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Exploring the anticancer effects of standardized extracts of poplar-type propolis: In vitro cytotoxicity toward cancer and normal cell lines

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ABSTRACT

Propolis was shown to exert antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. Its composition is influenced by seasonal, climatic and phytogeographic conditions. Further variability derives from the extraction methods. Multi Dynamic Extraction Method (MED) has been recently proposed to improve extracts reproducibility. Here, the cytotoxic/anticancer activity of three MED extracts of poplar-type propolis was assayed on human promyelocytic leukaemia HL60, human monocytic leukaemia THP-1, human osteosarcoma MG63, murine fibroblast L929 and human mesenchymal cells (hMSCs). As far as we are aware of, MG63 cells have never been challenged with propolis before, while few studies have so far addressed the effects of propolis on non-tumor cell lines. Consistent results were observed for all propolis preparations. The extracts turned out mildly cytotoxic toward cancer cells, in particular osteosarcoma cells (IC50: 81.9–86.7 μg/ml). Nonetheless, cytotoxicity was observed also in non-tumor L929 cells, with an even lower IC50. hMSCs demonstrated the lowest sensitivity to propolis (IC50: 258.3–287.2 μg/ml). In THP-1 cells, extracts were found to stimulate apoptosis caspase 3/7 activity. The IC50 values observed with osteosarcoma and leukaemia cells do not support a relevant cytotoxicity (as the figures abundantly exceeded 30 μg/ml), despites some selective activity exhibited with HL60 cells. The results confirm the validity of the extraction method, emphasizing the need to assess the selectivity of the interaction with cancer cells when screening for anticancer-drug candidates.

1. Introduction

Alternatively know as bee wax, propolis is a complex resinous material that bees collect from vegetable plants, elaborate and use as a sort of cementing substance to insulate and protect the beehives [1]. In addition to functioning as a sealing material, propolis exerts antimicrobial activity acting as a natural ant-infective material. Given its vegetable origin, propolis composition is strongly influenced by the flora inhabiting the area of production. Thus, phytogeography, but also climatic and seasonal conditions affect its final chemical composition [2–4]. Primarily consisting of resins and wax, together representing the most conspicuous fraction (respectively up to about 50% and 30%), propolis contains about 10% of essential oils and fatty acids, and, in minor proportion, pollen, organic acids, amino acids, vitamins and minerals [3,5]. Polyphenols such as flavonoids, flavones, flavanols, and phenolic acids are an important fraction of the approximately three hundred chemical substances contained in propolis.

The rich composition in active phytocompounds has been found to

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confer to propolis several interesting properties. Over the last decades, propolis was demonstrated to possess protective antioxidant and reactive oxygen species (ROS)-scavenging properties [6–9], to be anti-inflammatory [10,11], to exhibit antiparasitic [12] and antimicrobial activity against a broad spectrum of bacteria [13–16], fungi [15,17] and viruses [18], to be neuroprotective [19], to be a wound healing promoter [20], and to have several anti-cancer functions, not only being selectively cytotoxic, anti-proliferative and pro-apoptotic against tumor cells, but also anti-metastatic, anti-mutagenic, anti-invasive and anti-angiogenic [10,21,22]. Moreover, propolis lends itself to being incorporated or encapsulated with pectin, a plant cell wall polysaccharide acting as non-toxic drug delivery [23,24]. Wound healing patches composed of pectin and propolis [25] and chitosan-pectin hydrogels encapsulating propolis [26] are suggestive examples.

Besides various terpenes, sterols, aldehydes and tannins, other phytocompounds occur in propolis including: caffeic acid, caffeic acid phenethyl ester (CAPE), artepillin C, quercetin, myricetin, pinocembrin, pinobanksin, apigenin, luteolin, naringenin, kaempferol and chrysin, among many others [22,27–30].

The type of propolis is often described referring to its colour (e.g. brown, red, green), the species of producer bees, the plants used by the bees (e.g. poplars, conifers and pines), and the geographic origin, all these variables contributing to distinct physical characteristics, chemical composition, and biological properties. In 2015, Silva-Carvalho et al. [29] reviewed the many different qualities of propolis, discussing the respective chemical characteristics and biological activities. The variable propolis composition is a largely debated, hard to circumvent, limitation for an as-it-is therapeutic product. However, the composition of propolis extracts is significantly influenced not only by the starting row material, but also by the solvents and the procedures followed for the extraction of its active fraction. To overcome some of these limitations, many efforts have been paid to develop well- standardized extraction methods. In a previous work of our research group [16], different batches of pooled raw poplar-type propolis from Europe, America, and Asia propolis were extracted by the Multi Dynamic Extraction method (MED). After the extraction procedure, all three product batches exhibited standardized polyphenolic mixtures from propolis, with reproducible chemical composition, and equivalent anti-microbial activity, with no relevant differences in their effects against antibiotic-susceptible and antibiotic-resistant bacterial strains. Here, in view of the great interest evoked by the toxicological properties of propolis toward cancer cells, further investigations were conducted to ascertain the activity of propolis extracts against the human osteosarcoma MG63 cell line, the leukaemia HL60 and the THP-1 cell lines, the normal murine fibroblasts L929 and human mesenchymal cells (hMSCs).

2. Materials and methods

2.1. Materials

This study was conducted on three distinct batches of propolis extract preparations, named Sample A, Sample B and Sample C, respectively. Propolis extracts were prepared combining a European, an American, and an Asian poplar-type raw propolis using Multi Dynamic Extraction (N. 001425516)1 by B Natural srl (Corbetta, MI, Italy), as reported in [16]. The propolis extract preparations had the following composition: water (25.5%); cane sugar (24.5%); propolis extract (24%); corn maltodextrins (9%); saccharose (8%); Arabic gum (6%); caramel (3%). The vehicle without propolis extracts was also used for control in the various tests (Vehicle Control). It had a similar composition than the propolis extracts preparations, except for the propolis extract component that was replaced by sterile deionized water. The chemical composition of the tested MED propolis extracts is reported in [16].

2.2. Cell cultures

Five eukaryotic cell lines were used for this study on the cytotoxicity of propolis extracts, respectively: the human osteosarcoma cell line MG63 (ATCC, Rockville, MD, USA), the murine immortalized fibroblast line L929 (ATCC, Rockville, MD, USA), the human promyelocytic leukaemia HL60 cells (ATCC, Rockville, MD, USA), the human monocytic leukaemia THP-1 cells (Sigma-Aldrich, Milan, Italy) and the bone marrow-derived, human mesenchymal cells (hMSCs, BioWhittaker Inc., Walkersville, MD, USA). Both MG63 and L929 cell lines were routinely cultured in MEM growth medium (Invitrogen Ltd, Paisley, UK), supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen Ltd, Paisley, UK), and penicillin/streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich, Milan, Italy), under standard culture conditions. Cells were regularly sub-cultured using a Trypsin-EDTA solution (Sigma-Aldrich, Milan, Italy) to detach adhered cells. For this investigation, hMSCs cells were thawed from a frozen stock and sub-cultured in Alfa-MEM medium (Lonza, Euroclone S.p.A, Pero, MI, Italy) supplemented with Ultra Glutamine (Lonza), 10% FBS and penicillin/streptomycin, twice a week. Both leukaemia-derived cell lines were cultured in suspension in RPMI (Life Technologies), which was supplemented as described above for MEM medium.

2.3. Resazurin cytotoxicity assay

The cytotoxicity of the propolis extracts was assayed on the human osteosarcoma cell line MG63 and the murine cell line L929, by the resazurin test [31,32]. For the test, following a similar scheme adopted for both cell lines, 96-well tissue-culture treated plates (96-Well CytoOne® Plate, STARLAB S.r.l., Milano Italy) were prepared by adding to each well 100 μl of a suspension of either the MG63 cells or the L929 cells in MEM medium at a concentration of 5×10^4 cells/ml. The propolis extracts preparations (Samples A, B and C) and, in parallel, the Vehicle Control were diluted 1–12 (w/v) in MEM supplemented with FBS and antibiotics.

In the case of three different propolis extracts batches, this initial stock solution corresponded to a concentration of 20 mg/ml of propolis extract. Starting from this stock solution, serial dilutions were prepared for each propolis extract batch. Parallel dilutions in medium were also prepared for the Vehicle Control.

Further controls used in each plate of the cytotoxicity tests included the medium control, consisting just of MEM medium fully supplemented with FBS, and antibiotics) and a positive control solution, consisting of 4% Tween 20 (Sigma-Aldrich, Milan, Italy) in MEM medium.

The culture plates seeded with the cells were incubated overnight at 37 °C. Volumes of 100 μl of test samples and control solutions were subsequently added to each well following the scheme reported in Fig. 1. After this 1:1 serial dilution, the concentrations of the propolis extracts

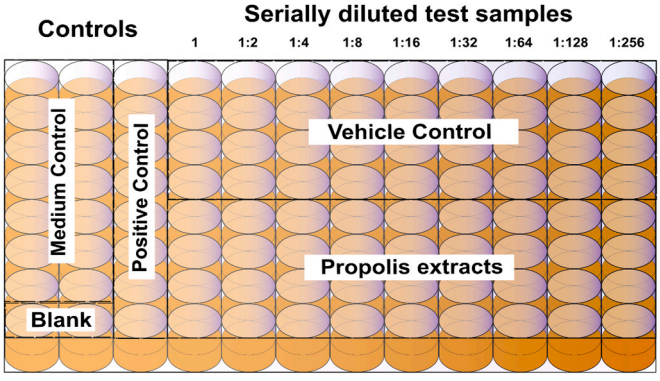

Fig. 1. The illustration reports a typical microplate scheme adopted to assay the cytotoxicity of the different test treatments and control solutions.

actually assayed ranged from 10 mg/ml to 39 μg/ml. Each dilution of samples and of the control vehicle was tested in quadruplicate within each microplate.

The test was repeated in at least three independent experiments. After 1 day of incubation under standard culture conditions, 20 μl of a filtered 2 mg/ml solution of resazurin sodium salt (Sigma- Aldrich, Milan, Italy) were added to each well (final concentration 100 mg/l).

Microtiter plates were then incubated at 37 °C for 4 h in the dark. After the incubation, 150 μl of solution were transferred from each well of the microplate to a new Greiner LUMITRAC™ 200 microplate for fluorescence reading. A Modulus II Multifunction Plate Reader (Turner BioSystems, Sunnyvale, CA, USA) was used to read the microplate in green fluorescence modality. The results were normalized and expressed as percent of cell activity with respect to the medium control within each plate, which was equated to 100% cell activity.

2.4. Cell viability by bioluminescent ATP assay

The effects of propolis extract on hMSCs, HL60 and THP-1 cells were assayed by a bioluminescent ATP assay. Briefly, for hMSCs, 96-well tissue-culture treated plates (96-Well CytoOne® Plate, STARLAB S.r.l., Milano Italy) were prepared by adding to each well 100 μl of a suspension of hMSCs in alfa-MEM complete medium at a concentration of 5×10^4 cells/ml. After 1 day of incubation under standard cell culture conditions, 96-well plates were treated with propolis extracts and control solutions as earlier described for the resazurin assay and illustrated in Fig. 1. After further 24 h of incubation at 37 °C, the 96-well plates were processed as follows: a volume of 100 μl of medium was removed from each well and replaced by a corresponding volume of solution of the CellTiter-Glo 2.0 Assay (Promega, Milano, Italy). Following 10 min of incubation at room temperature in the dark, the plates were processed for bioluminescence reading by the Modulus II Multifunction Plate Reader (Turner BioSystems). The readings in relative luminescence units (RLU) were log-transformed. For the extrapolation of the IC50 values of the vehicle, after subtraction of the blank all data were normalized by equating the Medium Control to 1. Conversely, for the IC50 values of the extracts, the $\log_{10}$(RLU) value of each dilution of the extracts was normalized by the mean of the corresponding dilution of the Vehicle Control equated to 1. Conversely, for both leukaemia cell lines grown in suspension, 96-well non-tissue-culture treated plates were prepared by adding to each well 100 μl of 10×10^4 cells/ml in medium. All other steps were the same as described for hMSCs. At least two independent experiments were performed with each propolis extract sample.

2.5. Observation of cytomorphology under light microscopy

2.5.1. Phase contrast microscopy

For the observation under phase contrast microscopy, MG63 and L929 cells were seeded and cultured on 4-well chamber slides (Thermo Scientific Nunc, Waltham MA, USA). Briefly, the two cell lines were prepared at a concentration of about 6.4×10^4 cells/ml in medium and a volume of 500 μl of cell suspension was added to each well. After 24 h in culture, cells MG63 and L929 were treated adding 500 μl the propolis extracts or control solutions. Propolis extracts were tested at a concentration of 39 μg/ml. Treated cells were incubated under standard culture conditions and examined under microscopy the following day. The phase contrast imaging was applied in this study by using an optical microscope (Eclipse Ti, Nikon, Nikon Instruments S.p.A, Campi Bisenzio, Italy). A 10 × phase contrast lens (Plan Ph1 0.25 NA, Nikon) and a CCD camera (DS-Qi1Mc, Nikon) were used for image acquisition. The image acquisition was implemented through NIS-Elements Advanced Research software (Version 5.20 64 bit edition, Nikon). The pixel size for all grabbed images was 1280 × 1024 and 4096 gray levels.

2.5.2. Confocal laser scanning microscopy (CLSM)

For the observation of the cytomorphology under CLSM using fluorescence labelling, MG63 and L9292 cells were cultured on sterile round glass coverslips. Briefly, suspensions of each cell line were prepared at a concentration of about 6.5 × 104 cells/ml.

Sterile coverslips were deposited on the bottom of the wells of 12-well tissue culture plates (Cat. 83.3921.500, Sarstedt, Nümbrecht, Germany) and 1 ml of cell suspension was added to each well. The plates were organized with negative and positive controls and treatments (vehicle and the three propolis extracts) tested in duplicate. Following a scheme similar to the one used for the cytotoxicity tests, the wells of the negative control were treated with complete MEM medium and those of the positive control with a solution of 4% Tween 20 in MEM medium.

The treatments with the vehicle and with propolis extracts were prepared as earlier explained and tested at a concentration corresponding to 39 μg/ml. After 24 h of treatment, the coverslips were stained for CLSM microscopy. All the wells of the 12-well plates were washed trice with 2 ml of PBS with calcium and magnesium (Cat. 14080–048, Life Technologies, Monza, Italy). The PBS solution was removed, and the wells were treated with 300 μl of a 1x solution (5 μg/ml) of CellMask™ Deep Red Plasma Membrane Stain (Deep Red: Excitation/Emission 649/666 nm) (Cat. C10046, Life Technologies) for 10 min at 37 °C, following the indications of the producer.

CellMask™ Deep Red Plasma Membrane Stain was used to stain cell membranes. The wells were washed once with PBS and treated cells on the coverslip were subsequently fixed by 3.75% paraformaldehyde at a temperature of about 37 °C for 10 min. Finally, cell nuclei of cultured cells were stained by a 10 mg/ml solution of Hoechst 33342 in water (Blue: Excitation/Emission 350/461 nm) (Cat. H3570, Life Technologies) for 15 min. The stain was removed, and cover slips were mounted using the Vectashield HardSet™ Mounting Medium (Cat: H-1400, Vector Lab, Burlingame, CA). Confocal imaging was performed using a Nikon A1 confocal laser scanning microscope, equipped with a 60 × objective (Plan Apo VC 1.4 NA, Nikon) and with 405 and 647 nm laser lines. Z-stacks were collected at optical resolution of 210 nm/pixel, stored with pixel size of 512 × 512 at 12-bit with 4096 different gray levels. The pinhole diameter was set to 1 Airy unit and z-step size to 300 nm. All image analyses and 3D rendering were performed using NIS-Elements Advanced Research software (Version 5.20 at 64 bits, Nikon).

2.6. Apoptosis assessment

The Caspase-Glo® 3/7 kit (Cat: G-8091, Promega Corporation, Milano, Italy) was used to assess the activity of caspase 3/7 in THP-1 cells, which were challenged with propolis extracts at two different concentrations, respectively 442 μg/ml (corresponding to more than twice the IC50) and 1250 μg/ml. This experimental part of the study was performed only with one propolis extract preparation, i.e. Sample A. 100 μl of a suspension of THP-1 cells in completely supplemented RPMI were added to the wells of a 96-well plate. After 24 h of cell culture, the cells were treated as follows. The negative control was obtained by adding to the cells 100 μl of RPMI medium. For the positive control 100 μl of a fresh 10 μM staurosporine solution (Cat: S6942, Sigma-Aldrich, Milan, Italy) were added to triplicated wells. Triplicate wells containing RPMI medium w/o cells were used for the blank. Vehicle control treatments and propolis extracts treatments were tested at the same final dilution of the propolis extracts mentioned above. After 2.5 h of incubation of the plate under standard culture conditions, the reconstructed substrate of the Caspase-Glo® 3/7 kit was added to each well, following the indications of the manufacturer. After 1.5 h of incubation in the dark, the bioluminescence was read by Modulus II Multifunction Plate Reader (Turner BioSystems). Two independent experiments with triplicate samples were performed.

2.7. Statistics

The statistical analysis performed by ANOVA followed by

Bonferroni/Dunn test was used to check the different cell activity exhibited with the resazurin test by Samples A, B and C at the same dilution factor (StatView, version 5.0.1, Sas Institute Inc.). Differences across the three preparations of propolis extracts were never found to be statistically significant. The same analysis was also applied to assess the results concerning the activation of caspase 3/7 activity. The IC50 values for each extract preparation were obtained using the Quest Graph™ IC50 Calculator, AAT Bioquest, Inc (https://www.aatbio.com/tools/ic50-calculator) online resource. In this case, the data were normalized equating the values for each corresponding concentration of the vehicle to 1 within each plate. To take in account even the mild toxicity of the vehicle and avoid major interferences, the IC50 values were calculated using exclusively dilutions that were in a range of low toxicity of the vehicle. Conversely, the IC50 of the Vehicle Control had to be calculated normalizing the data by equating the Medium Control to 1.

3. Results

3.1. Resazurin assay

The study of the cytotoxicity based on the resazurin assay revealed that the vehicle of the propolis extract itself expressed some cytotoxicity toward osteosarcoma cells MG63, with an IC50 approaching a concentration of 1.042 mg/ml, which was achieved at a dilution corresponding to 250.8 μg/g for the propolis extracts. The low cytotoxicity of the vehicle medium may simply derive from a hyperosmolarity of the vehicle or from other factors that could affect this type of preparation, usually not for internal use. Honey itself has been reported to be cytotoxic when tested in vitro on L929 cells [33], with an IC50 of 3.1%.

The three propolis preparations showed similar cytotoxicity curves. Even at the lowest concentration tested (39 μg/ml), the treatment of cells MG63 with propolis extracts resulted in a significantly reduced cell activity with respect to both the Medium Control and the Vehicle Control, suggestive of metabolic inhibition and mild cytotoxicity. In detail, at such concentration, the mean reduction in cell activity observed for the three propolis samples was of 36.5% ± 3.3% (mean ± standard deviation) with respect to the Medium Control and 38.4% ± 4.9% with respect to the Vehicle Control. Fig. 2 illustrates the variation of cell activity as a function of the concentration of propolis extracts. The IC50 values calculated for the three different preparations were of 86.7 μg/ml for Sample A, 81.9 μg/ml for Sample B and 84.0 μg/ml for Sample C.

When the tests were performed on the murine fibroblast cell line L929, the cytotoxic effects were more pronounced not only for all three preparations of propolis extracts, but also for the Vehicle Control (Fig. 3).

At the lowest concentration tested (39 μg/ml), the mean reduction in cell activity with respect to the Medium Control observed for the three samples was of 59.5% ± 2.7% (mean ± standard deviation). These findings are suggestive of a greater susceptibility of the murine fibroblasts to the toxicity of the preparations containing the propolis extracts. The three preparations of propolis showed similar, well aligned, cytotoxicity curves and never reached a cell activity level of 50% than the medium control, even at the highest dilution used in the set of experiments.

A further set of experiments was conducted just to enable to assess the IC50 for the three different preparations. In this further set of experiments, treatments were tested in quadruplicate in 4 independent microplates down to a concentration of 9.8 μg/ml. As expected, the IC50 values for Samples A, B and C were found to be very similar, in the range of 17.9–18.6 μg/ml.

These rather low values imply that, for all three batches of propolis extracts, the selectivity index (SI: calculated as the ratio: IC50 value L929 cells/IC50 value of MG63 cells) was always <0.5, thus much lower than the threshold of 2 required for a significant selectivity in cell inhibition of cancer cells [34].

3.2. ATP bioluminescence cell viability assay

hMSCs, THP-1 and HL60 viability after treatment with the three propolis extracts was assessed by ATP bioluminescence assay. hMSCs exhibited a much lower susceptibility than the other two cancer cell lines both to the effects of the Vehicle Control and of the propolis extracts (Fig. 4). The Vehicle Control appeared to be non-cytotoxic as its IC50 value exceeded 4 mg/ml (4.127 mg/ml). However, hMSCs were found to be less sensitive also to all propolis extracts. Once again, the calculated IC50 values for Sample A, B and C resulted relatively close, respectively of 263.2, 258.3 and 287.2 μg/ml.

Previous work by Miret et al. had earlier documented that the results of cytotoxicity tests such as resazurin and the ATP bioluminescence cell viability assay, both based on metabolic markers, are generally well

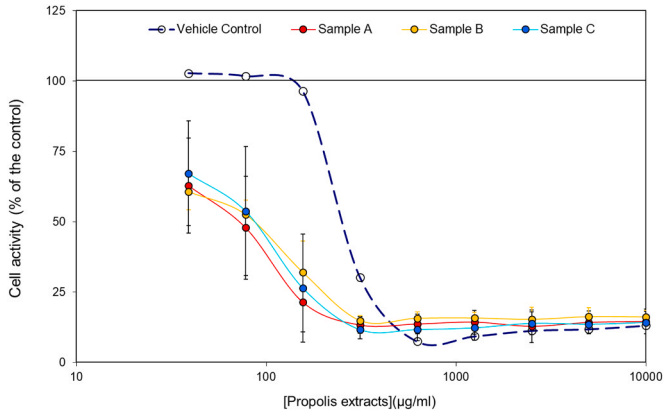

Fig. 2. Cytotoxicity of the different propolis preparation batches as a function of the different concentration when tested on MG63 cells. The cell activity was normalized within each microplate by equating the mean value found for the Medium Control to 100%. Mean cell activities ± standard deviations are shown for propolis extracts (N = 12). A curve based on the mean values is shown for the Vehicle Control. This control consisting of the vehicle lacking propolis extracts was itself to some extent cytotoxic. The three different propolis-containing preparations appeared all similarly cytotoxic down to the lowest concentrations tested, without statistically significant differences at each single point.

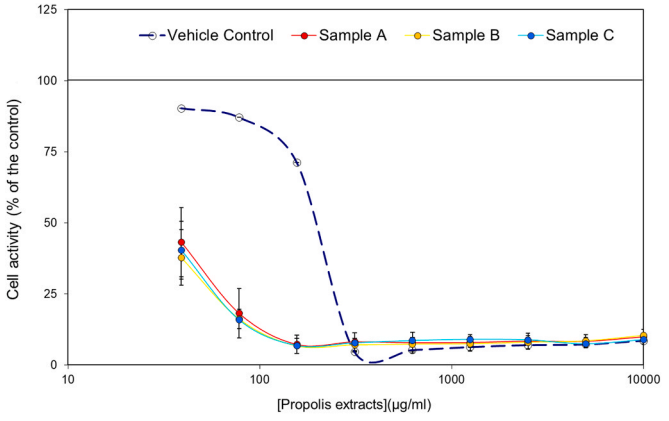

Fig. 3. Cytotoxicity of the different propolis preparation batches as a function of the different concentration when tested on fibroblast cells L929. Mean cell activity ± standard deviation is shown for propolis extracts (N = 16). A curve based on the mean values is shown for the Vehicle Control. The control, consisting of the vehicle without propolis extracts, was itself found to affect the cellular metabolism and exhibited an IC50 of 695.6 mg/ml (the same dilution corresponding to 166.9 μg/ml of propolis extracts for the treatments). Nonetheless, all three batches of propolis-containing preparations appear cytotoxic up to the lowest concentrations, without statistically significant differences at each single concentration.

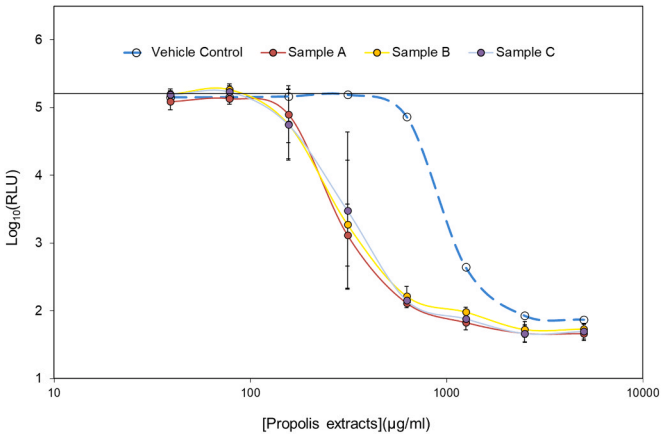

Fig. 4. Cytotoxicity of the different propolis preparation batches as a function of the different concentration when tested on hMSCs. The metabolic activity of the hMSCs is expressed as logarithm of relative luminescence units (RLU). Mean cell activity ± standard deviation is shown for propolis extracts (N = 8).

correlated [35] and provide similar values in terms of 50% inhibitory concentrations. These results would support a dissimilar behaviour of primary hMSCs with respect to the normal murine fibroblasts L929, which are commonly used as a reference cell line to assess anticancer drugs selectivity [36,37] and, in this study, appeared particularly sensitive even to low propolis extracts concentrations. However, direct comparisons of IC50 values obtained by different cytotoxicity methods should probably be avoided.

The cytotoxicity of the propolis extracts on THP-1 cells was found to be slightly greater than that observed in the case of hMSCs (Fig. 5) and the IC50 values measured for Sample A, B and C were respectively of 187.4, 203.2 and 164.4 µg/ml. Interestingly, these results would suggest a greater cytotoxicity of the propolis extracts towards THP-1 cells than hMSCs. This is also reflected by an SI ranging from 1.27 to 1.75 for the three different samples, which anyway never reached the fixed threshold for significance of 2. Furthermore, the same cells were also more susceptible to the Vehicle Control as the IC50 calculated for the Vehicle Control was 2.66 mg/ml, and, thus, they appear more generally susceptible to cytotoxic stimuli.

Leukemic HL60 cells exhibited a susceptibility similar or even greater than THP-1 cells to propolis extracts (Fig. 6). IC50 values measured for Sample A, B and C were respectively of 126.0, 185.8 and

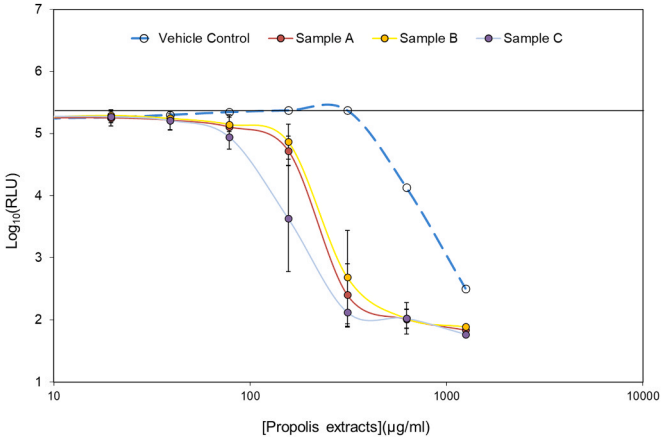

Fig. 5. Cytotoxicity of the different propolis preparation batches as a function of the different concentration when tested on THP-1 cancer cells. The metabolic activity of the THP-1 cells is expressed as logarithm of relative luminescence units (RLU). Mean cell activity ± standard deviation is shown for propolis extracts (N = 12).

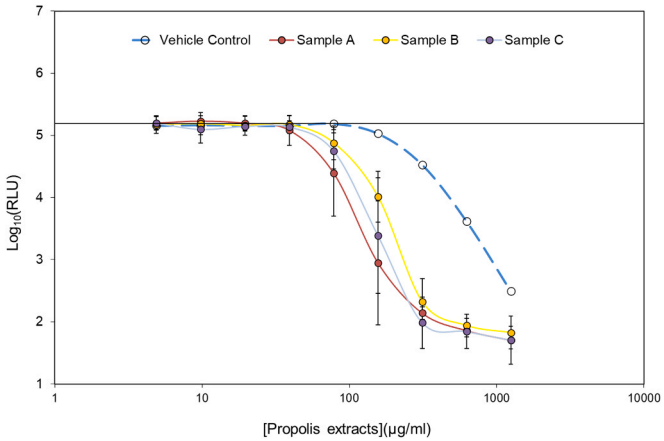

Fig. 6. Cytotoxicity of the different propolis preparation batches as a function of the different concentration when tested on HL60 cancer cells. The metabolic activity of the HL60 cells is expressed as logarithm of relative luminescence units (RLU). Mean cell activity ± standard deviation is shown for propolis extracts (N = 8).

149.6 µg/ml. In the case of HL60 cells, the IS of propolis extracts reached the value of 2.1 for Sample A and 1.9 for Sample C. Although, these IS values would be suggestive of some selective effect, the IC50 values far exceed the concentration of 30 µg/ml considered indicative of significant cytotoxicity. As observed for the THP-1 cells, even the susceptibility of HL60 cells to the Vehicle Control slightly varied (IC50 of 2.92 mg/ml) with respect to that of hMSCs.

3.3. *Microscopic observations*

3.3.1. *Phase contrast microscopy*

The observations of cell cultures under phase contrast microscopy were consistent with the results of the resazurin test. In the case of the Medium Control, cultures of both cell lines appeared just slightly subconfluent or nearly confluent and fibroblasts and osteosarcoma cells exhibited a typical morphology, well adhered on the tissue culture surface of the microtiter plates and with regular nuclei and nucleoli. With fibroblasts L929, dividing round cells were also noticeable (Fig. 5). Conversely, MG63 cells generally appeared well spread and tightly adhering to the substrate.

In the case of the Positive Control, both fibroblasts and osteoblast-like cells lost their adherence and often clustered together, exhibiting an irregular, grossly round morphology and signs of cell suffering with extensive alterations.

For the control performed using the dilutions of the vehicle as well as for all three propolis extracts preparations, insoluble sedimented microparticles, probably consisting of the only known, partly soluble, component, i.e. the Arabic gum, were observable even at the highest dilutions of the treatments. In order to exclude bacterial contamination, at the end of the microscopic observation, random samples of the culture medium from the various treated wells were plated on Tryptone Soy Agar (MEUS Srl, Piove di Sacco, Italy) and checked for bacterial growth, but colony formation was never noticed. The observation under CLSM further excluded the presence of microbial contamination, as the solid particles were not stained by the Hoechst 33342, a DNA-staining dye.

The treatment with the three distinct preparations of propolis extracts produced comparable effects. The cell number appeared decreased and a consistent fraction of fibroblast cells L929, much larger than in the Medium Control, was loosely attached and with a round morphology. Nonetheless, spread viable cells with a regular morphology were still observable. Similarly, even in the case of the osteosarcoma cells MG63, a fraction of cells rounded up and lost their adhesion to the substrate. Overall, for both cultured cell lines, at the low propolis extract

concentration of 39 μg/ml, some viable cells with visible, apparently regular, nuclei and nucleoli were present, but the extension of cell spreading appeared generally diminished. (Fig. 7).

3.3.2. Confocal laser scanning microscopy (CLSM)

The observations under fluorescence microscopy further supported the findings of the resazurin test and of the phase contrast microscopy. In the Medium Controls, cultures of both cell lines appeared just slightly sub-confluent, with a typical morphology and well adhering to the tissue culture surface of the glass coverslips (Fig. 8). In the case of the Positive Control, having lost their adherence to the substrate, both fibroblast and osteosarcoma cells were mostly removed from the glass surface by the washes during the preparation of the slides. Rare still adhering cells exhibited an irregular morphology with signs of advanced detachment from the substrate, extensive cellular damage and altered nuclei. The cultures treated with the Vehicle Control and the propolis extracts at a concentration of 39 μg/ml did not exhibit the presence of visible insoluble microparticles of the vehicle under phase contrast, perhaps for their removal during washing, but, more likely, due to their negativity to fluorescence stains. The treatment with the three distinct preparations of propolis extracts produced similar effects. The number of the adhered cells appeared reduced to about one- third in the case of cells L929 with respect to the Vehicle Control and most loosely attached cells with round morphology were probably removed during the preparation. However, the adhered cells showed a regular morphology as those of the Medium and the Vehicle Controls. Similarly, even in the case of the osteosarcoma cells MG63, adhered cells appeared less numerous than the Medium and the Vehicle Control but exhibited a regular well spread morphology.

3.4. Caspase-3/7 activities in THP-1 cells treated with propolis extracts

The measurement of the activity of caspase 3 and 7, two central effector caspases, revealed that propolis extracts in Sample A could induce apoptosis in THP-1 cells (Fig. 9), but just at the low concentration of 442 μg/ml. Noticeably, after 2.5 h of incubation with the cells, the propolis extracts were found to activate the same level of response observed for the Positive Control, consisting of 5 μM staurosporine. At greater concentration (1250 μg/ml), propolis extracts showed just a slight, not statistically significant, increase in caspase 3/7 activity with respect to the Reference Control. On the contrary, the Vehicle Control with the highest concentration exhibited a slightly decreased activity with respect to the Reference Control, where the cells were treated just with RPMI medium, but, also in this case, the difference was not significant.

4. Discussion

Apart from the interest in achieving propolis-based products with well-tailored therapeutic properties, the investigation of the activities of different propolis types enables a rapid screening for the identification of active phytocompounds and the development of new drugs of pharmaceutical importance. It has to be emphasized that, in literature, there is still much need for good-quality work that meets high research standards [38]. In many studies on anti-cancer activities of propolis, the selectivity of cytotoxicity of propolis extracts has been often overlooked and so have been relevant recommendations such as those of the National Cancer Institute (NCI) [39]. Here, we aimed at ascertaining both the existence of an anticancer activity of the propolis extracts on an osteosarcoma and two reference leukaemia cell lines and the consistency of the results between different batches of propolis obtained with the same extraction method. To ascertain if the effects toward the cancer cells were specific, the toxicity of the propolis extracts was also assayed on a reference cell line of mouse connective tissue and on primary hMSCs. The results obtained in terms of IC50 for all the tested cell lines are reported in Table 1, where they are compared to those reported in literature by similar previous studies.

The IC50 values in the range of 17.9–18.6 μg/ml observed with L929 in this work are just slightly lower than the values reported by Machado et al. [8] (2016) for the ethanol and methanol extracts of yellow propolis, respectively of 31.6 and 30.1 μg/ml (with 21.3–46.9 and 23.5–38.7 confidence intervals). In their study, the authors reported that green propolis extract samples exhibited insignificant toxicity towards all cancer cell lines tested and yellow Brazilian propolis exhibited relevant cytotoxicity only against ovarian carcinoma. Conversely, brown and red propolis had the highest cytotoxic potential and selectivity index, with significantly greater IC50 values, when tested on L929 cells.

Sadeghi-Aliabadi et al. [33] (2015) investigated the cytotoxic effects of *Astragalus* honey and propolis extracts on two human cancer cell lines, the human bladder cancer cell line 5637 and the hepatic cancer cell line HepG2, and on L929 cells, exploring also the effects on their oncogenic and proapoptotic gene expression profiles. The authors reported that honey was two-fold more cytotoxic towards cancer cells than normal L929 cells. Moreover, only in the two cancer cell lines, honey was found to decrease the expression of the Bcl-2 oncogene, whose overexpression is known to inhibit apoptosis, but did not upregulate the tumor-suppressor gene p53. Conversely, although propolis demonstrated a selective cytotoxicity against the two cancer cell lines with respect to the normal fibroblast L929 cells, it did not alter the expression of the Bcl-2 and p53 genes. The authors reported for propolis extracts an IC50 value of 58 μg/ml when tested against L929 cells and of 30 and

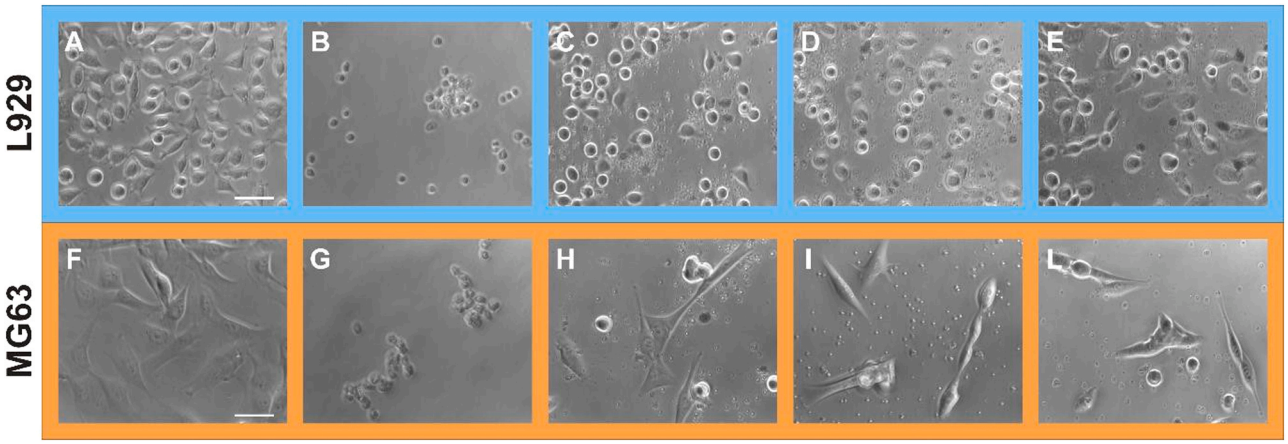

Fig. 7. Representative phase contrast micrographs illustrating density and morphology of cells L929 and MG63 after 1-day treatment with propolis extracts at the concentration of 39 μg/ml. Corresponding micrographs of the Medium Control and the Positive Control are also shown (bar = 50 μm). Legend: A-E, L929 cells; F-L, MG63 cells; A and F, Medium Control; B and G, Positive Control; C and H, Sample A; D and I, Sample B; E and L, Sample C.

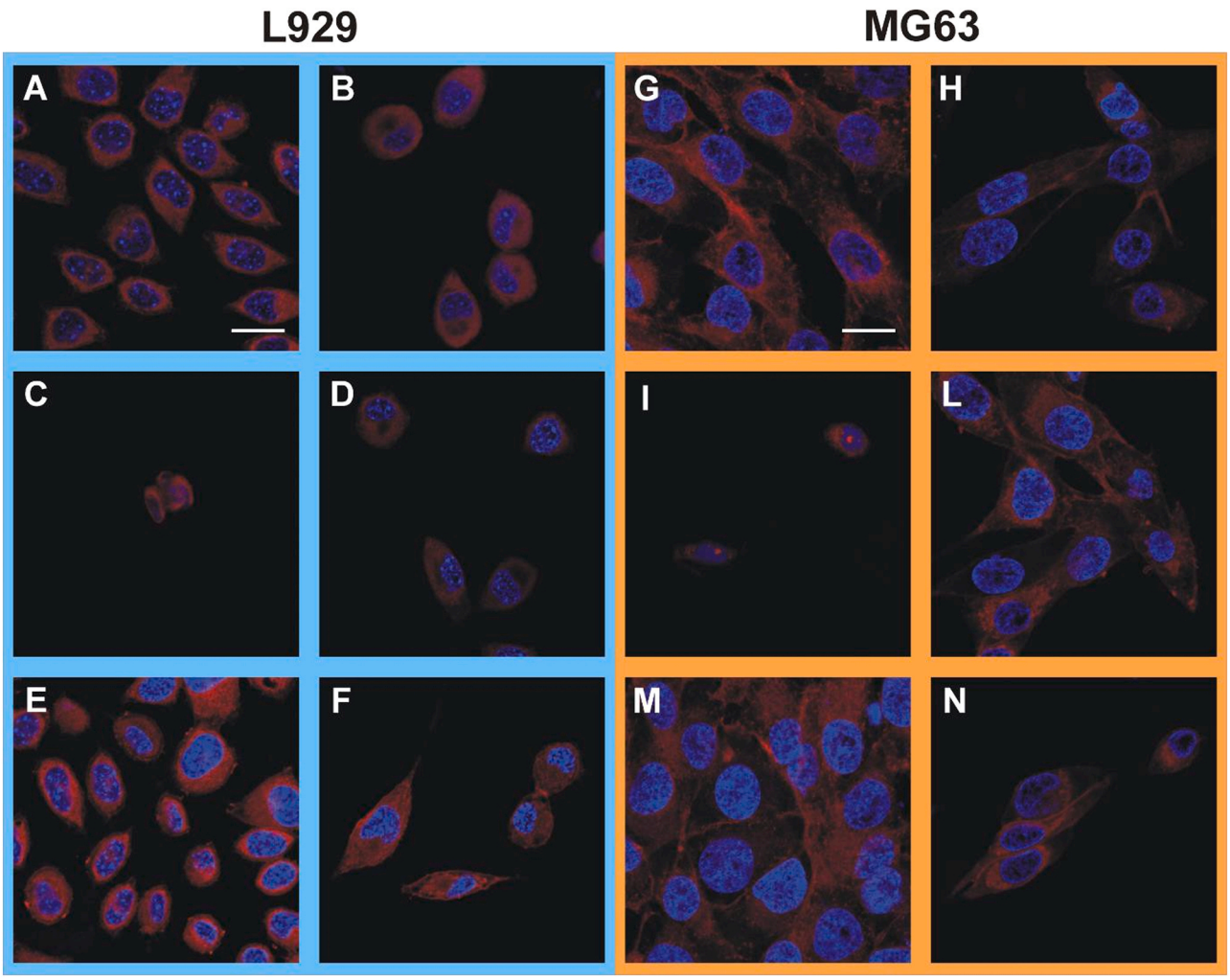

Fig. 8. CLSM micrographs of cultures of cells L929 and cells MG63 seeded on glass coverslip surfaces and treated with diluted propolis extracts at the concentration of 39 μg/ml, with a corresponding dilution of the Vehicle Control or with positive and negative control solutions. Legend: A-F, L929 cells; G-N, MG63 cells; A and G, Medium Control; C and I, Positive Control; E and M, Vehicle Control; B and H, Sample A; D and L, Sample B; F and N, Sample C. Bar = 20 μm.

15 μg/ml, respectively for the HepG2 and 5637 cell lines. Thus, only for the human bladder cancer cell line the selectivity index could strictly meet the criterion of a SI > 2.

Xavier et al. [44] (2017) screened the cytotoxicity of ethanol extracts of two Brazilian propolis types on a panel of tumor cell lines. The cytotoxicity tests were conducted over a period of cell culture of 72 h. The authors implemented the recommendations of Sforcin & Bankova [38] (2011) that, for crude plant extracts, cytotoxicity towards human cancer cells should be considered significant with IC50 values ≤ 30 μg/ml and fixed an even lower IC50 threshold of 25 μg/ml. The propolis extracts were found cytotoxic only when tested on leukaemia cell lines but not on other tumor and normal (PBMC, V79 and L929) cell lines, which exhibited an IC50 > 25 μg/ml. However, the level of selectivity was not actually assessed in terms of selectivity index. Using the bioluminescence method, we observed that two different leukaemia cell lines appeared more susceptible to the cytotoxic effects of propolis extracts than normal hMSCs, in some cases approaching or reaching an IS of 2. Nonetheless, the IC50 values far exceeded 100 μg/ml.

Lopez et al. [46] (2015) gave great emphasis to the limited existing data on the cytotoxicity of propolis preparations towards normal human cells. They highlighted as most of the studies on anticancer properties and cytotoxic activities of propolis on tumor cells miss appropriate reference controls to assess the levels of selectivity. It has however to be said that many recent studies are not any longer conducted on propolis extracts, but on the purified active phytocompounds.

In their conclusions, Lopez et al. [46] (2015) stated that, to have a non-cytotoxic, and thus, safe use of red propolis, it is necessary to use a concentration of red propolis below 50 μg/ml. Indeed, they themselves detected a certain degree of cytotoxicity of the extracts on L929 cells. The data reported by Machado et al. [8] (2016) and our findings indicate that, depending on the propolis extracts, the IC50 value for normal L929 cells can be as low as 30 μg/ml or even lower. These common observations further strengthen the need for regularly including the use of normal cells when testing propolis activities, as variations in propolis composition can affect even parameters such as the level of toxicity and safety of this type of products.

L929 cells have been broadly adopted for testing biomaterials cytotoxicity and they are recommended by international standards in view of their sensitivity to toxic effects and of their reproducible responses. However, some authors have reported as the L929 cell line tends to exhibit a relatively high sensitivity compared to other cell lines of fibroblasts, epithelial cells, astrocytes and mesenchymal stem cells [47–49]. This observation is in line with our findings. Here, L929 cells were found particularly sensitive to the effects of all three propolis extracts. Conversely, although their viability was tested by a different technique, hMSCs definitively appeared less susceptible to the effects of all three propolis extracts, showing more than a log higher IC50 value.

Interestingly, Sample A was found capable of inducing apoptosis in

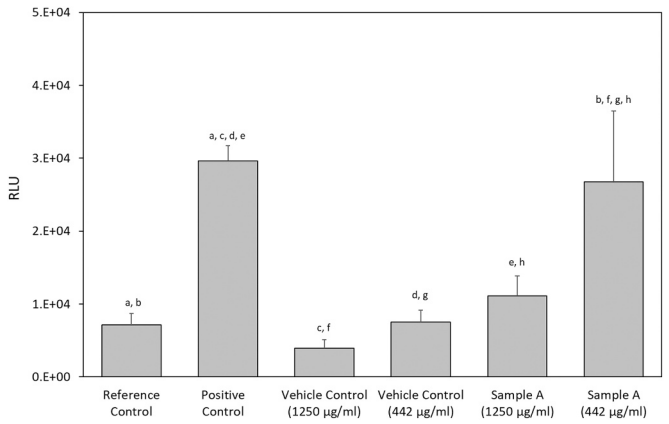

Fig. 9. Apoptosis caspase 3 and 7 activity in THP-1 cells exposed for 2.5 h to Sample A at two distinct concentrations: 1250 µg/ml and 442 µg/ml, the latter concentration corresponding to more than twice the IC50. For the positive control, staurosporine was used at a 5 µM final concentration. Vehicle controls were prepared at the same corresponding dilutions of the propolis extracts treatments. The real vehicle concentration used in the controls was of 3125 µg/ml and 1842 µg/ml, respectively for the high and the low concentration of propolis extracts. Bioluminescence measurements obtained by the Caspase-Glo™ 3/7 assay are expressed in RLU. At this early time point, the lowest concentration of Sample A emerged to activate caspase 3 and 7 to the same extent of the positive control. Mean ± standard deviation (N = 6). Letters indicate statistically significant differences emerged from cross comparisons performed by ANOVA followed by Bonferroni/Dunn test. All indicated comparisons exhibited a p-value <0.0001 (for significance p-values were required to be less than 0.0033).

THP-1 cells after a 2.5-hour treatment to a similar extent than the positive control, but only at a low concentration.

In the present work, the values of IC50 observed with osteosarcoma MG63 cells do not support a relevant cytotoxicity, as the figures abundantly exceeded 30 µg/ml, nor a selective activity towards cancer cells, as the SI was lower than 1. This said, the selectivity criteria were nearly met with leukaemia cells in comparison with normal hMSCs, as the SI approached and, in one case, even reached the expected threshold. Nonetheless, IC50 were exceedingly high. The value of IC50 estimated for the different propolis extracts was in general rather consistent. Even though the value of IC50 estimated for the different propolis extracts showed some slight variation, the coefficient of variation calculated for the three batches was lower than 3% for L929 and MG63 cells, less than 6% for hMSCs, 10.6% for THP-1 cells and only in the case of HL60 cells exceeded 15% (19.6%). The overall findings of this work further confirm the validity and standardization of the extraction method.

5. Conclusions

Propolis is certainly a natural product with a complex composition that includes numerous organic compounds, many of which are active and capable to interact with cells and influence their metabolic functions. Propolis components have been shown to be cytotoxic and induce cell apoptosis in cancer cells, others have been found capable of preventing cell damage and mutagenesis or exhibit antioxidant, antibacterial, cell protective or anti-inflammatory properties. Often extracts derive from pools of propolis from different origin and with unpredictable composition both in terms of types of compounds and of their concentration. However, the interesting properties of some of its ingredients, in particular anticancer and bactericidal properties, attract great attention for broader applications in other more specialized medical areas. A reliable control over propolis composition from batch to batch is of outmost importance and the standardization of the extraction conditions appears crucial. Similarly, it might be desirable to reach a point in which the exact thresholds of cytotoxicity for the most critical compounds will become known, having in mind the variable sensitivities exhibited by different cell types.

In the present study, the three propolis extracts resulted to be modestly cytotoxic against an osteosarcoma and two leukemic reference cell lines. A greater level of toxicity was ascertained against the normal murine connective tissue cells L929, which are among the most used cells for testing the cytotoxicity of biomaterials and are commonly considered when assessing the selectivity of anticancer drugs. These

Table 1
Cytotoxicity of propolis extracts.

Cell line	Cell type	$^{IC}50$	N.E. (type*)	Reference
L929	Mouse fibroblasts (N)	17.9–18.6 µg/ml	3 (MDE)	PS
		58 µg/ml	1 (E)	[33]
		31.6–859 µg/ml	3 (E)	[8]
		30.1 µg/ml	1 (M)	
HepG2	Hepatocellular carcinoma	30 µg/ml	1 (E)	[33]
		3 µg/ml	(M)	[40]
		6.3 µg/ml	(A)	
		47.4 µg/ml	1 (M)	[41]
		43.7 µg/ml	1 (H)	
hMSCs	Normal bone marrow-derived MSCs	258.3–287.2 µg/ml	3 (MDE)	PS
MG63	Osteosarcoma cells	81.9–86.7 µg/ml	3 (MDE)	PS
OSA	Osteosarcoma cells	20.3–49.6 µg/ml	(48 h)	[42]
MCF-7	Estrogen receptor positive (ER+) breast cancer cells	145 µg/ml	1 (M)	[41]
		92.8 µg/ml	1 (H)	
		108.9 µg/ml	1 (E)	[43]
MDA-MB-231	Estrogen receptor negative (ER-) Breast cancer cells	91.3 µg/ml	1 (M)	[41]
		38.7 µg/ml	1 (H)	
MCF7	Brest cancer	>25 µg/ml (72 h)	2 (E)	[44]
Hek-293	Human kidney epithelial cells (N)	>150 µg/ml	1 (E)	[45]
HEp-2	Epithelial carcinoma	63.5 µg/ml	1 (E)	[45]
HeLa	Epithelial cervix cancer	81.4 µg/ml	1 (E)	[44]
MDA-MB435	Melanoma	>25 µg/ml (72 h)	2 (E)	[45]
LoVo	Human colorectal adenocarcinoma	77.9 µg/ml	1 (M)	[41]
		38.7 µg/ml	1 (H)	
HCT-116	Colorectal carcinoma	19.4->50 µg/ml	3 (E)	[8]
		43.7 µg/ml	1 (M)	
		>25 µg/ml (72 h)	2 (E)	[44]
		479.2 µg/ml	1 (E)	[43]
SF-295	Human glioblastoma	27.9–71 µg/ml	3 (E)	[8]
		16.4 µg/ml	1 (M)	
		>25 µg/ml (72 h)	2 (E)	[44]
OVCAR-8	Ovary carcinoma	17.9–53.5 µg/ml	3 (E)	[8]
		20.7 µg/ml	1 (M)	
		>25 µg/ml (72 h)	2 (E)	[44]
A549	Lung carcinoma	37 µg/ml	(M)	[40]
		1.8 µg/ml	(A)	
JURKAT	Acute T cell leukaemia	70 µg/ml	(M)	[40]
		3.2 µg/ml	(A)	
		14.9–17.3 µg/ml (72 h)	2 (E)	[44]
MOLT-4	Acute T cell leukaemia	14.1–16.3 µg/ml (72 h)	2 (E)	[44]
HL-60	Promyelocytic leukaemia	9.4->50 µg/ml	3 (E)	[8]
		31.6 µg/ml	1 (M)	
		15.4–19.24 µg/ml (72 h)	2 (E)	[44]
		126.0–185.8 µg/ml	3 (MDE)	PS
THP-1	Human monocytic leukaemia	50.5 µg/ml	1 (E)	[43]
		164.4–203.2 µg/ml	3 (MDE)	PS

Legend: *, extraction solvent/method; E, ethanol extracts; M, methanol extract; A, L-acetate fraction; H, hexane fraction; MDE, multi dynamic extraction; N, normal cell line; N.E., number of extracts tested; PS, the present study.

results point out the variability shown by the various types of propolis and their extracts in the different studies. It follows the need to develop a more extensive knowledge on the safety and toxicological profile of each single active constituent of propolis, to establish specific qualitative analytical standards.

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CRediT authorship contribution statement

Davide Campoccia: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Project administration. **Stefano Ravaioli:** Investigation, Project administration. **Spartaco Santi:** Investigation. **Valentina Mariani:** Investigation. **Cristina Santarcangelo:** Investigation. **Anna De Filippis:** Writing - original draft. **Lucio Montanaro:** Conceptualization, Methodology, Validation, Project administration. **Carla Renata Arciola:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Project administration. **Maria Daglia:** Conceptualization, Methodology, Validation, Resources, Project administration. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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