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Original article

Tamarix gallica phenolics protect IEC-6 cells against H_2O_2 induced stress by restricting oxidative injuries and MAPKs signaling pathways

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ABSTRACT

Polyphenolic compounds gained interest in the pharmaceutical research area due to their beneficial properties. Herein, antioxidant and cytoprotective capacities of *T. gallica* extract on H_2O_2-challenged rat small intestine epithelial cells were investigated. To set stress conditions, IEC-6 cultures were challenged with numerous H_2O_2 doses and durations. Then, 40 μM H_2O_2 during 4 h were selected to assess the cytoprotective effect of different *T. gallica* extract concentrations. Oxidative parameters, measured through CAT and SOD activities as well as MDA quantification were assessed. In addition, the expression of possibly involved MAPKs was also valued. Main results reported that *T. gallica* was rich in polyphenols and exhibited an important antioxidant activity (DPPH$^{\bullet}$ Assay, IC_{50} = 6 μg mL^{-1}; $ABTS^{\bullet+}$ test, IC_{50} = 50 μg mL^{-1}; Fe-reducing power, EC_{50} = 100 μg mL^{-1}). The exposure of IEC-6 cultures to 40 μM H_2O_2 during 4 h caused oxidative stress manifested by (i) over 70% cell mortality, (ii) over-activity of CAT (246%), (iii) excess in MDA content (18.4 nmol mg^{-1}) and (iiii) a trigger of JNK phosphorylation. Pretreatment with *T. gallica* extract, especially when used at 0.25 μg mL^{-1}, restored cell viability to 122%, and normal cell morphology in H_2O_2-chalenged cells. In addition, this extract normalized CAT activity and MDA content (100% and 14.7 nmol mg^{-1}, respectively) to their basal levels as compared to control cells. Furthermore, stopping cell death seems to be due to dephosphorylated JNK MAPK exerted by *T. gallica* bioactive compounds. In all, *T. gallica* components provided a cross-talk between regulatory pathways leading to an efficient cytoprotection against harmful oxidative stimulus.

1. Introduction

Over-expression of reactive oxygen species (ROS) induces severe oxidative threats to cell components including: protein, lipid, DNA and RNA associated with cell structural damage, tissue injury and gene mutation [1]. ROS excess is harmful for transcriptions factors, ion channels, phosphatases activity and can lead to inappropriate cell signaling. The mitogen-activated protein kinases (MAPKs) pathways are triggered in response to stress-associated stimuli, resulting in growth arrest or even apoptosis [2]. These disorders play a causative role in aging and are often associated with several physiological disruptions such as cognitive dysfunction, cancer, atherosclerosis, heart disease, and inflammation injury [3].

To deal with detrimental effects of ROS, cells possess a number of compensatory mechanisms such as the induction of superoxide

Abbreviations: BSA, bovine serum albumin; CAT, catalase; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; DW, dry weight; EC_{50}, effective concentration at which the absorbance was 0.5; FBS, fetal bovine serum; GAE, gallic acid equivalent; IC_{50}, inhibition concentration at 50%; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPKs, mitogen-activated protein kinases; MDA, malondialdehyde; NBT, Nitrotetrazolium blue chloride; IEC-6 cells, rat small intestine epithelial cells; SOD, superoxide dismutase; TgE, *Tamarix gallica* extract; TBARS, thiobarbituric acid reactive substances.

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dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [4]. *In vivo* as well as *in vitro*, superoxide anion, a major free radical generated in oxidative stress, is catalyzed into H_2O_2 by SOD. Consecutively, the produced H_2O_2 is detoxified into water and molecular oxygen by CAT and GPx. It is commonly assumed that ROS–antioxidant balance and membrane integrity are maintained when the activities of these detoxifying enzymes are privileged [5].

Otherwise, phenolics, flavonoids, tannins and anthocyanidins are proved to be natural, efficient and safe antioxidants [3]. These bioactive compounds can delay the lipids and proteins oxidation by inhibiting the initiation or/and propagation of oxidative chain reaction. Thus, they may prevent or repair cell damage caused by oxygen [6]. In recent years, natural biophenols, such as resveratrol, caffeic and rosmarinic acids were highlighted as efficient protectors against the oxidative cytotoxicity of hydrogen peroxide by the regulation of the endogenous antioxidant defense system and the modulation of signaling pathways [7]. In this context, bioactive compounds such as stilbene and resveratrol were described for their capacity in regulating CAT, SOD and GPx activities as well as for their ability to restrict JNK and p38 MAPKs phosphorylation induced by H_2O_2 stress [5,7,8].

In Tunisia, a considerable diversity of halophytic species of multiple interests including therapeutic practices occurs, such as *Tamarix gallica*. This medicinal halophyte was studied by our laboratory for its important phenolic content; high antioxidant and antimicrobial activities and it even showed efficient antitumoral capacity [6,9,10]. Following these works schedule, this species is further investigated for its cytoprotective effect against oxidative stress.

In that framework, antioxidant capacities of *T. gallica* were further evaluated. Then, cytoprotective effect of several *T. gallica* extract concentrations on H_2O_2-induced insult within IEC-6 cells was explored. SOD and CAT activities as well as MDA levels were assessed and the possible underlying mechanism involving JNK and p38 MAPKs was investigated.

2. Materials and methods

2.1. Reagents

IEC-6 cells (rat-derived intestinal epithelial cell line) were purchased from Public Health England (88071401). DMEM (Dulbecco's Modified Eagle Medium), fetal bovine serum (FBS), antibiotics (100 μg/mL of streptomycin and 100 UI/mL of penicillin) and trypsin/EDTA were obtained from Lonza (France). Hydrogen peroxide 3% (w/w) was procured by Laboratoire Gilbert (France). Phosphate buffered saline (PBS) was furnished by Dominique Dutscher (France). Solvents (ethanol, DSMO), MTT, protease and phosphatase inhibitor cocktail, riboflavin, methionine, NBT, hydrochloric acid, trichloroacetic acid, thiobarbituric acid, BSA and primary antibodies β-actin were purchased from Sigma Aldrich (Germany). Anti-p38, pp38 and horseradish peroxidise-conjugated secondary antibody were acquired from Abcam (England). SAPK/JNK and phospho SAPK/JNK were from Cell Signaling Technology. ECL detection agents were acquired from Amersham International.

2.2. Extract preparation

Extract of *T. gallica* shoots were obtained by maceration of powdered samples (3 g) during 30 min in 30 mL of water–ethanol solvent (1:1, v/v) at room temperature. After 24 h in obscurity at 4 °C, supernatants were recovered by centrifugation at 1250 × *g* for 5 min at 4 °C. *T. gallica* extract (TgE) was stored at 4 °C until use [6]. For the cytoprotective effect analysis, TgE was prepared as described above and then concentrated by vacuum-evaporation until dryness. Dry extracts were dissolved in DMSO to get 10% stock concentration. Obtained extract was stored at −20 °C until analysis.

2.3. Characterization of phenolic levels and antioxidant activities in plant extract

2.3.1. Determination of total polyphenol content

The amount of total phenolics in TgE was determined with the Folin–Ciocalteu reagent [11]. An aliquot of 125 μL of diluted extract was added to 500 μL of distilled water and 125 μL of the Folin–Ciocalteu reagent. The mixture was shaken, before adding 1250 μL of Na_2CO_3 (7%) and adjusting with distilled water to a final volume of 3 mL. After incubation for 90 min at 23 °C in the dark, the absorbance versus prepared blank was read at 760 nm. Total phenolic content was expressed as mg GAE (Gallic Acid Equivalent)/g DW (Dry Weight) using a calibration curve with gallic acid, ranged from 0 to 400 $\mu g\,mL^{-1}$. The sample was analyzed in triplicate.

2.3.2. Total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH [12]. An aliquot of sample extract was combined in an eppendorf tube with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g DW). The sample was analyzed in three replications.

2.3.3. DPPH scavenging activity

One milliliter of the extract at known concentrations was added to 0.5 mL of a DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature in the dark for 30 min. The absorbance was then measured at 517 nm and corresponds to the extract ability to reduce the radical $DPPH^{\bullet}$ to the yellow-colored diphenylpicrylhydrazine [13]. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A0 - A1)/A0^{*}100 \quad (1)$$

Where A0 is the absorbance of the control at 30 min, and A1 is the absorbance of the sample at 30 min. The antiradical activity was expressed as IC_{50} ($\mu g\,mL^{-1}$), the antiradical dose required to cause a 50% inhibition. The sample was analyzed in triplicate.

2.3.4. Scavenging ability on ABTS test

The $ABTS^{\bullet+}$ was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate solution, stored in the dark at room temperature for 16 h. Before usage, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. In a final volume of 1 ml, the reaction mixture comprised 950 μL of $ABTS^{\bullet+}$ solution and 50 μL of the TgE at various concentrations. These mixtures were homogenised and its absorbance was recorded at 734 nm. All measurements were done after at least 6 min. Similarly, the reaction mixture of standard group was made with 950 μL of $ABTS^{\bullet+}$ solution and 50 μL of BHT [14]. As for the antiradical activity, ABTS scavenging ability was expressed as IC_{50} ($\mu g\,mL^{-1}$). The inhibition percentage of ABTS radical was calculated using formula (1).