Propolis from Poland versus propolis from New Zealand - chemical composition and antiproliferative properties on glioblastoma cell lines.

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Abstract

Background Several studies have previously reported that propolis and its ingredients inhibit glioma cancer cell lines. The chemical composition and antiproliferative activity of propolis from Poland (PPE) and propolis from New Zealand (MPE) were compared in this study. Methods The chemical composition was investigated by gas chromatography-mass spectrometry. Antiproliferative activity of PPE and MPE was determined by a cytotoxicity test and DNA binding by $[^{3}H]$-thymidine incorporation on Human Diffuse Astrocytoma cell line (DASC) derived from a patient with a Grade II glioma and glioblastoma multiforme T98G and LN-18 cell lines from American Type Culture Collection. Results The chemical composition of both propolis was comparable, with marginal differences in the amount of some compounds. Flavonoids and chalcones, of which pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin and galangin showed the highest level, were the main components of both examined propolis (PPE–49.4% and MPE–52.1%). The performed cytotoxicity test showed powerful activity of PPE and MPE propolis on DASC, T98G and LN-18 cells. The degree of the antiproliferative activity was similar in the case of both propolis (viability after 72 h for 30 µg/mL ranged from 22.0% to 51.6% and proliferation inhibition after 72 h approximately was from 18.6% to 75.6%). Conclusions These results are the first to show that propolis from Poland and propolis from New Zealand have a strong cytotoxic and antiproliferative effect on DASC (Grade II glioma) derived from a patient and glioblastoma multiforme T98G and LN-18 cell lines. This activity may be associated with the high content of polyphenolic compounds in both propolis. These findings suggest that Polish and New Zealand propolis shows promising anticancer activity in the treatment of glioblastoma. However, further studies are required.

Background

A number of studies have focused on the composition and properties of propolis. Propolis is a natural product composed of tree and plant resin, bee wax, pollen and gland secretions of bees. When compared to other natural products, propolis is unique since it is of both plant and animal origin. Propolis contains a wide range of active ingredients, whose concentration depends primarily on the origin, geographical provenance, season of the year and the breed of bees. There are several types of propolis: “Poplar” (European, Chinese, North and South American, including Manuka propolis from New Zealand, “Brazilian green” (containing artepillin-C), “Red” (from Cuba, Brazil, Mexico), “Brich” (from Russia), “Mediterranean” (Greece, Crete, Sicily, Malta), “Pacific” (from Okinawa, Taiwan, Indonesia) and “Clusia” (from Cuba and Venezuela) [1]. Hence, different biological activity of propolis has been reported by different authors. The most active compounds are flavonoids (e.g. chrysin, apigenin, pinocembrin, pinobanksin, kaempferol), aromatic acids (e.g. p-coumaric, ferulic) and esters (caffeic acid phenethyl ester – CAPE) [2, 3]. A number of studies concerning the anti-cancer activity of propolis on various cancer cell lines such as human colorectal cancer (DLD-1) [1], human lung cancer (A549) [4], gastric cancer (HGC27) [5] and human prostate cancer (PC3) [6] have been published. The antiproliferative potential of propolis from Poland on the human glioblastoma multiforme cell line U87MG has been confirmed in our previous studies [2, 7, 8].
The present study is the first to compare the chemical composition and antiproliferative activity of propolis from Poland and propolis from New Zealand on Human Diffuse Astrocytoma cell lines (DASC) derived from a patient with Grade II glioma and glioblastoma multiforme T98G and LN-18 cell lines.

**Methods**

**Materials**

DMEM/Ham's F12 with L-glutamine was purchased from PAA Laboratories GmbH (Pasching, Austria). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), minimal essential medium eagle (MEM) with L-glutamine, trypsin-EDTA, penicillin, streptomycin were purchased from Gibco (Thermo Fisher Scientific, Waltham, USA). Calcium-free phosphate buffered saline (PBS) was received from Biomed (Lublin, Poland). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with an addition of 1% trimethylchlorosilane, C\textsubscript{10}–C\textsubscript{40} n-alkane standard solution, methylthiazolyl diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), pyridine, trichloroacetic acid, trizma base were obtained from Sigma-Aldrich (St. Louis, USA). Ethanol 95% (AWW Group, Poland). The scintillation cocktail was purchased from PerkinElmer (Boston, MA). Methyl-3H thymidine from MP Biomedicals, Inc. (Irvine, USA).

**Sample preparations**

Propolis of Apis mellifera was collected in the Podlasie region (northeastern Poland). To prepare the ethanolic extract of Polish propolis (PPE), 20 g of crushed propolis was extracted on a shaker with 80 g of 70% ethanol for 12 h in a darkened place. The extract was centrifuged at 2500 rpm for 10 min at 20°C, evaporated (40°C) in a rotary evaporator (Rotavapor R-3, Buchi, Switzerland) and lyophilised. The dry Polish propolis extract (PPE) was protected from light and kept frozen at −20 °C. The yield of the prepared extracts (% w/w) in terms of the starting material was 47.6.

Propolis Manuka Health New Zealand (Bio 30) ethanolic tincture was purchased from the manufacturer. The tincture was evaporated (40°C) in a rotary evaporator (Rotavapor R-3, Buchi, Switzerland) and lyophilised. The dry Manuka Propolis extract (MPE) was protected from light and kept frozen at −20 °C.

The extracts were dissolved in DMSO and prepared as 1 mg/mL stock solution (calculated as dry extracts) in the culture medium.

**Gas chromatography-mass spectrometry (GC-MS) analysis**

5 mg of PPE and MPE were diluted with 220 µL of pyridine and 80 µL of BSTFA with an addition of 1% trimethylchlorosilane. The reaction mixture was sealed and heated for 0.5 h at 60 °C to form trimethylsilyl (TMS) derivatives.

GC-MS analyses of PPE and MPE were performed using GC–MS on a HP 6890 gas chromatograph with a mass selective detector MSD 5973 (Agilent Technologies, USA) equipped with a ZB-5MSi fused silica column (30 m, 0.25 mm i.d., 0.25 µm film thickness), with electronic pressure control and a split/splitless
injector. Helium flow rate through the column was 1 mL/min in a constant flow mode. The injector worked at 250°C in the split (1:50) mode. The initial column temperature was 50°C, rising to 310°C at 5°C/min and the higher temperature was maintained for 15 min. MSD detector acquisition parameters were as follows: transfer line temperature 280°C, MS Source temperature 230°C and MS Quad temperature 150°C. The EIMS spectra were obtained at the ionisation energy of 70 eV. The MSD was set to scan 41–600 a.m.u. Following the integration, the fraction of each component in the total ion current was calculated. Hexane solutions of C_{10}–C_{40} n-alkanes were separated under the above conditions. Gas chromatographic linear programmed retention indices (I_T) were calculated on the basis of the retention times of the n-alkanes hexane solution and separated components of the extract samples.

To identify the separated components, two independent analytical parameters were used: mass spectra and calculated retention indices. The mass spectrometric identification of non-derivatised components was performed with an automatic system for GC-MS data processing supplied by the NIST 14 library (NIST/EPA/NIH Library of Electron Ionization Mass Spectra). The mass spectra and retention indices of the components registered in the form of TMS derivatives were compared with those presented in a recently published database [9] and a private mass spectra library. Identification was considered reliable if the results of the computer search of the mass spectra library were confirmed by experimental RI values, i.e. if their deviation from the published database values did not exceed ± 10 u.i. (the average quantity of inter-laboratory deviation for non-polar stationary phases).

**Total phenolic content analysis**

Total phenolic content (TPC) was measured using the Folin–Ciocalteu colorimetric method (FC). Absorbance versus a prepared blank was read at 760 nm using Cintra 3030 (GBC Scientific Equipment, Australia). The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of a dry extract. The concentration of samples equalled 2 mg/mL (extract dissolved in 70% ethanol). Assays were performed in triplicate. Data were expressed as mean ± SD.

**Cell culture**

The study was performed using Diffuse astrocytoma steam-like cells (DASC) and glioblastoma multiforme (T98G and LN-18) cell lines. DASC cell line was derived from a 43-year-old patient with diffuse astrocytoma (Grade II), which was described in our previous research [10]. The study was approved by the local Ethics Committee [10]. T98G and LN-18 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in a humidified incubator at 37 °C and 5% CO₂ atmosphere, in MEM (DASC and T98G) or DMEM (LN-18) supplemented with 10% heat inactivated FBS; 100 U/mL penicillin and 0.1 mg/mL streptomycin. Subconfluent cells were detached with a trypsin-EDTA solution in PBS and counted in a Neubauer hemocytometer.

**Cytotoxicity assay**

Cell viability was measured using an MTT assay, as previously described for glioma cells [10]. The effects of PPE and MPE extracts on DASC, T98G and LN-18 cell lines were studied after 24 h, 48 h and
72 h of the treatment. The cells were cultured in a humidified incubator at 37 °C and 5% CO₂ atmosphere; in MEM or DMEM supplemented with 10% heat inactivated FBS; 100 U/mL penicillin and 0.1 mg/mL streptomycin. Doses of propolis (10, 20, 30, 50, 100 µg/mL) were selected in our previous experiments [8]. Cells at a density of 1 × 10⁵ cells/mL were seeded onto 96-well plates at a volume of 200 µl per well and grown for 22 h at 37°C in a humidified 5% CO₂ incubator. The data were expressed as a percentage of the control (0.1% DMSO).

DNA synthesis assay

[³H]-thymidine assays were performed to study DNA synthesis in the cells after the treatment, as described in our previously published study [10]. The cells were seeded (1.5 × 10⁵ cell/well) on 24-well plates in MEM or DMEM supplemented with 10% heat inactivated FBS; 100 U/mL penicillin and 0.1 mg/mL streptomycin and exposed to the treatment medium containing DMSO (0.1% - control), PPE and MPE (30 µg/mL). The cells were cultured for 44 h prior to adding 0.5 µCi of [³H]-thymidine per well. After 4 h of incubation, the medium was removed and the cells were washed twice with cold 0.05 M Tris-HCl and 5% trichloroacetic acid, then scraped and transferred to a scintillation cocktail. The level of [³H]-thymidine incorporated in the newly synthesised DNA strand was assessed by a scintillation counter in relation to the number of cells proliferating during the S phase of the cell cycle.

Statistical analysis

All data were analysed using Dell Inc. (2016). Dell Statistica (data analysis software system), version 13. software.dell.com. The results were expressed as mean ± SD and statistically compared to the control. Values were tested for a normal distribution using the Shaprio-Wilk test. Differences between two groups were analyzed using Student's t-test or U Mann-Whitney test. P < 0.05 was considered to be statistically significant.

Results And Discussion

Chemical composition of Polish and Manuka propolis

The complex chemical composition of propolis is associated with the quality of the resinous materials gathered by honey bees from different floral sources available around the hive, which has a direct impact on the quality and bioactivity of propolis. In this study, more than 100 individual compounds in PPE and more than 150 compounds in MPE were identified. A list of these constituents is presented in Table 1. Flavonoids and chalcones were the main components of both examined propolis (PPE–49.4% and MPE–52.1%) (Table 2). The main representatives of this group of compounds in PPE and MPE were, respectively, pinocembrin (8.16% and 14.64%), pinobanksin (4.25% and 4.70%), pinobanksin 3-acetate (11.27% and 9.21%), chrysin (5.33% and 5.73%), galangin (8.95% and 9.60%) and their derivatives. (Table 1). These compounds are characteristic of propolis originating from bud exudates of Populus nigra [11, 3]. Our analysis also confirmed research results published by other authors who have demonstrated that New Zealand propolis has very high levels of pinocembrin and pinobanksin-3-O-
acetate [1]. Cinnamic acid derivatives such as esters: 3-methyl-2-bytenyl (E)-caffeate, benzyl (E)-caffeate, benzyl (E)-p-coumarate, 2-phenylethyl p-coumarate, benzyl (E)-ferulate, CAPE, cinnamyl (E)-p-coumarate and others were the second significant group of compounds in PPE and MPE (19.8% and 14.5%) (Table 2). Considerable quantities of aromatic acids were present in both studied propolis extracts, although propolis from Poland (PPE–18.3%) contained twice as great a quantity of aromatic acids as propolis from New Zealand (MPE–7.8%) (Table 2). The main representatives of this group were p-coumaric acid, (E)-ferulic acid and (E)-caffeic acid. PPE contained high levels of p-coumaric acid (9.80%) (Table 1). TPC determination confirmed that PPE and MPE are rich in phenolic compounds. Their levels were calculated to be 243.7 ± 9.0 in PPE and 245.6 ± 5.9 mg GAE/g in MPE (Table 3). Other authors have demonstrated higher or lower TPC in propolis. The values ranged from 14.6 to 150.8 mg GAE/g in Polish propolis [12] and from 99 ± 4.0 to 775 ± 8.5 mg GAE/g in Manuka propolis [13]. TPC value depended on the extraction method utilised.

Comparison of the chemical composition of the tested propolis revealed that both PPE and MPE had similar quantities of the identified active components and the total content of phenols, which is consistent with the classification of propolis from New Zealand as the “Poplar” type. According to Kumazawa et al. [14], comparison of the antioxidant activity and composition, total phenol and flavonoid content in individual samples of ethanolic extracted propolis from 14 countries showed that New Zealand-sourced propolis was similar in composition to propolis from Bulgaria, Uzbekistan and Hungary, and to propolis from three South American countries: Chile, Uruguay and Argentina.
# Table 1

Chemical composition of the ethanolic extracts of propolis from Poland (PPE) and New Zealand (MPE)

| Components                                           | CAS       | $I_T^{Exp}$ | $I_T^{Lit}$ | PPE [%] | MPE [%] |
|------------------------------------------------------|-----------|-------------|-------------|---------|---------|
| Benzyl alcohol, mono-TMS                             | 14642-79-6 | 1152        | 1155        | trace*  | 0.09    |
| 2-Phenyl ethanol, mono-TMS                           | 14629-58-4 | 1225        | 1227        | trace   | 0.04    |
| Benzoic acid, mono-TMS                               | 2078-12-8 | 1244        | 1247        | 1.80    | 0.33    |
| Diethylene glycol, di-TMS? (73,117,103,147,191)       | 16654-74-3 | 1250        | 1239        | -       | 0.07    |
| $H_3PO_4$, tri-TMS                                    | 10497-05-9 | 1289        | 1289        | -       | 0.02    |
| Glycerol, tri-TMS                                     | 6787-10-6 | 1291        | 1293        | 0.22    | 0.57    |
| Succinic acid, di-TMS                                | 40309-57-7 | 1321        | 1324        | -       | trace   |
| Ethyl dihydrocinnamate                               | 2021-28-5 | 1345        | 1349        | -       | trace   |
| Hydroquinone, di-TMS                                  | 2117-24-0 | 1406        | 1410        | 0.04    | trace   |
| 3-Hydroxy acid, di-TMS? (147,73,103,233)              | -         | 1410        | -           | -       | trace   |
| Hydrocinnamic acid, mono-TMS                          | 21273-15-4 | 1414        | 1418        | trace   | 0.06    |
| Cinnamoyl alcohol, mono-TMS                           | N/A**     | 1425        | 1427        | trace   | 0.03    |
| 3-Hydroxy acid, di-TMS? (73,131,147,233)              | -         | 1461        | -           | -       | 0.02    |
| 4'-Hydroxyacetophenone, mono-TMS                      | 18803-29-7 | 1467        | 1471        | -       | trace   |
| Malic acid, tri-TMS                                   | 38166-11-9 | 1507        | 1511        | -       | 0.03    |
| Vanillin, mono-TMS                                    | 6689-43-6 | 1534        | 1533        | trace   | trace   |
| Erythritol, tetra-TMS                                 | 25258-02-0 | 1536        | 1535        | -       | 0.06    |
| Cinnamic acid, mono-TMS                               | 2078-20-8 | 1542        | 1546        | 0.20    | 1.82    |
| 7-Phenyl-5-hepten-2-one? (130,129,91)                 | 33046-89-6 | 1567        | -           | -       | 0.03    |
| Protocatechuic aldehyde, di-TMS                       | N/A       | 1619        | 1620        | trace   | 0.07    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                      | CAS       | $I_{Exp}$ | $I_{Lit}$ | PPE [%] | MPE [%] |
|------------------------------------------------|-----------|-----------|-----------|---------|---------|
| NN (73 > 130,75,233,248)                      | -         | 1626      | -         | -       | 0.15    |
| 4-Hydroxybenzoic acid, di-TMS                 | 2078-13-9 | 1632      | 1636      | 0.16    | 0.03    |
| Docosanoic acid, mono-TMS                     | 55520-95-1| 1661      | 1658      | trace   | -       |
| Guaiol, mono-TMS                              | N/A       | 1682      | 1685      | trace   | 0.07    |
| Acorenol, mono-TMS                            | N/A       | 1720      | 1723      | -       | 0.02    |
| Agarospirol, mono-TMS                         | N/A       | 1737      | 1734      | -       | 0.01    |
| γ-Eudesmol, mono-TMS                          | N/A       | 1740      | 1739      | -       | 0.11    |
| α-Bisabolol, mono-TMS                         | N/A       | 1747      | 1752      | trace   | 0.05    |
| β-Eudesmol, mono-TMS                          | N/A N/A   | 1749      | 1751      | -       | 0.09    |
| Arabinitol, penta-TMS                         | 25138-28-7| 1755      | 1760      | -       | 0.07    |
| Benzyl benzoate                               | 120-51-4  | 1764      | 1763      | 0.29    | -       |
| Vanillic acid, di-TMS                         | 2078-15-1 | 1776      | 1776      | trace   | -       |
| (Z)-p-Coumaric acid, di-TMS                   | N/A       | 1799      | 1798      | 0.12    | -       |
| Methylfuranoside, tetra-TMS                   | 30788-71-7| 1811      | 1813      | -       | 0.11    |
| p-Methoxycinnamic acid, mono-TMS              | 25436-23-1| 1827      | 1830      | -       | 0.11    |
| Cinnamylideneacetic acid, mono-TMS            | N/A       | 1835      | 1840      | trace   | 0.63    |
| α-Fructofuranose, penta-TMS                   | N/A       | 1841      | 1845      | 0.26    | 0.69    |
| β-Fructofuranose, penta-TMS                   | N/A       | 1850      | 1854      | 3.67    | 7.32    |
| α-Mannofuranose, penta-TMS                    | N/A       | 1872      | 1874      | -       | 0.35    |
| α-Glucofuranose, penta-TMS                    | 66807-66-7| 1884      | 1885      | -       | 0.11    |
| NN (73,131,204,368,203)                       | -         | 1896      | -         | -       | 0.06    |
| α-Glucopyranose, penta-TMS                    | N/A       | 1929      | 1930      | 0.91    | 4.02    |
| p-Coumaric acid, di-TMS                       | 10517-30-3| 1944      | 1947      | 9.77    | 0.87    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                      | CAS   | $I_T^{Exp}$ | $I_T^{Lit}$ | PPE [%] | MPE [%] |
|------------------------------------------------|-------|-------------|-------------|---------|---------|
| Sesquiterpenol C15H26O-TMS? (131 > 73...279,103) | -     | 1948        | -           | -       | 0.26    |
| NN (131,73,249,179...399,355)                   | -     | 1956        | -           | -       | 0.04    |
| Mannitol, hexa-TMS                              | 14317-07-8 | 1970     | 1972        | -       | 0.04    |
| Sedoheptulose, hexa-TMS                        | 74987-26-0 | 1974     | 1972        | -       | 0.16    |
| Ethyl hexadecanoate                             | 628-97-7 | 1990       | 1994        | -       | 0.03    |
| NN (73,147,289,248,319...379)                   | -     | 2007        | -           | -       | 0.05    |
| β-Glucopyranose, penta-TMS                     | 2775-90-8 | 2028     | 2032        | 0.99    | 5.25    |
| 3,4-Dimethoxycinnamic acid, mono-TMS           | 27750-71-6 | 2030    | 2034        | -       | 1.51    |
| Gluconic acid, hexa-TMS                        | 34290-52-3 | 2041    | 2045        | -       | 0.04    |
| Hexadecanoic acid, mono-TMS                    | 55520-89-3 | 2049    | 2052        | 0.27    | 0.11    |
| (E)-1,4-Diphenyl-3-buten-1-one                  | 32363-55-6 | 2072    | -           | -       | trace   |
| Isoferulic acid, di-TMS                        | 32342-04-4 | 2087    | 2088        | 0.95    | 0.82    |
| Ethyl caffeate, di-TMS                         | N/A   | 2092       | 2091        | -       | 0.04    |
| NN (131,73 > 162,143)                          | -     | 2096        | -           | -       | 0.26    |
| (E)-Ferulic acid, di-TM                        | 10517-09-6 | 2101    | 2101        | 3.22    | 0.15    |
| myo-Inositol, hexa-TMS                         | 2582-79-8 | 2124    | 2125        | trace   | 0.04    |
| NN (73 > 157,156)                              | -     | 2148        | -           | -       | 0.06    |
| 3-Methylbutanyl (E)-p-coumarate, mono-TMS      | N/A   | 2152       | 2145        | 0.65    | -       |
| (E)-Caffeic acid, di-TMS                       | 10586-03-5 | 2155    | 2155        | 2.10    | 1.53    |
| 3-Methyl-3-butenyl p-coumarate, mono-TMS       | N/A   | 2159       | 2159        | 0.18    | 0.11    |
| NN (247 > 73,131...358)                        | -     | 2169       | -           | -       | 0.10    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                      | CAS   | $I_{\text{Exp}}$ | $I_{\text{Lit}}$ | PPE [%] | MPE [%] |
|------------------------------------------------|-------|------------------|------------------|---------|---------|
| 2-Methyl-2-butenyl p-coumarate, mono-TMS       | N/A   | 2205             | 2201             | 0.97    | trace   |
| 3-Methyl-2-butenyl p-coumarate, mono-TMS       | N/A   | 2212             | 2216             | 0.23    | 0.07    |
| Linoleic acid, mono-TMS                        | 56259-07-5 | 2217         | 2215             | trace   | 0.03    |
| Oleic acid, mono-TMS                           | 21556-26-3 | 2222             | 2222             | 0.33    | 0.21    |
| NN (73 > 156,244,143,93,147...381)             | -     | 2234             | -                | 1.31    | 0.17    |
| Octadecanoic acid, mono-TMS                    | 18748-91-9 | 2249             | 2252             | trace   | 0.06    |
| 3-Methyl-3-butenyl isofurulate, mono-TMS       | N/A   | 2303             | 2304             | -       | 0.38    |
| 3-Methyl-3-butenyl (E)-ferulate, mono-TMS      | N/A   | 2318             | 2319             | 0.07    | 0.16    |
| Benzyl (Z)-p-coumarate, mono-TMS               | N/A   | 2323             | 2329             | 0.07    | -       |
| Eicosanoic acid, mono-TMS                      | 55530-70-6 | 2349             | 2349             | -       | 0.03    |
| NN (335,73,446,147,69,41,147)                  | -     | 2346             | -                | -       | 0.04    |
| 3-Methylbutanyl (E)-caffeate, di-TMS           | N/A   | 2358             | 2358             | 0.06    | 0.15    |
| 3-Methyl-2-butenyl (E)-isoferulate, mono-TMS   | N/A   | 2365             | 2365             | -       | 0.05    |
| 3-Methyl-3-butenyl (E)-caffeate, di-TMS        | N/A   | 2371             | 2367             | 1.18    | 3.39    |
| 3-Methyl-2-butenyl (E)-caffeate, di-TMS        | N/A   | 2374             | 2375             | 0.50    | 0.44    |
| NN (397,369,73,91)                             | -     | 2384             | -                | -       | 0.03    |
| Pinostrobin chalcone                           | 18956-15-5 | 2392         | -                | 0.16    | 0.07    |
| NN (73,75,55,143,207,129,41)                   | -     | 2404             | -                | -       | 0.02    |
| Cinnamyl cinnamate                             | 122-69-0 | 2408             | 2391             | -       | 0.29    |
| 2-Methyl-2-butenyl (E)-caffeate, di-TMS        | N/A   | 2414             | 2413             | 0.09    | 0.24    |
| 2',6'-Dihydroxy-4'-methoxydihydrochalcone, di-TMS | N/A | 2418             | 2416             | 0.46    | 0.02    |
| 3-Methyl-2-butenyl (E)-caffeate, di-TMS        | N/A   | 2425             | 2421             | 1.65    | 2.36    |
| NN (143,73,81,95,121,151)                      | -     | 2444             | -                | -       | 0.02    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                                                 | CAS     | $I_{T}^{Exp}$ | $I_{T}^{Lit}$ | PPE [%] | MPE [%] |
|----------------------------------------------------------------------------|---------|---------------|---------------|---------|---------|
| NN (287,372,357,263,73)                                                   | -       | 2450          | -             | -       | 0.18    |
| NN (262,73,247,460,375,287,445)                                           | -       | 2452          | -             | -       | 0.25    |
| Pinocembrin, mono-TMS                                                     | N/A     | 2460          | 2461          | 1.14    | 0.46    |
| 2',6',α-Trihydroxy-4'-methoxychalcone, tri-TMS                           | N/A     | 2491          | 2492          | 0.14    | -       |
| (Z)-Coniferyl benzoate, mono-TMS                                          | N/A     | 2494          | 2495          | -       | 0.15    |
| n-Pentacosane                                                             | 629-99-2| 2500          | 2500          | trace   | trace   |
| Pinostrobin chalcone, di-TMS                                              | N/A     | 2506          | 2508          | 0.26    | 0.04    |
| Pinostrobin, mono-TMS                                                     | N/A     | 2512          | 2512          | 0.66    | 0.84    |
| Benzyl (E)-p-coumarate, mono-TMS                                          | N/A     | 2516          | 2515          | 3.78    | 0.37    |
| 1-p-Coumaroyl glycerol, tri-TMS                                           | N/A     | 2528          | 2528          | 0.06    | -       |
| Pinocembrin chalcone, tri-TMS                                             | N/A     | 2542          | 2541          | 0.09    | 0.08    |
| Pinocembrin, di-TMS                                                       | N/A     | 2551          | 2552          | 6.93    | 14.10   |
| NN (73,75,121,81,95,143...)                                               | -       | 2555          | -             | -       | 0.05    |
| NN (303 > 73,95,147,213,225)                                              | -       | 2563          | -             | -       | 0.81    |
| NN (262,73,247,460,375)                                                   | -       | 2569          | -             | -       | 0.09    |
| 2-Acetyl-1-p-coumaroyl glycerol, di-TMS                                   | N/A     | 2578          | 2578          | 0.12    | -       |
| 1-Acetyl-3-p-coumaroyl glycerol, di-TMS                                   | N/A     | 2581          | 2580          | 0.19    | -       |
| Chalcone, TMS? (192,73,311,238)                                           | N/A     | 2586          | -             | trace   | 0.26    |
| 2-Phenylethyl p-coumarate, mono-TMS                                       | N/A     | 2603          | 2603          | 1.02    | 0.11    |
| Pinobanksin, tri-TMS                                                      | N/A     | 2613          | 2611          | 4.25    | 4.73    |
| 3-Hydroxyeicosanoic acid, di-TMS                                          | N/A     | 2623          | 2620          | -       | 0.03    |
| Pinobanksin 3-acetate, mono-TMS                                           | N/A     | 2634          | 2632          | 1.26    | 0.21    |
| Coniferyl benzoate, mono-TMS                                              | N/A     | 2637          | 2640          | trace   | -       |
| Chrys, mono-TMS                                                           | N/A     | 2655          | 2648          | 1.95    | 0.42    |
| Benzyl (E)-isoferulate, mono-TMS                                          | N/A     | 2659          | 2659          | -       | 0.26    |
| 2',6'-Dihydroxy-4,4'-dimethoxydihydrochalcone                             | N/A     | 2659          | 2659          | trace   |         |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                                                 | CAS       | $I_{Exp}^T$ | $I_{Lit}^T$ | PPE [%] | MPE [%] |
|---------------------------------------------------------------------------|-----------|-------------|-------------|---------|---------|
| NN (238,385,325,73,43,341)                                               | -         | 2666        | -           | 0.34    | 0.21    |
| Pinobanksin x-acetate, TMS? (296,443,73,383)                              | -         | 2671        | -           | -       | 0.58    |
| 5,7-Dihydroxy-3-methoxyflavanone                                          | N/A       | 2675        | 2673        | 2.02    | 2.04    |
| Benzyl (E)-ferulate, mono-TMS                                             | N/A       | 2680        | 2680        | 1.64    | 0.45    |
| Pinobanksin 3-acetate, di-TMS                                             | N/A       | 2694        | 2693        | 10.01   | 9.00    |
| NN (325 >> 282,155,73)                                                   | -         | 2706        | -           | 0.14    | 0.85    |
| Sucrose, octa-TMS                                                         | 19159-25-2| 2714        | 2714        | 0.25    | 0.33    |
| Galangin, di-TMS                                                          | N/A       | 2719        | 2717        | trace   | trace   |
| Benzyl (E)-caffeate, di-TMS                                               | N/A       | 2723        | 2722        | 3.79    | 2.70    |
| 2',6',4-Trihydroxy-4'-methoxydihydrochalcone, tri-TMS                    | N/A       | 2636        | 2637        | 0.14    | -       |
| Pinobanksin 3-propanoate, di-TMS                                          | N/A       | 2737        | -           | -       | 0.06    |
| Isosakuranetin, mono-TMS                                                  | N/A       | 2740        | 2742        | trace   | -       |
| Chrysine, di-TMS                                                          | N/A       | 2746        | 2745        | 5.33    | 5.73    |
| 5,7-Dihydroxy-3-methoxyflavone, di-TMS                                    | N/A       | 2755        | 2750        | 0.67    | 0.60    |
| 1-Acetyl-3-caffeoyl glycerol, tri-TMS                                     | N/A       | 2761        | 2768        | 0.06    | -       |
| Galangin, tri-TMS                                                         | N/A       | 2767        | 2769        | 8.95    | 9.60    |
| Disaccharide, TMS                                                         | -         | 2775        | -           | -       | 0.12    |
| Pinobanksin 3-isobutanoate, di-TMS                                       | N/A       | 2788        | 2791        | 0.52    | 0.51    |
| β-Maltose, octa-TMS                                                       | N/A       | 2797        | 2800        | 0.07    | 0.14    |
| CAPE, di-TMS                                                              | N/A       | 2805        | 2805        | 1.29    | 1.15    |
| Isosakuranetin, di-TMS + disaccharide                                     | -         | 2816        | 2820        | 1.34    | -       |
| Isosakuranetin, di-TMS                                                    | N/A       | 2816        | 2820        | -       | 0.18    |
| Dihydroxymethoxyflavone, di-TMS                                          | -         | 2821        | 2820        | 0.39    | 0.40    |
| Cinnamyl (E)-p-coumarate, mono-TMS                                        | N/A       | 2836        | 2833        | 1.91    | 0.23    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                      | CAS         | $I_T^{Exp}$ | $I_T^{Lit}$ | PPE [%] | MPE [%] |
|------------------------------------------------|-------------|-------------|-------------|---------|---------|
| Tetracosanoic acid, mono-TMS                    | 74367-37-6  | 2844        | 2845        | 0.53    | -       |
| Pinobanksin-3-n-butanoate, di-TMS               | N/A         | 2848        | 2849        | 0.17    | 0.13    |
| Disaccharide, TMS (73,361,217)                  | -           | 2857        | -           | -       | 0.09    |
| Sakuranetin chalcone, tri-TMS                   | N/A         | 2871        | 2871        | -       | 0.05    |
| Sakuranetin, di-TMS                             | N/A         | 2877        | 2880        | 0.55    | 0.05    |
| Pinobanksin 5-pentanoate, di-TMS                | N/A         | 2885        | 2884        | 0.19    | 0.58    |
| β-Cellobiose, octa-TMS                          | N/A         | 2889        | 2888        | -       | 0.12    |
| Naringenin, tri-TMS                             | N/A         | 2895        | 2895        | 0.23    | 0.06    |
| NN (191,117,91)                                 | -           | 2933        | -           | -       | 0.03    |
| Disaccharide, TMS (204,73,361)                  | -           | 2956        | -           | -       | 0.03    |
| Pinobanksin 5-pentenoate, di-TMS                | N/A         | 2965        | 2964        | -       | 0.03    |
| NN (73,299,305,147,129,233...445)               | -           | 2968        | -           | -       | 0.05    |
| Cinnamyl (E)-isoferulate, mono-TMS              | N/A         | 2980        | 2975        | -       | 0.56    |
| Cinnamyl (E)-ferulate, mono-TMS                 | N/A         | 2990        | 2997        | 0.09    | -       |
| NN (356,341,75,135)                             | -           | 2995        | -           | 0.26    | -       |
| β-Isomaltose, octa-TMS                          | N/A         | 3005        | 3005        | -       | 0.05    |
| 3,5,7-Trihydroxy-4′-methoxyflavone, tri-TMS     | N/A         | 3015        | 3015        | 0.31    | -       |
| Pinobanksin 3-hexanoate, di-TMS                 | N/A         | 3037        | 3032        | -       | 0.04    |
| Cinnamyl (E)-caffeate, di-TMS                   | N/A         | 3044        | 3043        | 0.41    | 0.96    |
| Kaempferide, tri-TMS                            | N/A         | 3052        | 3050        | 0.33    | 0.02    |
| 9-Hentriacontene                                | -           | 3076        | 3075        | 0.14    | -       |
| Kaempferol, tri-TMS                             | N/A         | 3082        | 3078        | 0.29    | 0.04    |
| NN (414,399)                                    | -           | 3086        | -           | 0.41    | 0.03    |
| NN (444,401,73,429)                             | -           | 3096        | -           | 0.18    | 0.02    |
| 3',4',7-Trihydroxyisoflavone, tri-TMS           | N/A         | 3101        | 3098        | 0.14    | 0.09    |

* trace – below 0.01% of the total ion current. ** N/A - not available
| Components                                      | CAS      | $I_{T}^{\text{Exp}}$ | $I_{T}^{\text{Lit}}$ | PPE [%] | MPE [%] |
|------------------------------------------------|----------|----------------------|----------------------|---------|---------|
| Kaempherol, tetra-TMS                           | N/A      | 3114                 | 3114                 | 0.38    | 0.41    |
| NN (341,73,103,143...475,515)                    | -        | 3121                 | -                    | -       | 0.05    |
| 5,7,4'-Trimethyl-3-methoxyflavone, tri-TMS      | N/A      | 3141                 | 3139                 | -       | 0.09    |
| Apigenin, tri-TMS                               | N/A      | 3163                 | 3161                 | -       | 0.09    |
| Triterpenoid (189,73,129,143,305)                | -        | 3180                 | -                    | -       | 0.08    |
| Quercetine, penta-TMS                           | 4067-66-7| 3218                 | 3213                 | 0.11    | -       |
| Isorhamnetin, tetra-TMS                         | N/A      | 3245                 | 3245                 | -       | 0.22    |
| p-Coumatate or ferulate, TMS (219,205,249)      | N/A      | 3249                 | -                    | 0.21    | -       |
| NN (73,271,301,103,129,147...451,531)           | -        | 3259                 | -                    | -       | 0.11    |
| 7-Tritriacontene                                | N/A      | 3283                 | 3282                 | 0.22    | -       |
| Myricetin, hexa-TMS                             | N/A      | 3303                 | 3303                 | -       | 0.04    |
| Triterpenoid, TMS (73,189,271,375,129,143)      | -        | 3311                 | -                    | 0.28    | 0.14    |
| NN (73,301,299,461)                             | -        | 3436                 | -                    | -       | 0.11    |
| Triterpenoid, TMS (189,73)                      | -        | 3497                 | -                    | -       | 0.11    |
| NN (393,207,73,134,129)                         | -        | 3574                 | -                    | -       | 0.22    |
| 1,3-Di-p-coumaroyl glycerol, tri-TMS            | N/A      | 3869                 | 3869                 | 0.02    | -       |
| 2-Acetyl-1,3-di-p-coumaroyl glycerol, di-RMS    | N/A      | 3963                 | 3963                 | 0.89    | -       |
| **Total**                                       |          | **100.00**          | **100.00**           |         |         |

* trace – below 0.01% of the total ion current. ** N/A - not available
Table 2

| Group of compounds                  | PPE [%] | MPE [%] |
|------------------------------------|---------|---------|
| Flavonoids and chalcones           | 49.4    | 52.1    |
| Aromatic acids                     | 18.3    | 7.8     |
| Cinnamic acid esters               | 19.8    | 14.5    |
| Phenylpropenoid glicerydes         | 1.3     | 0.0     |
| Aliphatic and aromatic alcohol     | 0.2     | 0.8     |
| Aliphatic acids                    | 0.8     | 0.2     |
| Carbohydrates                      | 6.2     | 18.7    |
| Sesquiterpenoids                   | 0.0     | 0.2     |
| Other compounds                    | 4.0     | 5.7     |
| Total                              | 100.0   | 100.0   |

Table 3

| Lp. | Extracts | TPC [mg GAE/g] Mean ± SD |
|-----|----------|--------------------------|
| 1.  | PPE      | 243.7 ± 9.0              |
| 2.  | MPE      | 245.6 ± 5.9              |

Cytotoxicity and antiproliferative activity

Chemical compounds present in propolis offer powerful bioactive protection against pathogens and are therefore used by bees to immunise the hive environment [15]. For this reason, they may also serve as a significant source of bioactive substances for pharmaceutical purposes. A number of research studies have focused on the potential utilisation of propolis phenolic compounds in the development of new anticancer drugs [16, 17]. Our previous study revealed that Polish propolis has strong cytotoxic and antiproliferative activity and, additionally, cooperates with (TMZ) synergistically, enhancing its growth-inhibiting activity against glioblastoma U87MG cell line through the reduction of NF-κB activity [8]. In this study, cytotoxicity and antiproliferative activity was determined using DASC cell line derived from a patient and T98G and LN-18 cell lines from ATCC. Dose and time-dependent decreases in DASC viability
were observed after 24, 48 and 72 h of incubation with both PPE and MPE (compared to the control) (Fig. 1), and were comparable for both propolis. For DASC cell line we observed a significant reduction in cell numbers (p < 0.05) in all concentrations after 24, 48 and 72 h; for the dose 30 µg/mL, it was 77.9 ± 4.3% and 81.3 ± 4.0% after 24 h, 58.6 ± 0.3% and 63.4 ± 7.8% after 48 h, and 47.0 ± 3.2% and 51.6 ± 8.1% after 72 h for PPE and MPE, respectively (Fig. 1A,B,C). A significant, but lower than 10%, difference (p < 0.05) in the reduction of DASC cells treated with PPE in comparison to those treated with MPE was observed for the 100 µg/mL concentration after 48 h (approximately 7%) (Fig. 1B) and for 20, 50, 100 µg/mL concentrations after 72 h (8.4%, 6.9%, 3.0%, respectively) (Fig. 1C). For T98G cell line we observed a stronger significant reduction in cell numbers (p < 0.05), in all concentrations after 24, 48 and 72 h than DASC cell line; for the dose 30 µg/mL, it was 78.4 ± 3.0% and 75.2 ± 2.3% after 24 h, 62.8 ± 1.3% and 50.8 ± 7.2% after 48 h, and 30.7 ± 7.7% and 22.0 ± 8.3% after 72 h for PPE and MPE, respectively (Fig. 1D,E,F). Interestingly, dose-dependent decreases in T98G cells viability were observed after 24, 48 and 72 h but only for the 10–50 µg/mL dose range. After treatment 100 µg/mL dose we observed “reflection effect” because decrease viability was smaller than for 50 µg/mL dose. A significant, difference (p < 0.05) in the reduction of T98G cells treated with PPE in comparison to those treated with MPE was observed for the 50 µg/mL concentration after 24 h (Fig. 1D), for 10, 20, 30, 50 µg/mL concentration after 48 h (Fig. 1E) and for 20, 50, 100 µg/mL concentrations after 72 h (Fig. 1F). For LN-18 cell line we observed a significant reduction in cell numbers (p < 0.05) in all concentrations 20–100 µg/mL after 24, 48 and 72 h. For the dose 30 µg/mL, 81.6 ± 3.3%, 83.2 ± 0.9% 24 h, 49.1 ± 7.8, 65.7 ± 8.0 after 48 h, 40.8 ± 2.5, 41.1 ± 2.9 respectively PPE and MPE. A significant, difference (p < 0.05) in the reduction of LN-18 cells treated with PPE in comparison to those treated with MPE was observed for the 10, 30, 50, 100 µg/mL concentration after 48 h (Fig. 1H), for, 50 and 100 µg/mL concentration after 48 h (Fig. 1I). Interestingly, significantly stronger cytotoxic effect on LN-18 cells was observed after treatment with PPE than MPE.

The impact of PPE and MPE on DNA biosynthesis in the [³H]-thymidine incorporation assay was examined in order to confirm if the inhibition of cell viability was caused by a reduction in proliferation capacity. For DASC cell line we found that both PPE and MPE significantly inhibited proliferation—by approximately 10.2% and 13.2% after 48 h and by approximately 23.1% and 18.6% after 72 h, respectively (Fig. 2A,B,C). For T98G cell line we observed a significant reduction in proliferation capacity (p < 0.05) only for MPE, it was 18.4% after 24 h, 18.6% after 48 h and 39.6% after 72 h (Fig. 2D,E,F). For LN-18 cell line we found a significant reduction in proliferation capacity (p < 0.05) in both, PPE and MPE after 24, 48 and 72 h, approximately 40.6% and 44.5% after 24 h, 39.4% and 43.3% after 48 h and 67.6% and 75.6% after 72 h, respectively (Fig. 2G,H,I).

Figure. 2. [³H]-thymidine incorporation into DASC, T98G and LN-18 cells after treatment with PPE and MPE. Legend: [³H]-thymidine incorporation into DASC (A,B,C) and T98G (D,E,F) and LN-18 (G,H,I) cells after 24, 48, 72 h of incubation with PPE and MPE (in concentrations 30 µg/mL). The results are presented as a percentage of control. All statistical analyses were performed using Student-t test (significant changes: *p < 0.05 vs control).
Comparing the effect of both propolis on different glioma cell lines, we found a strong cytotoxic effect against DASC, T98G and LN-18 cells. According to our results, both PPE and MPE have a significant antiproliferative effect on DASC and LN-18 cell lines, while on T98G only MPE. It is worth noting that the cytotoxic and antiproliferative effect of PPE and MPE on DASC cells derived from patient was significant enough to suggest that it is a promising agent for use in supporting anticancer therapy.

Due to the presence of a large number of active substances, propolis exhibits powerful anticancer activity, which has been confirmed in many studies [16–18]. Catchpole et al. [1] demonstrated strong antiproliferative activity of propolis from New Zealand against DLD-1, HCT-116, KYSE-30, NCI-N87 gastrointestinal cancer cells, associated with high levels of phenolic compounds (pinocembrin, pinobanksin-3-O-acetate and others). Propolis from Brazil has been demonstrated to exert a strong inhibitory effect on cell growth in glioblastoma (U251 and U343) and fibroblast cell lines (MRC5), but had no effect on apoptosis, demonstrating a cytostatic action [19]. Many publications have explored significant anticancer properties of individual components of propolis. Szliszka and Krol [20] have suggested that polyphenols from propolis sensitise tumor cells to TRAIL-induced apoptosis. The compounds, in combination with TRAIL, exhibit a strong cytotoxic effect on cancer cells [21, 22]. CAPE inhibits NF-kB and enhances the extrinsic pathway of apoptosis in cancer cells induced by TRAIL and Fas receptor stimulation [23]. The most recent research has demonstrated that CAPE displays significant cytotoxicity towards two glioma cell lines Hs683 and LN319 [24]. Other authors have also confirmed that CAPE exhibits powerful antitumor effects on the following cancer cells: fibroblasts from oral submucous fibrosis (OSF), neck metastasis of Gingiva carcinoma (GNM) and tongue squamous cell carcinoma (TSCCa) [25]. Chrysin shows antiproliferative activity against human colorectal cancer cell line HCT-116, liver cancer cell line HepG2 and nasopharyngeal line CNE-1 to TNF-α induced apoptosis and HCT-116, HepG2, cervical cancer cell line HeLa and CNE-1 to TRAIL induced apoptosis [26]. Chrysin induces apoptosis in cancer cells by the activation of caspases, suppression of anti-apoptotic proteins such as IAP, c-FLIP, PI3K/Akt signal pathway, inhibition of IKK and NF-kB activity [27]. In our previous study we demonstrated that natural bee products such as bee bread, royal jelly and honey extract showed varied activity against U87MG and SVGp12 cell lines. Furthermore, the use of these bee products may increase the cytotoxic effect of TMZ on U87MG and SVGp12 cell lines. We also observed that U87MG cells were sensitive to natural bee products, but no impact of natural bee products on DASC cells was noted [10].

Conclusions

Summarising, these results are the first to show that propolis from Poland and propolis from New Zealand have a strong cytotoxic and antiproliferative effect on Human Diffuse Astrocytoma cell line (DASC) (Grade II glioma) derived from a patient and glioblastoma multiforme T98G and LN-18 cell lines from ATCC. This activity may be associated with the high content of polyphenolic compounds in both propolis. The chemical composition of both propolis was comparable, with marginal differences in the amount of some compounds. These findings suggest that Polish and New Zealand propolis shows promising anticancer activity in the treatment of glioblastoma. However, further studies are required.
**Abbreviations**

PPE  
Propolis from Poland  
MPE  
Propolis from New Zealand  
ATCC  
American Type Culture Collection  
CAPE  
Caffeic acid phenethyl ester  
DMEM  
Dulbecco's modified eagle medium  
MEM  
Minimal essential medium eagle  
FBS  
Fetal bovine serum  
PBS  
Phosphate buffered saline  
BSTFA  
Bis(trimethylsilyl)trifluoroacetamide )  
MTT  
Methylthiazolyl diphenyl-tetrazolium bromide  
DMSO  
Dimethyl sulfoxide  
TMS  
Trimethylsilyl  
GC-MS  
Gas chromatography-mass spectrometry  
MSD  
Mass selective detector  
EI MS  
Electron ionization mass spectra  
$I_T^{Lit}$  
Linear programmed retention indices  
$R_I^{Exp}$  
Experimental retention indices  
TPC  
Total phenolic content  
FC  
Folin–Ciocalteu colorimetric method
GAE
Gallic acid equivalent
SD
Standard deviation
TMZ
Temozolomide
NF-κB
Nuclear factor kappa-light-chain-enhancer of activated B cells
TRAIL
TNF-related apoptosis-inducing ligand
IAP
Inhibitors of apoptosis proteins
c-FLIP
FLICE-like inhibitory protein
PI3K
Phosphoinositide 3-kinase
Akt
Protein kinase B
IKK
IkB kinase

Declarations

Authors' contributions

J.M. and S.K.N. were responsible for conception, study design, obtaining funds, laboratory analysis, statistical analysis and writing the manuscript. V.I. was responsible for the GC-MS laboratory analysis and writing the manuscript. R.M.Ż. and K.J.G.K was responsible for performing laboratory analysis. M.H.B. was responsible for management of the study and was responsible for revising the manuscript critically for important intellectual content. The final manuscript was revised by all co-authors.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
Ethics approval and consent to participate

The study was approved by the Local Ethical Committee (R-I-002/346/2008).

Conflicts of Interest

The authors declare no conflict of interest.

Consent for publication

Not applicable.

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Figures

![Figure 1](image1)

**Figure 1**

Viability of DASC, T98G and LN-18 cells after treatment with PPE and MPE. Legend: Cytotoxicity effect of PPE and MPE (in concentrations 10, 20, 30, 50, 100 μg/mL) of DASC (A,B,C), T98G (D,E,F) and LN-18 (G,H,I) cells after 24, 48 and 72 h of incubation. The results are presented as a percentage of control. All statistical analyses were performed using Student-t or U Manna-Whitneya tests (significant changes: *p<0.05 vs control, # PPE vs MPE).
Figure 2

[3H]-thymidine incorporation into DASC, T98G and LN-18 cells after treatment with PPE and MPE. Legend: [3H]-thymidine incorporation into DASC (A,B,C) and T98G (D,E,F) and LN-18 (G,H,I) cells after 24, 48, 72 h of incubation with PPE and MPE (in concentrations 30 µg/mL). The results are presented as a percentage of control. All statistical analyses were performed using Student-t test (significant changes: *p<0.05 vs control).