Viability-reducing activity of *Coryllus avellana* L. extracts against human cancer cell lines

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Abstract

The increasing rate of cancer incidence has encouraged the search for novel natural sources of anticancer compounds. The presence of small quantities of taxol and taxanes in *Corylus avellana* L. has impelled new potential applications for this plant in the field of biomedicine. In the present work, the cell viability-reducing activity of stems and leaves from three different hazel trees was studied against three human-derived cancer cell lines (HeLa, HepG2 and MCF-7). Both leaf and stem extracts significantly reduced

viability of the three cell lines either after maceration with methanol or using taxane extraction methods. Since maceration reduced cell viability to a greater extent than taxane extraction methods, we scaled up the maceration extraction process using a method for solid/liquid extraction (Zippertex technology). Methanol leaf extracts promoted a higher reduction in viability of all cell lines assayed than stem extracts. Fractionation of methanol leaf extracts using silica gel chormatography led to the purification and identification of two compounds by HPLC-MS and NMR: (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-β-D-glucopyranoside and quercetin-3-O-rhamnoside. The isolated compounds decreased viability of HeLa and HepG2 cells to a greater extent than MCF-7 cells. Our results suggest a potential use of *C. avellana* extracts in the pharmacotherapy of cervical cancer and hepatocarcinoma and, to a lesser extent, breast cancer.

Key words

Anticancer activity; *Corylus avellana*; Human cancer cells; Plant extracts; Taxol.

Abbreviations

DCM, dichloromethane; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; DHHP, (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-β-D-glucopyranoside; DW, dry weight; ESI, Electrospray ionization; EtOH, ethanol; HeLa, human cervical cancer cells; HepG2, liver hepatocellular cells; HGF, normal human gingival fibroblast; HL-60, human promyelocytic leukemia cells; HPLC, high-performance liquid chromatography; HSC-2, oral squamous cell carcinoma; IC_{50}, half maximal inhibitory concentration; MCF-7, human breast adenocarcinoma cell line; MeOD, deuterated methanol; MeOH, methanol; MS, mass spectrometry; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide); NMR, nuclear magnetic resonance; PPARs, peroxisome proliferator-activated receptors; QR, quercetin-3-O-rhamnoside; SDS, sodium dodecyl sulfate; SK-Mes-1, human lung cancer cell line; THF-α, tumor necrosis factor alpha; NF-κβ, nuclear factor kappa-B

1. Introduction

Drug development has a long background of searching for active natural compounds in plants, many of which have been used in traditional medicine to treat a wide range of diseases and infections. Almost half the drugs approved since 1994 are based on natural products [1]. Concerning drugs used for cancer treatment, more than 60% have a natural origin and natural products are a major source for drug discovery [2].

The recent increase of cancer incidence has promoted a growing interest in finding new active compounds to be used to fight the disease. The discovery of taxanes in hazel plant extracts triggered an interest to study natural products from this plant and its secondary metabolites [3,4,5,6]. In *C. avellana*, over 100 compounds have been described and classified as organic acids, triacylglycerols, phytosterols, tocols, phenolic acids, diarylheptanoids, flavonoids, tannins, isoflavones, lignans, terpenes and taxanes.

Previous studies reported a taxol-dependent antiproliferative activity in extracts of *C. avellana* cell cultures. Treatment of SK-Mes-1 cells (a human cell line derived from lung cancer) with extracts from *C. avellana* cell culture medium blocked cancer cells at the metaphase/anaphase transition more effectively than those treated with yew extract [3]. Recently, the effect of a *C. avellana* cell culture extract was evaluated in the MCF-7 cancer cell line (derived from breast cancer). The study confirmed the aforementioned activity, and suggested that the *C. avellana* cell culture extracts were more effective than pure taxol. It was therefore hypothesized that *C. avellana* extracts may present compounds that could enhance its effects [7]. Moreover, the antiproliferative activity of extracts of *C. avellana* trees has not been previously studied.

In the present study, bioassay-guided experiments were carried out to isolate and characterize compounds from *C. avellana* with potential anticancer activity. For this purpose, compounds from *C. avellana* leaves and stems were extracted using different methodologies, and their anticancer activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in three human cancer cell lines: HeLa (derived from cervical cancer cells), HepG2 (derived from liver hepatocarcinoma) and MCF-7.

2. Materials and Methods

2.1 Mammalian cell lines

HeLa, HepG2 and MCF-7 cell lines were obtained from ATCC (ATCC nos.: CCL-2, HB-8065 and HTB-22, respectively). The cells were cultured in Dulbecco's Modified Eagle's (DMEM) medium without pyruvate, supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10 % (v/v) of heat inactivated fetal bovine serum. All the components were purchased from Gibco (Invitrogen, Barcelona, Spain). The cells were grown at 37 ºC in 5 % CO_2.

2.2 Plant material and extractions

Stems and leaves of *Corylus avellana* L. were collected in October 2014 from three mature trees in Catalonia: (I) wild type in Barcelona, (II) wild type in Terrassa, and (III) a cultivar in Tarragona. The plant materials were identified by Dr. Joan Vallès, Professor of Botany at the Faculty of Pharmacy at the University of Barcelona, and deposited in the herbarium of the Documentation Centre of Plant Biodiversity (CeDocBiV), University of Barcelona (voucher specimens: BCN 131396, BCN 131397 and BCN 131398, respectively). Plant material was air-dried overnight at 60 ºC and powdered.

2.2.1 Screening of extraction procedures

Two different extraction procedures were tested in the screening assays. 1) E-extraction: Taxane extraction from 1g freeze-dried leaves or stems using methanol:water (9:1,v/v) [8] and 2) M-extraction: Extraction by maceration from 1 g of freeze-dried leaves or stems in methanol (MeOH) 90%:H_2O 10% (40 mL, 24 h). All the extraction fractions were evaporated in a rotavapor (Buchi Labortechnik AG, Switzerland). Dry E-extracts (screening and Zippertex extractor) were resuspended in 0.5 mL of dimethyl sulfoxide (DMSO), and 1 mL of DMSO was necessary to resuspended the M-extracts. All samples were filtered through a DMSO-safe filter (0.22 μM; Tecknochroma).

2.2.2 Large-scale extractions

Following screening extraction assays, the tree whose leaf and stem extracts showed the highest antiproliferative activity was submitted to a scaled-up extract process using the accelerated solvent system, Zippertex extractor [9]. 25 g of stems and 40 g of leaves were extracted with dichloromethane (DCM) (2x100 mL), followed by MeOH (2x100

mL) at 100 bars. Afterwards, the solvents were evaporated and resuspended in DMSO at a concentration of 2 g dry weight (DW)/mL.

2.2.3 Plant extract fractionation

The MeOH extract from leaves was subjected to an additional fractionation procedure. Compounds of the extract were firstly separated by silica gel chromatography using a Combiflash-companion chromatograph (Serlabo) and ready-to-use RediSep column (40 g), obtaining 8 fractions which were resuspended in DMSO, at a concentration of 0.4 g DW /mL. The mobile phase consisted of DCM (A) and MeOH (B) with the following increasing polarity gradient (t (min), %B): (5, 0), (7-17, 5), (18-28, 10), (32-42, 15), (44-55, 20) in 60 min at 30 mL/min.

Flash chromatography was performed with two active fractions to purify the lead compounds. A Sunfire C_{18} III (10 x 250 mm) 5 µm column was used and the mobile phase consisted of water (A): acetonitrile (B), both added with 0.1% formic acid (v/v). A linear gradient of 100% A to 100% B was performed in 20 minutes with a flow of 30 mL/min.

2.3 MTT assay

2.3.1 MTT cell assay conditions

Cell viability was determined by the MTT assay [10]. The cell growth medium was removed and 0.63 mM of MTT and 18.4 mM of sodium succinate (from Sigma-Aldrich, Madrid, Spain) were added to 1 mL of fresh culture medium and the cells were incubated for 3 h at 37 ºC. Thereafter the medium was removed and formazan resuspended in DMSO supplemented with 0.57% CH_3COOH and 10% sodium dodecyl sulfate (SDS) (from Sigma-Aldrich, Madrid, Spain). Absorbance was measured at 570 nm in a UV2310 spectrophotometer (Dinko, Barcelona, Spain).

2.3.2 Vehicle validation

To determine the most suitable solvent and the appropriate volume to be used for the experiments DMSO and ethanol (EtOH) were analyzed at 0.01%, 0.02%, 0.1%, 0.2% and 1% (v/v) on HeLa cells. Cells were seeded at 3.4 $x10^4$ cells/well in 12 well plates and after 24 h of growth, DMSO or EtOH was added to the medium and the cell viability assay was carried out for 48 h.

2.3.3 Plant extract assays

HeLa, HepG2 and MCF-7 cells were seeded at a density of $3.4x10^4$ cells/well. Twenty-four hours later, different amounts of each plant extraction were added to the cell cultures: 15 mg DW/mL and 3.33 mg DW/mL obtained from E-extraction, 7.5 mg DW/mL and 1.66 mg DW/mL from M-extraction and 3.33 µg DW/mL from Zippertex extractor. For the different fractions obtained 3 mg DW/mL and 0.66 mg DW/mL were assayed. Cell viability was determined 48 h later.

2.3.4 Taxol toxicity

Taxol (ChromaDex, Irvine, CA, USA) was used as an antiproliferative control. The half maximal inhibitory concentration (IC_{50}) of taxol was determined in HeLa cell line. To this end, taxol was diluted in DMSO at the following concentrations: 0.5 nM, 5 nM, 50 nM, 500 nM and 1500 nM. $3.4x10^4$ cells/well were seeded and grown for 24 h. Taxol solution was added to the medium to analyze cell viability 48 h later.

2.3.5 (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-β-D-glucopyranoside and quercetin-3-O-rhamnoside assay

(3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-β-D-glucopyranoside (named as DHHP) and quercetin-3-O-rhamnoside (QR) were dissolved in DMSO at 1 mg/mL. Two concentrations (15 µg/ml and 7.5 µg/ml) were tested. $3.4x10^4$ cells/well were seeded and grown for 24 h. Each compound was added to the culture in the exponential phase and the effect was measured 48 h later.

2.4 Analytical procedures

2.4.1 HPLC-MS conditions

An Alliance Waters 2695 HPLC instrument (Waters) including autosample 717, with a pump 600 equipped with a photodiode array 2998 detector and an evaporative light-scattering detector 2420 was used. The HPLC analytical column used was Sunfire C_{18} III (4.6 x 150 mm) 3.5 µm operating at 0.7 ml/min. The mobile phase was water (A) and acetonitrile (B), both containing 0.1% of formic acid. A linear gradient from 100% of A to 100% of B was performed in 50 minutes. HPLC was fitted to a simple quadrupole Waters-Micromass®ZQ 2000 mass spectrometer. All analyses were done using electrospray ionization (ESI) in positive and negative mode with the following

MS conditions: capillary voltage: 3.5 kV; cone voltage: 25 V (+), 45 V (-); extractor voltage: 1 V; source temperature: 110 °C; desolvatation temperature: 250 °C; RF Lens: 0.1 V; desolvatation gas flow: 450 L/h; cone gas flow: 50 L/h; LM1/HM1 resolution: 15; ion Energy: 0.5 keV and Multiplier: 600 V.

2.4.2 Nuclear magnetic resonance

^{1}H spectra were recorded with a Brüker Avance-500 instrument operating at 500 MHz. Deuterated methanol (MeOD) was used as a solvent. The obtained ^{1}H chromatograms for each compound were compared with the corresponding simulated chromatogram estimated by ChemNMR software.

2.5 Statistical analysis

All the conditions were run in triplicate in at least two independent experiments. The statistical analysis was performed with SPSS (version 22.0). To analyze the effect of each treatment on cell viability, ANOVA analysis was performed. *p*-values lower than 0.05 (*) or 0.01 (**) were considered statistically significant.

3. Results

3.1 Effects of C. avellana extracts on the viability human cancer cell lines

The effects of different concentrations of DMSO or EtOH on the viability of HeLa cells were determined. In all conditions assayed, EtOH promoted a significant decrease in cell viability compared with non-treated cells ($p<0.05$; Figure 1). EtOH solutions presented more cytotoxicity than DMSO at all the concentrations tested, while DMSO treatment did not affect significantly the cell viability. Thus, DMSO was selected as the solvent for subsequent experiments at concentrations up to 1 % (v/v).

As a control for cytotoxicity, HeLa cells were incubated in the presence of taxol. At the concentrations tested, viability of HeLa cells showed a dose-response effect: the amount of taxol added to the culture medium correlated with cytotoxicity. IC_{50} for taxol was 5 nM in HeLa cells non-treated cells or cells in presence of DMSO as control (Figure 2).

Thereafter, screening experiments using leaf and stem extracts obtained from three different *C. avellana* trees were carried out. To this end, dry extracts were resuspended

211 in DMSO, at a concentration of 2 g DW/mL in the case of E-extraction and 1 g DW/mL
212 when the M-extraction procedure was followed. Twenty-four hours after seeding, HeLa,
213 HepG2 and MCF-7 cells were incubated for 48 h in the presence of leaf and stem E- and
214 M-extracts. HeLa cells treated with 15 mg DW/mL of E-extractions (Figure 3A) and 7.5
215 mg DW/mL of M-extractions (Figure 3B) showed a strong reduction of cell viability
216 compared to DMSO- and non-treated cells. Leaf and stem E- and M-extracts from trees
217 II and III reduced viability of HeLa cells more extensively than extracts from tree I. The
218 leaf M-extract from tree III was chosen to carry out a dose-response assay. The results
219 showed that when the M-extract concentration added to the cell culture was increased
220 (1.66, 3.75 and 7.50 mg DW/mL), the HeLa cell viability decreased (12.5, 2.4 and 1.9
221 %, respectively).

222 In HepG2 and MCF-7 cells, 3.33 mg DW/mL of leaf and stem E-extracts (Figures 3C
223 and 2E) and 1.66 mg DW/mL of leaf and stem M-extracts (Figures 3D and 3F) were
224 assayed. E- and M-extracts of trees II and III similarly reduced HepG2 cell viability and
225 exhibited more cytotoxic effects than extracts obtained from tree I. Compared to
226 HepG2, MCF-7 cells were more resistant to cytotoxic effects of *C. avellana* leaf and
227 stem extracts. However, leaf and stem extracts from tree III reduced cell viability to a
228 greater extent than trees I and II.

229 Since leaf and stem extracts from tree III reduced cell viability more than trees I and II
230 in the three tumour-derived cell lines, tree III was used for subsequent studies.
231 Concerning the type of extraction, M-extraction showed a significantly higher viability-
232 reducing effect than E-extraction ($p<0.01$).
233

234 *3.2 Identification of C. avellana compounds with cell viability-reducing activity*

235 To identify compounds responsible for the growth inhibitory activity of *C. avellana*
236 leaves and stems from tree III, a scaled up maceration-like extraction process was used,
237 using Zippertex technology and two different solvents, MeOH and DCM. The
238 anticancer activity of the resulting extracts was confirmed by assaying the viability of
239 HeLa, HepG2 and MCF-7 cell lines (Figure 4). For stem extracts, no differences in
240 viability were found for the three cell lines related to the solvent used for the extraction
241 process (MeOH or DCM). However, MeOH leaf extracts showed a marked higher
242 toxicity than DCM in all cell lines. Irrespective of the extraction solvent, the cell growth

243 inhibitory effect of leaf and stem extracts was similar for HeLa and HepG2 cells, while
244 MCF-7 exhibited greater viability. Compared to control cells, MeOH leaf extracts
245 significantly decreased HeLa, HepG2 and MCF-7 viability by 86, 89 and 71 %,
246 respectively.
247 Flash chromatography was used to fractionate the MeOH leaf extracts obtained using a
248 Zippertex extractor. The cell viability-reducing activity of eight different fractions (3
249 mg DW/mL and 0.66 mg DW/mL) were assayed in HeLa cells (Figure 5). Fractions 1,
250 2, 6 and 7 showed a significantly reduced cell viability at both concentrations assayed.
251 While fractions 1 and 2 promoted a dramatic reduction in cell viability, the effects of
252 fractions 6 and 7 were dose-dependent at the concentrations assayed. Fractions 3, 4, 5
253 and 8 showed only a statistically significant cell growth decrease at the higher
254 concentration assayed (3 mg DW/mL).

255 Fractions 6 and 7 were selected for further assays due to both their antiproliferative and
256 dose-response activity. The chromatograms obtained by HPLC-MS for fractions 6 (A)
257 and 7 (B) are presented in Figure 6. Spectra showed two masses, *m/z* 478 and *m/z* 448,
258 present in both fractions 6 and 7. ESI in negative mode and MS allowed us to determine
259 that the two masses corresponded to DHHP (C) and QR (D).
260
261 *3.3 Effects of DHHP and QR on the viability of human cancer cell lines*
262 HeLa, HepG2 and MCF-7 cells were incubated for 48 h in the presence of 7.5 µg/mL of
263 DHHP or QR to assay the effect of both compounds on cell viability (Figure 7). DHHP
264 and QR significantly decreased HeLa and HepG2 cell viability to similar levels: 51-40
265 % for DHHP and 31-20 % for QR respectively. However, MCF-7 cell viability was
266 hardly affected by any of the two compounds at the concentrations tested. Albeit not
267 significant, a slightly reducing trend of MCF-7 cell viability was observed when using
268 DHHP (8 %) and QR (2 %).
269
270 **4. Discussion**
271 Several studies have reported the content of growth inhibitory products, taxol and
272 taxanes, in *C. avellana* cell cultures [3,7,11] and trace amounts of taxol in *C. avellana*
273 tree extracts [4]. The *C. avellana* tree extracts used in this work showed trace amounts
274 of taxol (data not shown). In the present study, identification of other compounds with
275 anticancer activity in *C. avellana* tree extracts was addressed by assaying viability-

276 reducing activity in different tumour-derived cell lines. For testing the effect of *C.*
277 *avellana* extracts and isolated compounds on the viability of HeLa, HepG2 and MCF-7
278 cell lines, first we confirmed that DMSO was a suitable vehicle for our studies. DMSO
279 is an amphipathic molecule that is extensively used as a solvent for drug testing [12]. At
280 a concentration of 1 % (v/v), DMSO did not promote changes in cell viability in any of
281 the cell lines assayed.

282 As a control to identify other compounds in *C. avellana* leaf and stem extracts with
283 anticancer activity, we used taxol, a compound with anticancer activity in numerous
284 tumour-derived cell lines. The IC_{50} value of taxol in HeLa cells was 5 nM, which is
285 consistent with previously reported IC_{50} values for this compound in this cell line [13].

286 It is well known that secondary metabolite production and accumulation is dramatically
287 affected by the season, plant variety and other environmental conditions [14]. To avoid
288 seasonal variations, stems and leaves from *C. avellana* were collected in the same
289 month (October) from three different locations in Catalonia. Two extraction approaches
290 were applied: taxane (E-) and maceration (M-) extraction methods. The taxane
291 extraction method (E-extraction) was used due to the fact that previous results of our
292 group indicate its suitability for isolating active compounds from stems [8].
293 Furthermore, taxol and related taxanes were previously found in *C. avellana*. In
294 addition, we used maceration with the aim of extracting a wider range of active
295 compounds. In fact, we found that the M-extraction procedure was more suitable for
296 leaves.
297 The cell viability assays using leaf and stem extracts revealed that maceration was a
298 better procedure than taxane extraction. Therefore, we used a large-scale maceration
299 system using a Zippertex extractor. This extraction method allows isolation of enough
300 quantity of compounds, reducing time and costs [9], and offered us an optimal
301 procedure to obtain high yields of the compounds produced or accumulated in *C.*
302 *avellana*. The growth inhibitory effects of leaf and stem Zippertex extracts was
303 comparable to that observed during the screening experiments. Leaf extracts using
304 MeOH as solvent produced the greater reduction of viability in the three cell lines
305 assayed (reduced viability by 86, 89 and 71 % in HeLa, HepG2 and MCF-7
306 respectively). This is consistent with the fact that leaves accumulate a great variety of
307 secondary metabolites and MeOH is a very efficient universal solvent.

Identification of compounds with cell viability-reducing activity was conducted after silica gel chromatography fractionation of MeOH leaf extracts. The fractions that significantly reduced cell viability of HeLa cells in a dose-dependent manner were selected for subsequent analysis. The main compounds found in the selected fractions were two polyphenols: quercetin-3-O-rhamnoside, a flavonoid also named quercitrin (QR), and (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-ß-D-glucopyranoside, a diarylheptanoid (DHHP).

Polyphenols, a large group of ubiquitous and abundant natural compounds, present low toxicity, high structural diversity and exert a wide range of biological activities. Quercitrin is the rhamnose C-3 glycosylate form of quercetin, a flavonoid widely produced by a huge number of plants. Quercitin can be found in different glycoside forms and exhibits potential anticancer activity [15, 16]. High rates of quercitrin in *C. avellana* leaves (3235.5 mg/Kg DW) have been reported using a flavonoid-extraction methodology [17]. Several studies reported a significant role of flavonoids in growth inhibition of breast, colon, gastric, prostate, ovary, endometrium, and lung tumour-derived cell lines [15, 18, 19].

As a diarylheptanoid, DHHP contains two aromatic rings linked by a seven-carbon aliphatic chain. This type of metabolite is mainly found in species from the Betulaceae, Zingiberaceae, Leguminoseae and Taccaceae families. The potential activity of these compounds has been described as antinflammatory, antioxidant, antitumor, estrogenic, hepatoprotective and neuroprotective [20, 21, 22]. Specifically, (3R,5R)-3,5-dihydroxy-1,7-bis(4- hydroxyphenyl) heptanes 3-O-ß-D-glucopyranoside has been described by Yokosuka [23] in *Tacca chantrieri*, Quang [24] in *Tacca plantaginea*, and Riethmüller [25] in *C. avellana*. At a concentration of 21 nM, no cytotoxic activity of this compound was found in HL-60 cells (human promyelocytic leukemia cells), while an IC_{50} of 384 and 445 nM was reported for HSC-2 (oral squamous cell carcinoma) and HGF (normal human gingival fibroblast) cells, respectively [23]. Moreover, Quang [24] reported that (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptanes 3-O-ß-D-glucopyranoside inhibits tumor necrosis factor alpha (TNF-α)-induced nuclear factor kappa-B (NF-κβ) transcriptional activity and activates the transcriptional activity of peroxisome proliferator-activated receptors (PPARs), both in a dose-dependent manner in the HepG2 cell line, suggesting diarylheptanoids as a new target for the prevention and treatment of metabolic and inflammatory diseases.

Our results suggest that the presence of DHHP and QR in *C. avellana* confers a marked cell viability-reducing activity of leaf extracts from this tree. Although the chromatographic fractions analyzed by HPLC-MS did not present traces of taxol, we cannot rule out the presence of taxol or other compounds with anticancer activity in other non-analyzed fractions. Indeed, the presence of very low quantities of taxanes in *C. avellana* has been reported [3, 6, 7, 26].

Notably, the growth inhibitory effects of DHHP and QR depended on the cell line analyzed. At the concentrations assayed, both compounds reduced viability of HeLa and HepG2 cells more intensively than that of MCF-7 cells, which suggests the potential use of DHHP and QR as part of a multi-drug therapy against specific tumours, such as in the treatment of cervical cancer and liver carcinoma. Both bioactive compounds have been reported as pro-apoptotic when added to the cultured cancer cell lines [27, 28]. It is well known that multi-drug therapies improve the effectiveness of treatment in many diseases. In this regard, combinations of polyphenols with commercial drugs have been reported [29, 30, 31, 32, 33].

5. Conclusion

In conclusion, this is the first report showing anticancer activity of *C. avellana* tree extracts against human cancer cell lines. In addition, we identified (3R,5R)-3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-ß-D-glucopyranoside and quercetrin as contributors to the strong cell viability-reducing activity of *C. avellana* leaf extracts. Our findings open new insights into the anticancer activity of *C. avellana* in human cancer cell lines and highlight greater sensitivity of HeLa and HepG2 cells to the isolated compounds from *C. avellana* leaf extracts. Further research is needed to analyze the interaction among active substances present in *C. avellana* extracts and study their effect on primary cultures of cancer cells in *in vivo* assays, with the ultimate aim of developing new and more effective cancer therapies.

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Conflict of interest statement

The authors confirm that there are no conflicts of interest associated with this publication.

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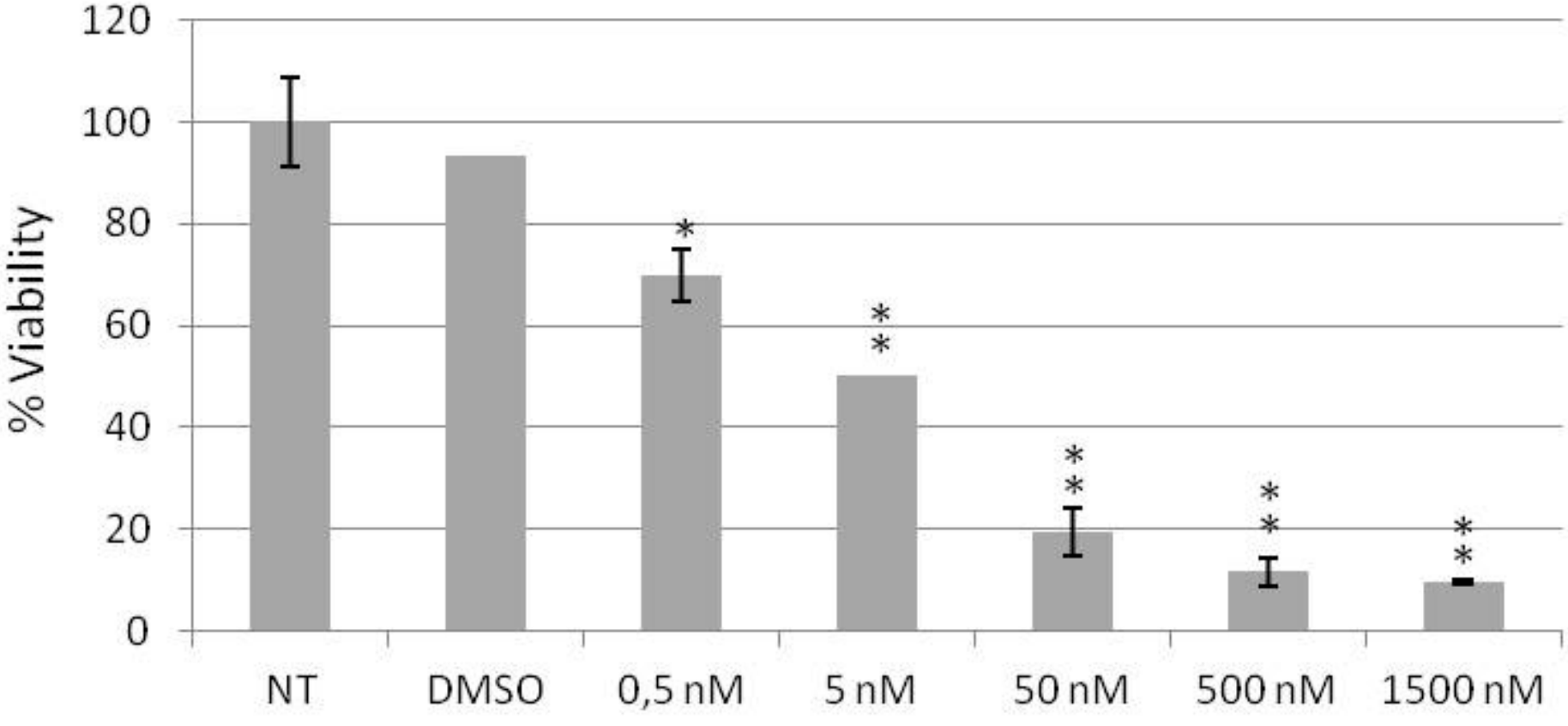
Figure legends

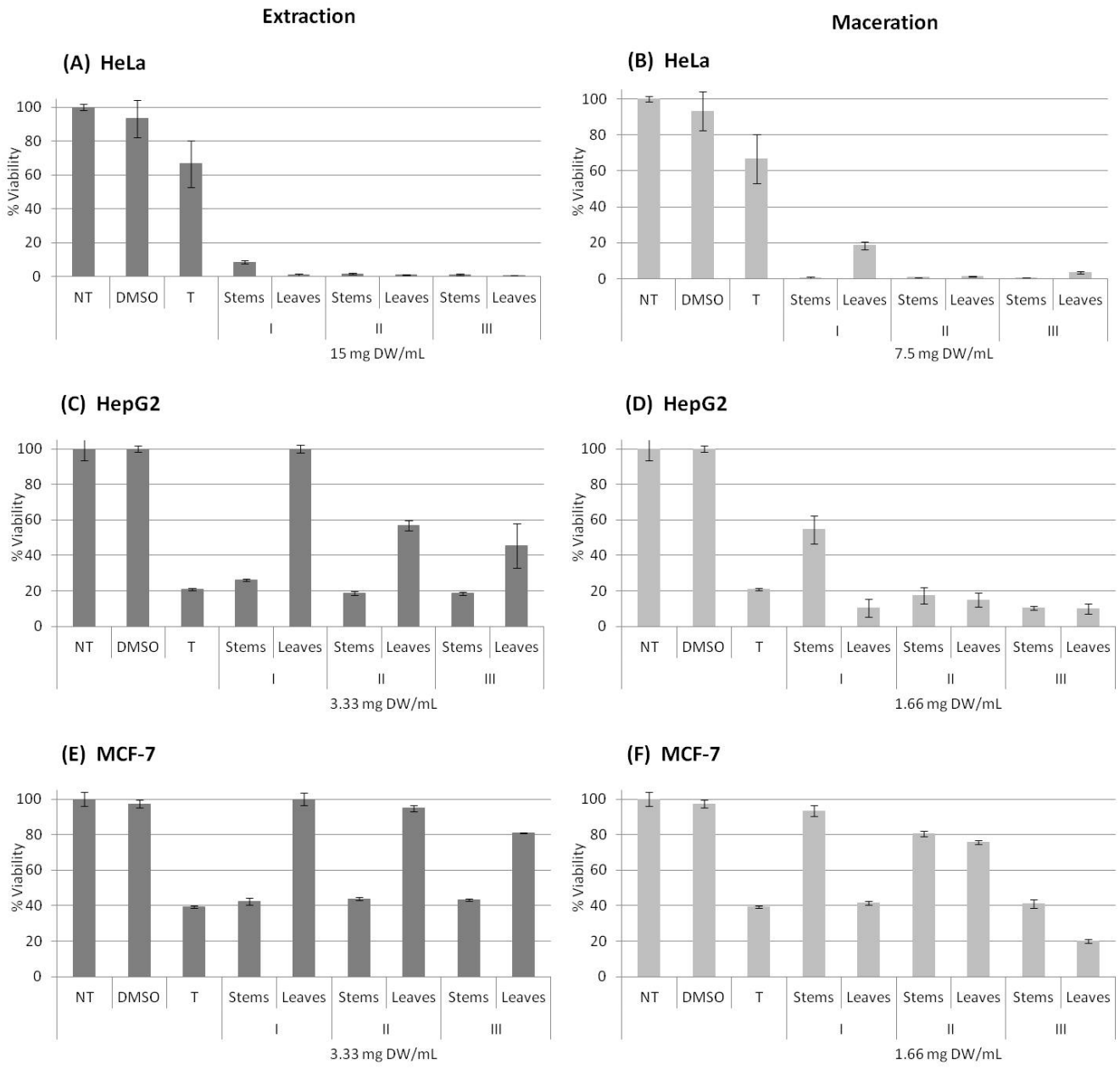
Figure 1. Effect of DMSO and EtOH on cell viability of HeLa cells. Viability of HeLa cells was assayed 48 h after treatment with DMSO or EtOH at concentrations ranging from 0 (NT) to 1%. The results are presented as mean values ± SD from a representative experiment run in triplicate. NT: non-treated cells; DMSO: cells incubated in the presence of DMSO; EtOH: cells incubated in the presence of EtOH.

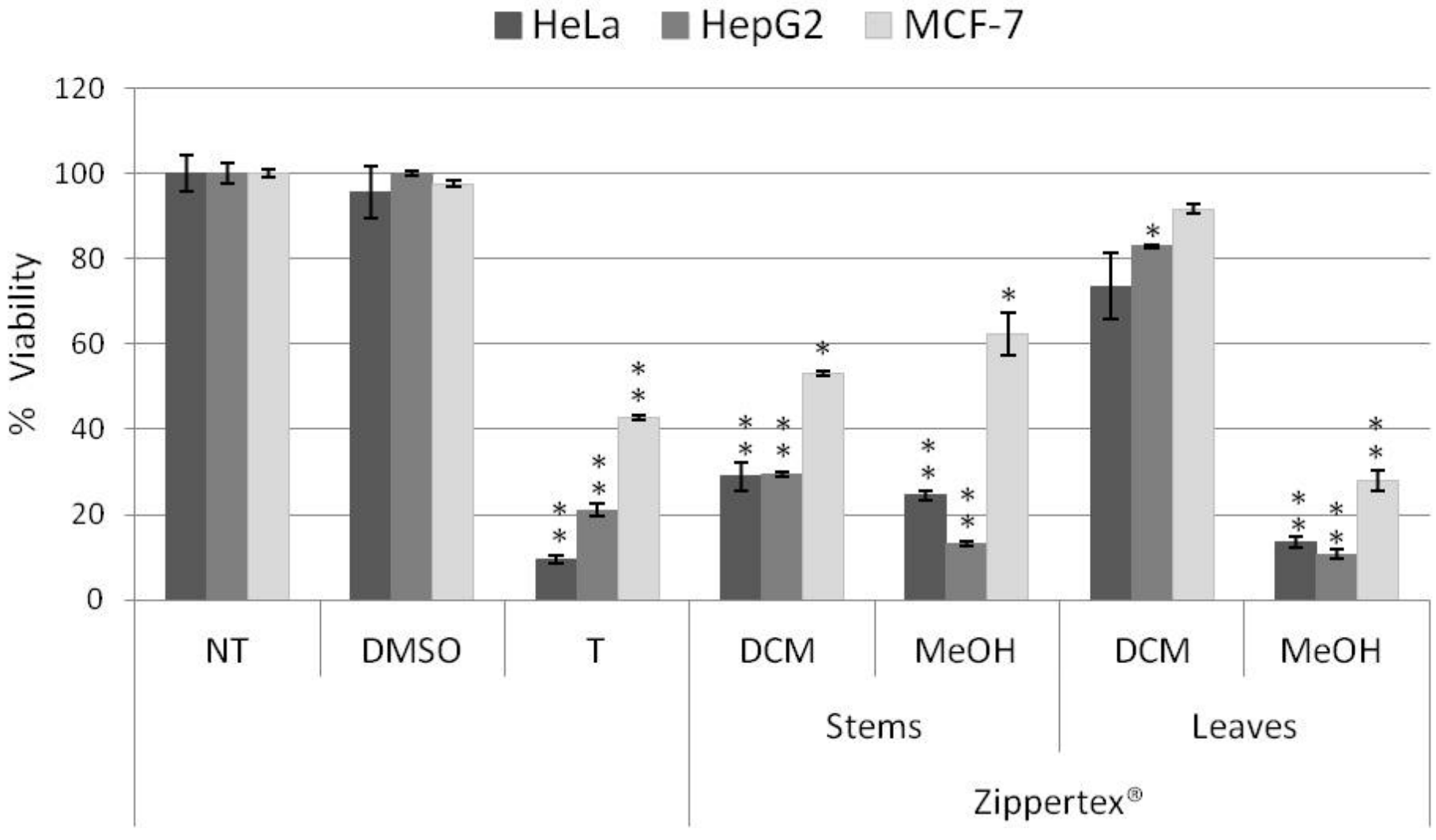
Figure 2. Effect of taxol on cell viability of HeLa cells. Viability of HeLa cells was assayed 48 h after treatment with taxol at concentrations ranging from 0 (NT and DMSO) to 1500 nM. Each bar represents the mean ± SD of a representative experiment run in triplicate. Statistically significant differences between treatments and DMSO-treated cells (control) is presented as $*p<0.05$ and $**p<0.01$. NT: non-treated cells; DMSO: cells incubated in the presence of 1% DMSO.

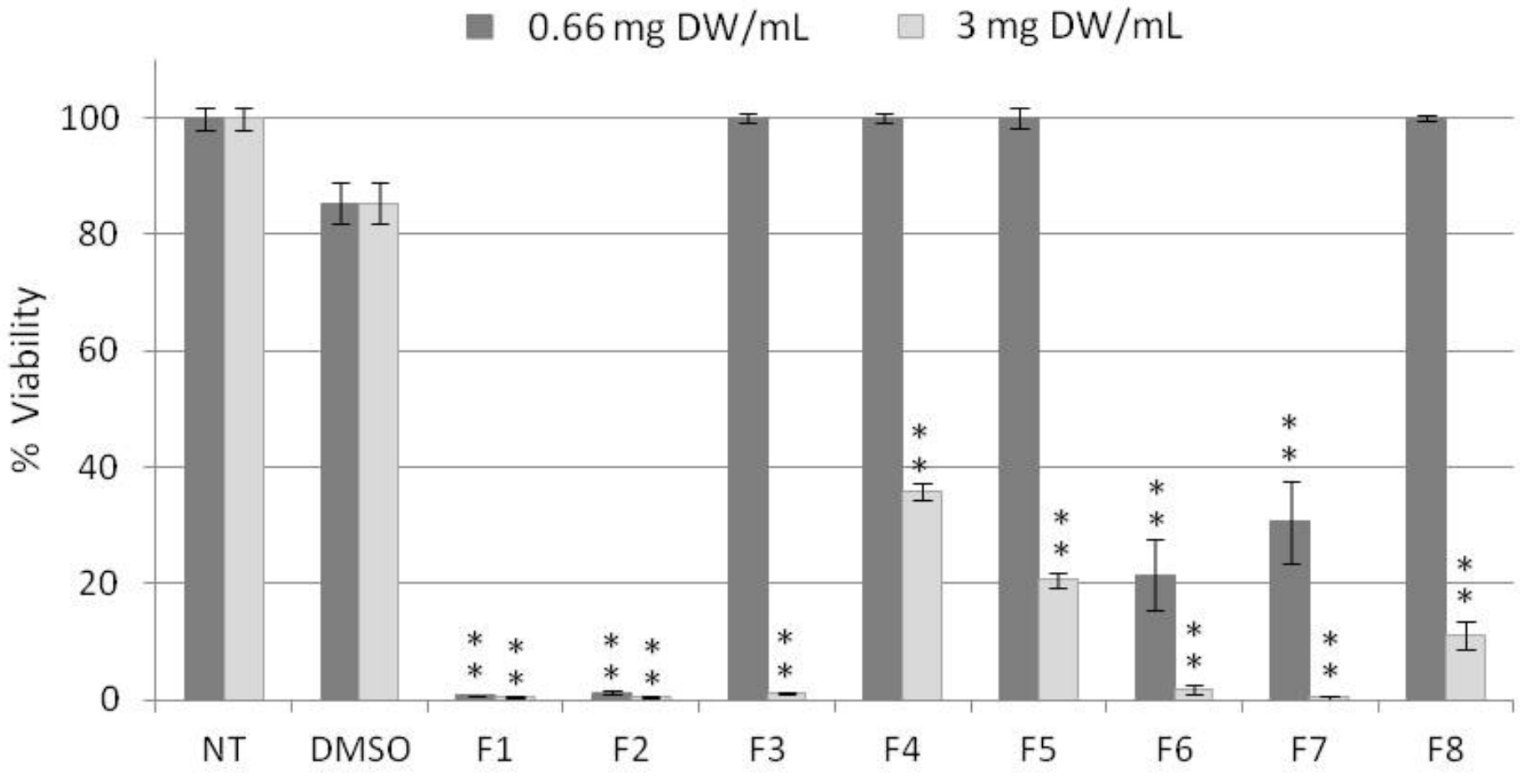
Figure 3. Cell growth inhibitory effects of extracts prepared from leaves or stems of three different *C. avellana* trees (I, II, III) by taxane extraction (E) or maceration extraction (M). Viability was assayed in HeLa cells after 48 h of treatment with 15 mg DW/mL for E-extracts (A) and 7.5 mg DW/mL for M-extracts (B), and HepG2 and MCF-7 cells 48 h following treatment with 3.33 mg DW/mL for E-extracts (C, E) and 1.66 mg DW/mL for M-extracts (D, F), using 1% DMSO as a solvent. The results are

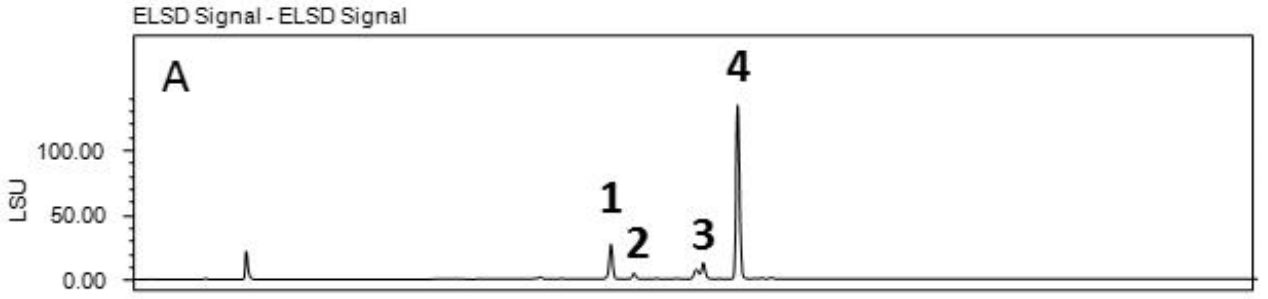
503 expressed as % (mean ± SD of a representative experiment run in triplicate) of the values
504 found in non-treated cells. NT: non-treated cells; DMSO: cells incubated in the presence
505 of 1% DMSO; T: cells treated with 5 nM taxol.
506
507 **Figure 4.** Cell growth inhibitory activity of Zippertex extracts from *C. avellana* stems
508 and leaves on HeLa, HepG2 and MCF-7 cell lines. Viability of all cell lines was assayed
509 48 h after no treatment in absence (NT) or presence of 1% DMSO (DMSO) and treatment
510 with 5nM taxol (T), DCM or MeOH Zippertex extracts from stems and leaves, using 1%
511 DMSO as a solvent. The results are presented as mean values ± SD from a representative
512 experiment run in triplicate. Statistically significant differences compared to the
513 respective control (DMSO) are presented as *p<0.05; **p<0.01.
514
515 **Figure 5.** Effect of eight chromatographic fractions obtained from MeOH *C. avellana*
516 leaf extracts on viability of HeLa cells. Viability was assayed in HeLa cells after 48 h of
517 no treatment in absence (NT) or presence of 1% DMSO (DMSO) and treatment with 0.66
518 and 3 mg dry weight /mL, using 1% DMSO as a solvent. The results presented are mean
519 values ± SD from a representative experiment run in triplicate. Statistically significant
520 differences compared to controls (DMSO) are presented as *p<0.05; **p<0.01.
521
522 **Figure 6.** HPLC-MS profile of fraction 6 (A) and fraction 7 (B) of MeOH *C. avellana*
523 leaf extracts obtained with a Zippertex extractor. MS chromatograms (ESI^-) of compound
524 3: DHHP (C) and 4: QR (D) are shown.
525
526 **Figure 7.** Cell growth inhibitory activity of quercetin-3-O-rhamnosid (QR) and (3R,5R)-
527 3,5-dihydroxy-1,7-bis(4-hydroxyphenyl) heptane 3-O-β-D-glucopyranoside (DHHP) on
528 HeLa, HepG2 and MCF-7 cells. Viability was assayed in all cell lines after 48 h of no
529 treatment in absence (NT) or presence of 1% DMSO (DMSO) and treatment with 7.5
530 mg/mL QR or DHHP, using 1% DMSO as a solvent. The results are presented as mean
531 values ± SD from a representative experiment run in triplicate. Statistically significant
532 differences compared to the corresponding control (DMSO) are presented as *p<0.05;
533 **p<0.01.


120
100
80
60
40
20
0
% Viability
*
**
***
***
***
NT
DMSO
0,5 nM
5 nM
50 nM
500 nM
1500 nM

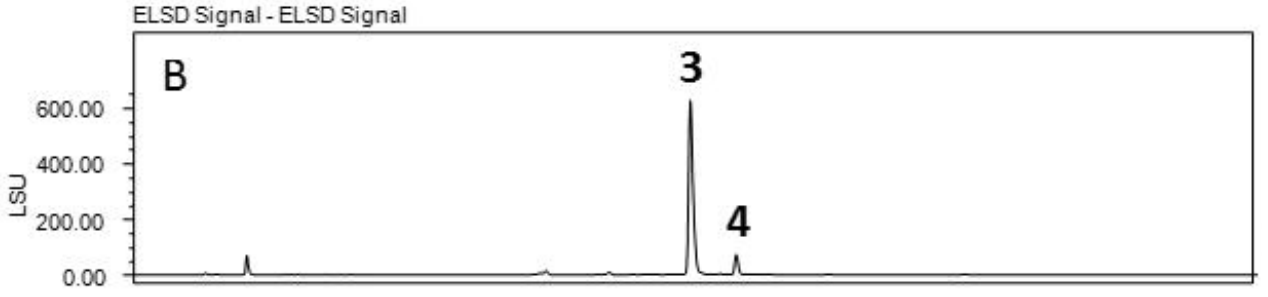

Extraction
Maceration
(A) HeLa
(B) HeLa
(C) HepG2
(D) HepG2
(E) MCF-7
(F) MCF-7
% Viability
0
20
40
60
80
100
NT
DMSO
T
Stems
Leaves
I
II
III
15 mg DW/mL
7.5 mg DW/mL
3.33 mg DW/mL
1.66 mg DW/mL


HeLa
HepG2
MCF-7
% Viability
120
100
80
60
40
20
0
NT
DMSO
T
DCM
MeOH
DCM
MeOH
Stems
Leaves
Zippertex®

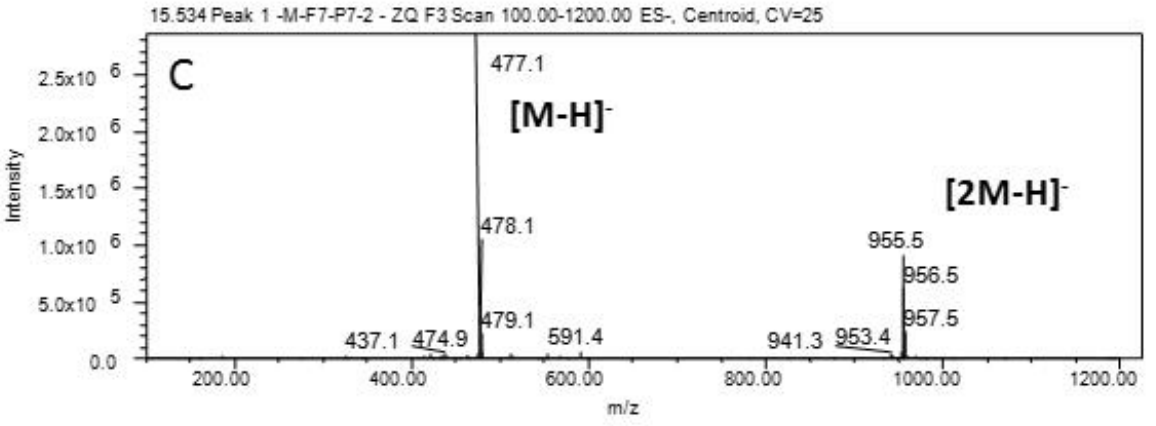

0.66 mg DW/mL
3 mg DW/mL
% Viability
100
80
60
40
20
0
NT
DMSO
F1
F2
F3
F4
F5
F6
F7
F8

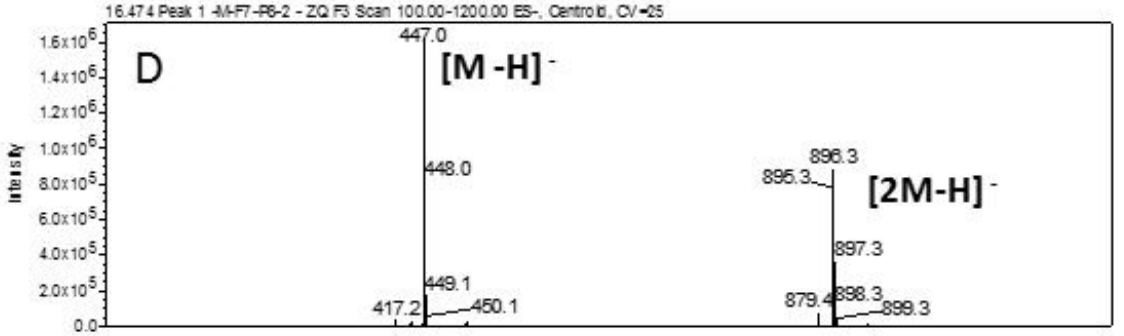


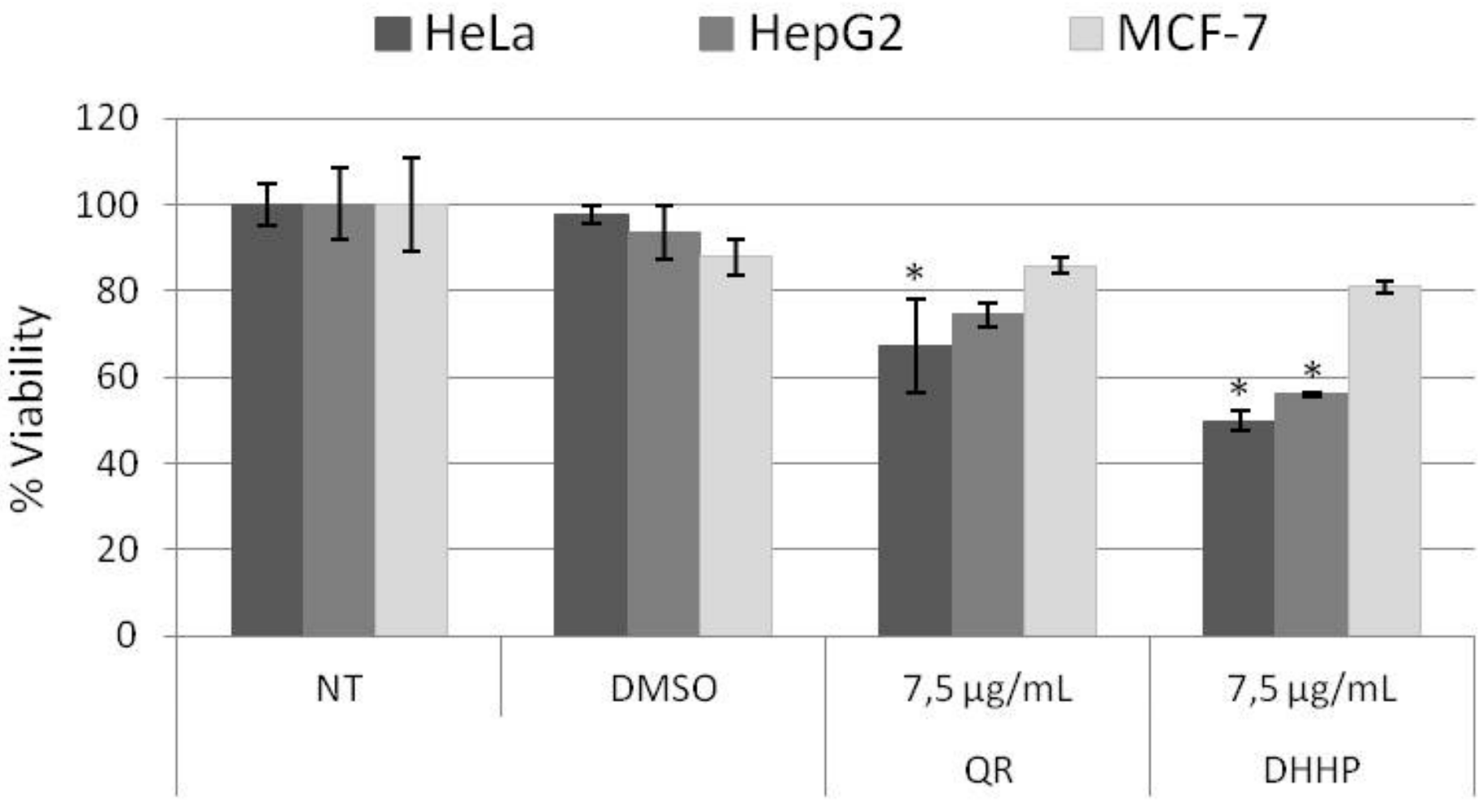
1. *m/z 386* Cholesterol
2. *m/z 388* Cholestanol
3. *m/z 478* Hydroxyphenyl-4-hepten-3-one hexoside familly
4. *m/z 448* Quercetin 3-O-Rhamnoside



3. *m/z* 478 Hydroxyphenyl-4-hepten-3-one hexoside familly
4. *m/z* 448 Quercetin 3-O-Rhamnoside






HeLa
HepG2
MCF-7
120
100
80
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40
20
0
% Viability
*
*
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NT
DMSO
7,5 µg/mL
QR
7,5 µg/mL
DHHP