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Original article

Turkish propolis supresses MCF-7 cell death induced by homocysteine

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ABSTRACT

Elevated plasma homocysteine (Hcy) level is a most important risk factor for various vascular diseases including coronary, cerebral and peripheral arterial and venous thrombosis.

Propolis is produced by honeybee from various oils, pollens and wax materials. Therefore, it has various biological properties including antioxidant, antitumor and antimicrobial activities.

This study investigated the effects of propolis and Hcy on apoptosis in cancer cells. According to our findings, Hcy induced apoptosis in human breast adenocarcinoma (MCF-7) cells by regulating numerous genes and proteins involved in the apoptotic signal transduction pathway. In contrast, treatment with propolis inhibited caspase- 3 and −9 induced by Hcy in MCF-7 cells. It can be concluded that Hcy may augment the activity of anticancer agents that induce excessive reactive oxygen species (ROS) generation and apoptosis in their target cells. In contrast to the previous studies herein we found that propolis in low doses protected cancer cells inhibiting cellular apoptosis mediated by intracellular ROS-dependent mitochondrial pathway.

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1. Introduction

Homocysteine (Hcy) is known as a transforming non-protein amino acid from methionine by remethylation or trans-sulphuriation mechanism. It completes its transforming cycle to cysteine or methionine with adequate amount of B_{12}, B_6 and folic acid [1]. Accumulated plasma levels of Hcy termed as hyperhomocysteinemia which induces apoptotic and cytotoxic effects on cells [2].

There has been increasing evidence that hyperhomocysteinemia is the most important risk factor for various vascular diseases including coronary, cerebral and peripheral arterial diseases [1,3,4]. Common molecular mechanisms involved in the cell death observed in the cardiovascular and cerebrovascular disorders include excitotoxicity, redox state alterations, endoplasmic reticulum stress, DNA damage, inflammation, endothelial dysfunction and apoptosis [5].

Hcy includes a thiol group that is readily oxidized and could lead to the generation of reactive oxygen species (ROS) which results in oxidative impairment to cells [6]. In addition, Hcy has been shown to inhibit the expression of antioxidant enzymes such as glutathione peroxidase (GSH-Px) and super oxide dismutase (SOD) which might decrease the toxic effects of ROS [7]. Furthermore, Hcy activates P38-MAPK, JNK and NF-kB signal pathways to induce intrinsic pro-apoptotic mechanisms which lead to cell death [8–10].

There are limited researches explaining the effects of Hcy on cancer cells [11] though numerous in vivo and in vitro studies have been performed to suggest its deleterious effects on normal cells [8,12,13]. It has been shown that Hcy accumulates in tumour sites because of dysfunction of methionine metabolism in cancer cells [14]. Thus, it can be considered as a biomarker of carcinoma [15]. The toxic effects of Hcy causing oxidative stress and apoptosis can be reduced by treatment with B12, B6 and folate [8]. However, clinical researches have demonstrated that recruitment of B12, B6 and folate would be incapable to re-establish due to the hypomethylation effects of Hcy on DNA [16].

Propolis is a natural product conventionally used with the purpose of treatment by folks. The usage of it is historically based on preferring an embalming material by ancient Egyptian, because

Abbreviations: DCFH-DA, 2′7′-dichlorodihydrofluorescin diacetate; CAPE, Caffeic Acid Phenethyl Ester; GSH-Px, Glutathione Peroxidase; Hcy, Homocysteine; H_2O_2, Hydrogen Peroxide; MDA, Malondialdehyde; MCF-7 cells, Michigan Cancer Foundation-7 Cells; μg/ml, Microgram/mililiter; μM, Micromolar; MPTP, Mitochondrial Membrane Permeability Transition Pore; NO, Nitric Oxide; ROS, Reactive Oxygen Species; SOD, Super Oxide Dismutase; TdT, Terminal deoxynucleotidyl Transferase; TBARS, Thiobarbituric Acid Reactive Substances; TBS-T, Tris Buffer Salined-Tween 20; WST-1, Water Soluble Tetrazolium-1.

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of being perfect plastic material to protect the mummy from bacteria, fungi and viruses [17]. It is produced by honeybee with various oils, pollens, special resins and wax materials collecting from tree cone and sprig of plants [18,19]. Its biological properties have been elicited by recent studies, including antimicrobial, antifungal, antiviral, antioxidant and antitumor [20].

Propolis consists of various components modified by kind of bees and gathering regions, climate and source of plants [21,22]. Therefore, it is difficult to standardize its content according to chemical compounds [23]. It is usually composed of flavonoids, phenolic acids and its esters, terpenoids, steroids, amino acids and inorganic compounds [24]. It has been shown that antitumor and antioxidant activity of propolis could be derived from flavonoids and phenolic acids in regard to dose [25].

Propolis can be diversely extracted in different soluble compounds such as ethanol, methanol and water due to its rich ingredient [26]. Its ethanolic extract is biologically most powerful owing to including abundant flavonoids and phenolic acids providing chemo-preventive and anticancer properties [27].

In recent years, the most considerable properties of propolis have been extensively recorded in various researches that have been studied in different culture cell lines such as human prostate cancer cells (PC3), human breast cancer cells (MCF-7, MDA-MB-231), and human lung carcinoma cells (A549) [11,28,29].

The numerous in vivo and in vitro publications displaying the diverse effects of propolis revealed that no side-effects [30,31] therefore, it could be potential as a new remedy agent in a dose-dependent manner [20]. Additionally, propolis has also antioxidant activity in lower doses to regulate apoptotic pathway [32].

In the present study, we investigated whether propolis interferes with Hcy treatment on MCF-7 cells. We analysed the impact of propolis on the induction of oxidative cell damage, therefore, changes in lipid peroxidation (LPO) and ROS generation were measured, the role of apoptosis- relevant pathways was examined, and the survival rate of breast cancer cells on treatment was determined.

2. Experimental procedures

2.1. Materials

DL-Homocysteine (PubChem CID:778), 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (PubChem CID:53705683), tris (PubChem CID:6503), sodium chloride (PubChem CID:5234), ethylenediaminetetraacetic acid (EDTA) (PubChem CID:6049), hydrogen peroxide (H_2O_2) (PubChem CID:784), tween 20 (PubChem CID:443314) were purchased from Sigma–Aldrich Chemical Co. (USA). Trichloro acetic acid (TCA) (PubChem CID:6421), 2-thiobarbituric acid (TBA) (PubChem CID:2723628), diaminobenzidine (DAB) (PubChem CID:7071) malondialdehyde bis (PubChem CID:67147) were purchased from Merck Company (Germany).

2.1.1. Extraction of propolis

Turkish propolis was gathered from the region of Bingol, Turkey. Crude propolis was shaken with ethanol for extraction that was maintained at room temperature. Then, it was filtered with Whatman No. 1 filter paper. The filtered propolis extract was dried with vacuum incubator. Later, the prepared propolis extracts were stored under a dry condition.

2.1.2. HPLC–MS/MS (Exactive Plus Orbitrap Mass)

The phenolics compounds; quercetin, acacetin, apigenin, galangin, kaempferide, pinocembrin, pinostrobin, sakuranetin, naringenin, chrysin and caffeic acid phenethyl ester (CAPE) were made ready in methanol as 1000 ppm solution. These stock solutions were utilised to prepare a standarts diluted with methanol to using volumes of 0.05, 0.10, 0.25, 0.50 and 1 ppm. Each solution was kept in the dark. Calibration curve was calculated by plotting the highest regions of the standards in contrast to their volume. Chromatographic segregation was completed working a HPLC method. An analytical Hypersilgold column (50 × 4.6 mm, 1.9 μm particle size) was performed for chromatographic separation (Thermo Fisher Scientific). (A) ultrapurewater with 1% formic acid and (B) acetonitrile with 1% formic acid were preferred as mobile phase. The multi-step gradient programme was followed as 0.0–1.0 min, 65–70% B; 1.0–3.0 min, 70–85% B; 3.0–5.0 min, 85–90% B; 5.0–8.0 min, 85–90; 8.0–10.0 min 95% B. The flow rate 300 μL/min and 20 μL injection volumes for each sample were performed. The mass spectrometer was applied in positive method. Limit of the ion source was applied: spray voltage 3.50 kV, capillary temperature 320 °C, sheath gas flow 40 and auxiliary gas flow 10 (au). The MS ranges were set by full-range acquisition including 250–310 m/z. Phenolics and flavonoids were recognised and measured in accordance with the corresponding spectral features that are mass ranges, exact mass, specific fragmentation and specific retention time. Data acquisition and analysis were made ready with Xcalibur software for instrument control. The "www.mzcloud.org" was preferred as references database to analyse the accurate mass of the constituents of sample. The sample for which no standard is present was identified with earlier described MS fragmentation records. Quantifications were performed in accordance with the accurate mass search technique (±5 ppm) by comparison the specific retention times and precise mass of present standards [33].

2.2. Cell culture

MCF-7 cells were kindly supplied by Advanced Technologies Research Center (Dumlupınar University, Kutahya) at passage 8 and cultured in RPMI 1640 complete media containing 10% fetal bovine serum and 1% penicilin-streptomycin (10,000 units/ml, 10 mg/ml streptomycin) solution. Cells at passage 11 through 15 were used in our studies. The cells were cultured in humidified incubator with 5% CO_2 and checked every two or three days. Cells were tested for mycoplasma contamination by using EZ-PCR mycoplasma test kit (Biological Industries, Israel).

2.2.1. Experimental grouping

Cells were separated into three groups. In control group, cells were treated with just complete media. In Hcy group, cells were exposed to 50 μM Hcy [34] in complete media for 24 h. In co-treatment group, cells were incubated with 50 μM Hcy and 50 μg/ml propolis [35] in complete media for 24 h.

2.3. Cell proliferation assay

Water Soluble Tetrazolium-1 (WST-1) cell proliferation assay kit (Clontech Laboratories, USA) was preferred to evaluate the effects of Hcy and propolis on MCF-7 cells proliferation. Experiments were carried out according to the protocol of the kit. MCF-7 cells were seeded in 96-well plate (5×10^3 cells/well). Cells were treated as stated in experimental grouping part. Later, WST-1 reagent (5 μl) was added to each well. After 4 h incubation, absorbance of each well were measured at 450 nm (reference: 630 nm) by SpectraMax Plus 384 Microplate Reader (Molecular Devices, USA).

2.4. Detection of lipid peroxidation

LPO assay was completed by measuring the amount of thiobarbituric acid reactive substances (TBARS) formed from malondialdehyde (MDA). Cells were treated as stated in

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