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Cytotoxic, proapoptotic and antioxidative potential of flavonoids isolated from propolis against colon (HCT-116) and breast (MDA-MB-231) cancer cell lines

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ABSTRACT

Isolated and structurally confirmed, eleven flavonoids from propolis were examined for their cytotoxicity toward human colon cancer and human breast cancer cells. Their effect on induction of apoptosis and their antioxidative activities were also evaluated. Six flavonoids induced cytotoxic effects in both cell lines. Luteolin had a marked effect on both cell lines, especially on HCT-116 cells (IC_{50} 72 h, 66.86 μM). Also, luteolin was observed to have the highest apoptotic potential after 72 h treatment of examined cell lines (27.13% and 37.09%, respectively). Myricetin exhibited selective inhibition of cell growth (IC_{50} 114.75 μM) and induced apoptosis in MDA-MB-231 cells only. Luteolin and galangin exhibited prooxidative properties 24 h after the treatment in HCT-116 cells, while myricetin induced prooxidative effects in MDA-MB-231 cells. On the other hand, selected flavonoids exhibited antioxidative properties 72 h after the treatment, decreasing superoxide anion radical and nitrite levels in both cell lines. Cytotoxic and proapoptotic effects on colon and breast cancer cell lines and the influence on their redox status make tested flavonoids good candidates for developing new anticancer drugs.

1. Introduction

Propolis is a resinous natural substance collected by bees, usually from tree buds and plants, used as bee glue in sealing holes, as a protective barrier and a sterilizing agent in beehives (Burdock, 1998; Marcucci, 1995). Propolis is a rich source of biologically active compounds, with > 300 identified constituents. Previously published data indicated that the most abundant bioactive compounds were galangin, pinocembrin, chrysin, quercetin, kaempferol and naringenin, with the fact that amounts of specific components vary depending on the place of origin (Bankova, Christoy, Stoev, & Popov, 1992; Pietta, Gardana, & Pietta, 2002). Also, some recent studies revealed that propolis and its constituents exert cytotoxic effects in cell lines *in vitro*, as well as antitumor effect *in vivo* (Banskota et al., 1998; Birt, Hendrich, & Wang, 2001; Orsolic, Knezevic, Sver, Terzic, & Basic, 2004).

Carcinogenesis is a multistage process, characterized by a progression of changes at the cellular, genetic and epigenetic level. This process consists of many phases during which intervention is possible, with the aim of preventing, slowing down or reversing the process. Intervention through modulation of various intracellular signaling pathways may provide molecular basis of chemoprevention with a wide variety of dietary phytochemicals (Khan, Afaq, & Mukhtar, 2007). Activation of programmed cell death (apoptosis) is considered to be the key molecular mechanism responsible for the anti-cancer activities of most of the currently studied potential anti-cancer agents (Lopaczynski & Zeisel, 2001).

Currently, many researches are focused on plant-derived bioactive compounds because of their low oral toxicity (generally recognized as safe) and the fact that they are common constituents of daily human diet.

Previous data indicated that among 155 small molecular anti-cancer drugs developed from the 1940s to 2006, 47% have natural origin (Fauzi, Norazmi, & Yaacob, 2011). Polyphenolics represent one of the most numerous class of compounds present in plants, with flavonoids being one of the main groups that occur ubiquitously in foods of plant origin. Over 4000 different naturally occurring flavonoids have been described that are common substances in the daily diet (Middleton & Kandaswami, 1994).

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Flavonoids (flavones, flavonols and flavanones) possess a wide range of biochemical and pharmacological activities that have an effect on proliferation and apoptosis of human colorectal, breast and stomach cancer cells (Graf, Milbury, & Blumberg, 2005; Sexton et al., 2006). On the other hand, due to their ability to scavenge and reduce the production of free radicals, these compounds exert considerable chemopreventive activity (Kampa, Nifli, Notas, & Castanas, 2007; Kundu & Surh, 2008).

In order to investigate exact influence of individual compounds on these processes, as well as to further elucidate possible molecular mechanisms, eleven isolated flavonoids from propolis were examined for their cytotoxicity, effects on induction of apoptosis and antioxidative activities on human colon cancer (HCT-116) and human breast cancer (MDA-MB-231) cells.

2. Materials and methods

2.1. Chemicals

Column chromatography was performed on silica gel (Silica gel 60, particle size 65 μm; 230 mesh, Fluka, St. Louis, MO, USA) and Sephadex LH-20 (GE Healthcare Biosciences, Uppsala, Sweden). Preparative TLC was performed on silica gel (MN-Kieselgel P/UV_{254} with $CaSO_4$, Mashery-Nagel, Germany), while analytical TLC was carried out on silica gel (Silica gel 60, layer 0.20 mm, Alugram Sil G, Mashery-Nagel, Düren, Germany). Visualization of TLC plates was performed by using UV lamp at 254 nm and 365 nm (VL-4.LC, 365/254, Vilber Lourmat, Marne-la-Vallée Cedex 1, France). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), Phosphate-buffered saline (PBS) and Trypsin-ethylene diamine tetraacetic acid (EDTA) were purchased from PAA (Linz, Austria). Nitro blue tetrazolium (NBT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Serva (Heidelberg, Germany). Dimethyl sulfoxide (DMSO), *N*-(1-naphthyl)ethylenediamine and 5-fluorouracil were obtained from Sigma-Aldrich (St Louis, MO, USA). Sodium nitrite ($NaNO_2$), sulfanilic acid and phosphoric acid were purchased from Zorka pharma (Sabac, Serbia).

2.2. Instrumentation

The NMR spectra were recorded on a Varian Gemini 200 spectrometer (^1H at 200 MHz and ^{13}C at 50 MHz) DMSO-d_6/$CDCl_3$ was used as the solvent mixture for all compounds. Chemical shifts were given in δ (ppm), *J*-coupling constants in Hertz (Hz), abbreviations: s-singlet, d-doublet, dd-doublet of doublet, m-multiplet, bs-broadend singlet. Microanalysis of carbon and hydrogen was carried out with a Carlo Erba 1106 microanalyser. Melting points of compounds were determined by using Kofler-hot stage apparatus.

2.3. Isolation of flavonoids from propolis

Amount of 75 g of crushed crude propolis sample (Miljevici, altitude 920 m, 43° 22′ 07″ N, 19° 35′ 25″ E, south west Serbia) and methanol (500 mL) were sonicated in ultrasonic bath at 40 °C for 8 h. The methanol solution obtained after sonication was removed from insoluble particles, evaporated to the half of volume under reduced pressure and frozen at 4 °C for 48 h. Insoluble part formed during freezing was removed from solution by filtration. This procedure was repeated three times. Defatted methanol solutions were combined and evaporated to dryness at 40 °C under reduced pressure, yielding 25.8 g of semisolid red to brown extract.

The extract was subjected to silica gel column chromatography, eluted with toluene, toluene: ethyl acetate (70:30, 50:50, 30:70), ethyl acetate, ethyl acetate:methanol (50:50, 30:70, 10:90) and methanol, to furnish 116 fraction. Further, fractions that showed similar pattern on TLC (toluen:ethyl acetate:formic acid = 40:40:5) after visualization with UV lamp at 254 nm and 365 nm were pooled and combined in six fractions (F1-F6) and evaporated under reduced pressure to dryness. Further, fraction F1 (2.65 g) was fractioned into two parts (FA1 and FA2) over silica gel column eluting with chloroform:methanol (30:1). Multiple development of preparative TLC of FA1 subfraction (1.76 g) (toluen:ethyl acetate:formic acid = 70:30:2) gave **1** (chrysin, 157 mg) and unresoluted fraction which was further separated on a Sephadex LH-20 column (methanol) yielding compound **2** (pinocembrin, 134 mg) and compound **3** (naringenin, 17 mg). By using preparative TLC (toluene:methanol = 10:1) and Sephadex LH-20 column (methanol), compound **4** (galangin, 87 mg) was isolated from FA2 subfraction (0.89 g). The F2 fraction (1.37 g) was subjected to silica gel column chromatography (chloroform, chloroform:methanol = 10:1 and methanol) to obtain compound **5** (tectochrysin, 27 mg) and compound **6** (apigenin, 38 mg). Fraction F3 (0.98 g) yielded **7** (kaempferol, 47 mg) and **8** (hesperetin, 18 mg) after multiple preparative TLC (toluen:-chloroform:methanol = 20:40:7.5). Separation of fraction F4 (0.74 g) on a preparative TLC (toluen:chloroform:methanol = 20:40:7.5) and Sephadex LH-20 column eluted with $CHCl_3$:MeOH (3:2, to pure MeOH) give **9** (isorhamnetin, 32 mg). From fractions F5 (0.81 g) and F6 (0.55 g), after series of column chromatography procedures on a Sephadex LH-20 ($CHCl_3$:MeOH = 2:2 to pure MeOH), compound **10** (luteolin, 24 mg) and compound **11** (myricetin, 13 mg) were obtained.

2.4. In vitro studies

2.4.1. Cell culture and treatment

Human colon cancer (HCT-116) and human triple negative breast cancer (MDA-MB-231) cell lines were obtained from American Type Culture Collection. The cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were grown in 75 cm^2 culture bottles supplied with 15 mL DMEM. The samples of all eleven flavonoids and 5-fluorouracil were prepared as stock solutions (1000 μM) in 0.1% DMSO (v/v). Working solutions (concentration range: 0.1 μM–500 μM) were prepared prior to testing. HCT-116 and MDA-MB-231 cells were seeded in 96-well microtitre plates (1×10^4 cells per well) for cell viability assay and in 6-well microtitre plates (1×10^6 cells per well) for apoptosis detection assay (exponentially growing viable cells were used throughout each assay); 24 h later, after cell adherence, the culturing medium was replaced with 100 μL of medium containing eleven flavonoid solutions in concentrations of 0.1μM, 1μM, 10μM, 50μM, 100μM and 500 μM for the cell viability assay and concentrations of three flavonoid solutions (galangin, luteolin and myricetin) in concentrations of 10 μM and 100 μM for apoptosis detection assay and concentrations 1 μM, 10 μM, 50 μM and 100 μM for determination of superoxide anion radical (O_2^-) and nitrite measurement, except in control wells, where only the nutrient medium was added to the cells. Cells were incubated with flavonoid solutions for 24 h and 72 h prior to testing.

2.4.2. Cell viability assay (MTT assay)

Cell viability was determined by the MTT assay (Mosmann, 1983). At the end of the treatment period, the medium was removed and 25 μL of MTT solution (5 mg/mL final concentration in PBS) was added to each well and incubated at 37 °C in 5% CO_2 for 3 h. Thus produced colored crystals of formazan were dissolved in 150 μL of DMSO. The absorbance at 570 nm was measured on a microplate reader (ELISA, RT-2100C, Rayto, Shenzhen, China). To determine cell viability (%), the absorbance of a sample with cells grown in the presence of various concentrations of the investigated flavonoids was divided by the absorbance of control cells grown only in culturing medium and multiplied by 100. It was implied that the absorbance of the blank was always subtracted from the absorbance of the corresponding sample with target cells. We also calculated the half-maximal inhibitory