humidity.

2.5. Cell viability assay

The cell viability was measured by MTT assay [16]. Briefly, cells were plated in 96-well culture plates (2×10^3 cells per well) and pre-incubated for 24 h. Then various concentrations of F2 were added, incubated for 12 h followed by treatment with 200 μM H_2O_2 for another 6 h. After treatment, 40 μL of MTT solution (5 mg/mL in PBS) was then added to each well and incubated for 4 h at 37 °C. DMSO (150 μL) was added to dissolve the formazan precipitate and the absorbance at 570 nm was determined using microplate reader (Infinite M200 Pro, Becton, USA).

2.6. Fractionation of extracts

Fresh radish leaves were identified and collected by Professor Duan Yuqing at the local farm located in Zhenjiang, China. The samples were washed, air dried, and followed by completely drying in an oven at 37 °C. Dried material of radish leaves (100 g) were ground into powder and extracted by petroleum ether (sample/solvent = 1:15) for three times. The residues (50 g) were extracted by subcritical water for three times in a polytetrafluoroethylene (PTFE) hydrothermal reactor (inner volume, 20 mL), with a stainless steel jacket [17]. The temperature was monitored and controlled by an air-dry oven. After extraction, extracts were immediately centrifuged (5000 rpm/min, 5 min). The extracts were decanted, filtered under vacuum, and then lyophilized. The dried extract was re-dissolved in distilled water (200 mL) and then the solution was partitioned successively with chloroform (3 × 200 mL), ethyl acetate (3 × 200 mL) and water saturated *n*-butanol (3 × 200 mL), respectively. The resulting extracts were evaporated to dryness under vacuum, subsequently freeze-dried to yield chloroform fraction (CLF), ethyl acetate fraction (EAF), water saturated *n*-butanol fraction (WBF) and the aqueous fraction (AQF), and stored at −20 °C until use. Then, the highest antioxidant activity fraction was loaded onto an AB-8 macroporous resins column and then washed successively using 0%, 20%, 40%, 60%, and 80% ethanol (v/v) for 4 times of column volume, respectively. The eluents were collected, lyophilized and named as F0, F1, F2, F3 and F4, respectively.

2.7. Identification of polyphenol compounds using LC/MS

The extracted samples were analyzed by a Shimadzu LC-20AT high