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Chemical characterization and cytotoxic activity evaluation of Lebanese propolis

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ABSTRACT

Chemical composition, anti-proliferative and proapoptotic activity as well as the effect of various fractions of Lebanese propolis on the cell cycle distribution were evaluated on Jurkat leukemic T-cells, glioblastoma U251 cells, and breast adenocarcinoma MDA-MB-231 cells using cytotoxic assays, flow cytometry as well as western blot analysis. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis revealed that ferulic acid, chrysin, pinocembrin, galangin are major constituents of the ethanolic crude extract of the Lebanese propolis, while the hexane fraction mostly contains chrysin, pinocembrin, galangin but at similar levels. Furthermore chemical analysis was performed using gas chromatography-mass spectrometry (GC–MS) to identify major compounds in the hexane fraction. Reduction of cell viability was observed in Jurkat cells exposed to the ethanolic crude extract and the hexane fraction, while viability of U251 and MDA-MB-231 cells was only affected upon exposure to the hexane fraction; the other fractions (aqueous phase, methylene chloride, and ethyl acetate) were without effect. Maximum toxic effect was obtained when Jurkat cells were cultivated with 90 µg/ml of both the crude extract and hexane faction. Toxicity started early after 24 h of incubation and remained till 72 h. Interestingly, the decrease in cell viability was accompanied by a significant increase in p53 protein expression levels and PARP cleavage. Cell cycle distribution showed an increase in the SubG0 fraction in Jurkat, U251 and MDA-MB-231 cells after 24 h incubation with the hexane fraction. This increase in SubG0 was further investigated in Jurkat cells by annexinV/PI and showed an increase in the percentage of cells in early and late apoptosis as well as necrosis. In conclusion, Lebanese propolis exhibited significant cytotoxicity and anti-proliferative activity promising enough that warrant further investigations on the molecular targets and mechanisms of action of Lebanese propolis.

1. Introduction

Natural products exhibiting anticancer properties attracted considerable attention as potential adjunctive complementary interventions to conventional treatment methods [1–3]. First, natural products are widely available and accessible, not expensive, can be prepared as easy-to-administer types of dosage forms (e.g *per os*), and widely consumed well-known popular natural products seems to be associated with low to moderate toxicity profile. However, the lack of strong clinical and experimental evidence is a major obstacle toward an established use of natural products as adjunct therapy to conventional regimens. Propolis is a glue-like resinous honey bee product collected by Apis mellifera from various plant sources [4]. A long time ago, propolis was used by human for embalming purposes to insulate and

Abbreviations: LC–MS/MS, Liquid chromatography-tandem mass spectrometry; GC–MS, Gas chromatography-mass spectrometry; PARP, Poly-ADP ribose polymerase; DAD, diode array detector; FACS, Fluorescence-activated cell sorting; EDTA, Ethylenediaminetetraacetic acid

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preserve cadavers and bodies as well as to prevent bacterial and fungal overgrowth and decomposition [5]. Additionally, propolis paste is included as an essential ingredient in various dosage forms such as tablets, capsules, toothpaste [6,7], solutions and mouthwash preparations [8,9], cream [10], and ointments [11]. More recently, a series of biological evaluations demonstrated that propolis exhibited several beneficial anti-inflammatory [12], antioxidant [13], cardioprotective [14], antimicrobial [15,16], antiviral [17,18], antiangiogenic [19], and importantly anticancer properties [20–22]. These various biological effects are mediated by a mixture of compounds found in the resin that can largely vary according to vegetation of the geographic areas [23–27]. To date, the major chemical compounds found in propolis are fatty acids, aliphatic and aromatic acids, flavonoids, alcohols, terpenes, sugars and various types of esters that include, but not limited to, Chrysin, apigenin, acacetin, galangin, kaempferol, kaempferid, quercetin, cinnanic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric caffeic acid and caffeic acid phenylethyl ester (CAPE). However, the list of chemicals and/or the level of each constituent can largely differ between the types of propolis [28]. Therefore, identification and characterization of new chemical compounds with important biological properties e.g., anti-inflammatory and/or anticancer, still emerge due to the heterogeneity in propolis sources. Several studies documented the anticancer activity of propolis, collected from various geographic locations, in various cancer cell lines such as colon and prostate cancer cells [29,30], MCF-7 (human breast cancer cells) [22,31], HT-29 (human colon adenocarcinoma) [29,32], Caco-2 (human epithelial colorectal adenocarcinoma) [32], B16F1 (murine melanoma) [32–34], and human lung and cervical cancer cell lines [33,35,36]. Propolis induced cancer cell death is thought to be mediated through induction of apoptosis, mitochondrial dysfunction and cell cycle arrest [30–32,36]. In this study, we investigated whether Lebanese propolis extracts exhibit potential anti-proliferative activity. Our results indicated that Lebanese propolis exhibited important anti-proliferative activity, and the viability of Jurkat, U251, MDA-MB-231 cells was significantly reduced upon exposure to Lebanese propolis hexane fraction. Furthermore, the toxic effect of Lebanese propolis was mediated by apoptosis as shown by apoptotic PARP cleavage, p53 protein induction and Annexin V positive cells. As a result, the cytotoxic activity of Lebanese propolis in different cancer cell lines is promising to warrant further investigation of its cytotoxic activity.

2. Materials and methods

2.1. Chemical evaluation

2.1.1. Preparation of the ethanolic crude extract and different fractions of Lebanese propolis

43 g of crude greenish powered propolis collected from the South of Lebanon were extracted with 50 ml of 70% ethanol by maceration for a week in a shaker regulated at a speed of 100 rpm and temperature of 30 °C. The insoluble portion was then separated by filtration and the filtrate was again extracted by maceration for a week in the same conditions. After the second filtration, the filtrate was kept in a freezer at −18 °C overnight and filtered again at this temperature to reduce the wax content of the extract. Part of the obtained extract was used as the crude extract. The other part of extract obtained was then used for a serial liquid–liquid fractionation process using 3 successive organic solvents from the less polar one to the more polar one: hexane, methylene chloride and ethyl acetate. Each fraction obtained was filtered, and concentrated at reduced pressure with a rotary evaporator at 40 °C. The crude extract and the aqueous phase were dried by lyophilization. Dry extracts of propolis were obtained and weighed: ethanolic crude extract (m = 18.5734 g), hexane (m = 1.5482 g); methylene Chloride (m = 0.5521 g), ethyl acetate (m = 0.4510 g) and aqueous phase (m = 5.761 g).

2.1.2. LC–MS/MS analysis

LC–MS/MS analysis was performed on an API 4000™LC/MS/MS system. Two phenolic acids (caffeic acid and trans-ferulic acid) and nine flavonoids including (rutin, quercetin, daidzein, genistein, apigenin, kaempferol, chrysin, pinocembrin and galangin) were investigation. The chromatographic system consisted of a pump LC-20AD (Flow: 0.3 ml/min, Pressure range: 0–276 bar), an autosampler, a degasser and an automatic thermostatic column compartment. The injection volume was 25 ml. The LC was run on a reversed phase octadecylsilane C18-column with standard mesures (250mḿ4 mm i.d., 5 mm particle diameter) and its temperature was maintained at 30 °C. The mobile phase was composed of solvent (A) 0.1% (v/v) formic acid in water, and solvent (B) acetonitrile, which were previously degassed and filtered. The solvent gradient started with 80% (A) and 20% (B), reaching 30% (B) at 10 min, 40% (B) at 40 min, 60% (B) at 60 min, 90% (B) at 80 min and followed by the return to the initial conditions. The freeze-dried extracts (10 mg of each) was dissolved in 1 ml of 80% of ethanol prior analysis. All samples were filtered through a 0.2 mm Nylon membrane (Whatman). The flow rate was 1 ml/min and split out 200 ml/min to MS. The MS used was an AB SCIEX triple quadrupole ion trap mass spectrometer MS equipped with an ESI source. Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative and positive ion modes, with ESI needle voltage set at 5.00 kV and the ESI capillary temperature at 325 °C. Multiple Reaction Monitoring (MRM) were used for targeted quantification and screening. The MS data were simultaneously acquired for the selected precursor ion. The collision induced decomposition and MS experiments were performed using helium as the collision gas, with a collision energy of 25–40 eV. Compounds were characterized based on their mass spectra, using the precursor ion, fragment ions, and comparison of the fragmentation patterns with the eleven standards investigated and molecules described in the literature. Quantification was established based on spectra of the analytical standard solutions showing the precursor ion.

2.1.3. Gas chromatography–mass spectrometry (GC–MS) analysis

2.1.3.1. Instruments. Gas chromatography-mass spectrometry was carried out on an Agilent GCMS, GC 7890 B MS in EI mode 5977 gas chromatograph with Auto-injector G4513A. The chromatographic column for the analysis was DB–5 ms Ultra Inert capillary column (30m × 250 µm i.d., 0.25 µm film thickness). The carrier gas used was high-purity helium (99.999%, UAE). The injection was performed in pulsed splitless mode (200 kPa until 0.5 min, Pressure 89.149 kPa), the injector temperature was maintained at 280 °C, the flow rate was of 1.2 ml/min for 16 min then 10 ml/min to 1.7 ml/min for 12 min, the purge flow was of 15 ml/min at 1 min. The injection volume was 1 µl. Samples were analyzed with the column held initially at 100 °C for 1 min and then increased to 300 °C with a 15 °C/min heating ramp for 14.3333 min. Run time was of 28.666 min. The interface temperature was 280 °C. Full-scan EI (Electron Impact) spectra were recorded from 30- 550 *m/z* (Mass/charge) with 2 scans per second. Peaks were identified by computer searches in reference libraries.

2.1.3.2. Sample preparation for injection. 3 mg of dry hexane propolis extract was diluted in 3 ml Ethyl Acetate then centrifuged for 10 min at 13k RPM. For underivatized free sample, 1 µl of the sample was directly injected into the GC–MS chromatograph. For derivatization, 100 µl of the diluted sample was evaporated under N2 Stream without heating, then reconstituted in 1000 µl of bis-(trimethyl-silyl) trifluoroacetamide (BSTFA) and incubated for 30 min at 80 °C. 1 µl of this derivatized sample was then injected into the GC–MS chromatograph.

2.2. Biological evaluation

2.2.1. Cell lines and culture conditions

Human Jurkat leukemic T cells, human glioblastoma U251 cells,