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Effects of elm bark extracts from *Ulmus laevis* on human chorion carcinoma cell lines

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Abstract
Purpose The potential of substances from elm bark extracts to affect cancer has been described in several studies. In this study, the anticancer effects of extracts from *Ulmus laevis* bark were tested in hormone-dependent gynecological tumours using human chorion carcinoma cell lines.
Methods The molecular-chemical composition of the bark extract was analysed by pyrolysis-field ionisation mass spectrometry. The influence of the extracts was determined on cell vitality and cytotoxicity in the human chorion carcinoma cell lines Jeg3 and BeWo in comparison with primary trophoblast cells.
Results The elm bark extract was mainly composed of triterpenes, phytosterols, free fatty acids and suberins with lower amounts of dilignols and lipids. The elm bark extract significantly inhibited the vitality of Jeg3 and BeWo cells but increased the vitality of primary trophoblast cells.
Conclusions Substances extracted from elm bark might have beneficial effects for the prevention of hormone-dependent tumours.

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Keywords *Ulmus laevis* bark extract · Py-FIMS · Trophoblast cells · Human chorion carcinoma cells · Vitality · Cytotoxicity

Abbreviations

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
LDH	Lactate dehydrogenase
E	17-β-estradiol
T	Tamoxifen

Introduction

Traditional Chinese medicine indicates that the bark of *Ulmus* sp. has positive effects against oedema, mastitis, inflammation and cancer [1, 2]. At present, the substances responsible for these effects are unknown. Naturally occurring substances such as terpenes [3], glycopeptides [4], polyphenols [5, 6] and phytoestrogens [7–9] have been considered to have anti-cancerogenic effects. The cytostatic agent Taxol A is a diterpene-polyester from the bark of *Taxus brevifolia* and has been successfully applied against mammary and ovarial carcinoma [10]. Bark of the regional elm (*Ulmus laevis*) may also contain substances with anti-cancerogenic potential against hormone-dependent gynecological tumours. Therefore, the aim of the present study has been to identify potentially active substances of extracts from bark of *Ulmus laevis* and to analyse their effects on cell vitality and cytotoxicity in human chorion carcinoma cell lines Jeg3 and BeWo. The placental cell culture model is suitable for the direct comparison of tumour cell lines with a primary cell culture under in vitro conditions [11].

Materials and methods

Extract preparation from elm (*Ulmus laevis*) bark

Bark was collected from the stem of *U. laevis* Pallas (identified by Prof. Porembski, Botany, University of Rostock) in a forest near Rostock (Mecklenburg-Western Pomerania, Germany). The extracts were prepared according to Luyengi et al. [12] as modified by Matscheski et al. [13]. In brief, 20 g of elm bark were ground mechanically and extracted with methanol (177.5 mL) in a water bath for 15 min at 70°C using a reflux condenser. The solution was cooled, filtered and evaporated to complete dryness. This extract was resuspended in 8 mL of distilled water and partitioned with ethyl acetate (five times) to give an ethyl acetate and a water fraction. The ethyl acetate soluble fraction was evaporated to complete dryness and dissolved in 100% ethanol to provide a stock solution of 100 mg/mL. This stock solution of the ethyl acetate fraction is hereafter referred to as 'elm bark extract'. Aliquots of the elm bark extract were added to the supplemented culture medium to give final concentrations of 0.5, 1, 5, 10, 50, 100, 250 and 500 μg/mL (1% final ethanol concentration).

Chemical analysis with pyrolysis-field ionisation mass spectrometry (Py-FIMS)

Pyrolysis-field ionisation mass spectrometry (Py-FIMS) is a rapid and comprehensive technique for molecular-chemical characterisation of biomaterials [14–16]. 5 μL of the elm bark extract was transferred to a quartz crucible that was placed in the micro-oven of the direct inlet system of a double-focusing Finnigan MAT 900 mass spectrometer (Finnigan, MAT, Bremen, Germany). The analyte was evaporated to dryness in the fore-vacuum (10^{-1} hPa). The micro-oven heated the sample from 110 to 700°C at 20 K-increments in 12 min, and 91 magnetic scans were recorded for the mass range 15–900 Dalton (single spectra). These were combined to obtain one thermogram of total ion intensity (TII) and an averaged Py-FI mass spectrum. For each of the single scans, the absolute and relative ion intensities of 14 classes of chemical compounds were calculated by summation of the ion intensities of 8–39 indicator signals [17], including the protonated molecule ion mass signals ($(M + H)^+$) if present. All Py-FIMS data were normalised per mg sample. This procedure was carried out for each of five replicate measurements per sample and the results were averaged for statistical analyses.

Cell culture

The chorion carcinoma cell lines Jeg3 and BeWo were obtained from LGC Standards GmbH, Wesel, Germany. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Bio Whittaker) with 10% inactivated foetal calf serum (FCS) and antibiotics (penicillin/streptomycin) and an antimycotic (amphotericin) at 37°C and 5% CO_2. The primary trophoblast cell culture was directly isolated from the placenta according to Jeschke et al. [11].

Cell vitality and cytotoxicity assay

As representative in vitro cell study tests, the cell vitality and cytotoxicity tests were performed using the MTT kit and the cytotoxicity detection kit (LDH kit) as recommended by the supplier (Roche, Germany).

Cell vitality and cytotoxicity were investigated with the chorion carcinoma cell lines Jeg3 and BeWo. For reproducible results, it was important to work with the same cell number in all tests. For this purpose, the optimal cell number was estimated in preliminary experiments. The following considerations were taken into account: (i) during the test phase the medium should not be consumed completely and hence should be added in excess, (ii) the cells should adhere to the plastic material and should not float, and (iii) a measurable absorbance to determine inhibitory effects should be reached. The optimal cell number was found to be 5×10^5 cells/mL, which was used in all tests. For the MTT test, measuring the activity of the mitochondrial dehydrogenase, Jeg3, BeWo cells and primary trophoblast cells were grown in 96-well tissue plates for 48 h in the absence (controls) and presence of the different concentrations of the elm bark extract at 37°C and 5% CO_2. After incubation with MTT for 4 h, solubilisation solution was added and the plates were incubated overnight. The spectrophotometric absorbance of the purple formazan crystals was measured at 570 nm using a microplate ELISA (enzyme-linked immunosorbent assay) reader (BioRad, Hercules, CA, USA). The reference wavelength was 670 nm.

For the LDH test measuring the cell death, the cells were grown in 96-well tissue plates for 24 h in an incubator (37°C, 5% CO_2). The elm bark extracts were then added and the cells were incubated for a further 24 h before the LDH activity contained in the supernatants was measured at 492 nm using a microplate ELISA reader (see above). The reference wavelength was 620 nm.

Two negative controls were examined in all tests: (i) cells in DMEM (control 1) and (ii) cells in DMEM and ethanol (final concentration of ethanol: 1%) (control 2). As positive controls, 17-β-estradiol (estrogen) and tamoxifen (anti-estrogen) dissolved in 100% ethanol (1% final ethanol concentration) were examined in all assays. 17-β-estradiol and tamoxifen were purchased from Sigma (Germany). Purity of 17-β-estradiol and of tamoxifen was $\geq$98 and =99%, respectively.

Statistical analysis

All in vitro cell study test procedures were repeated three till five times with four replicates of the negative and positive controls and the different concentrations of elm bark extracts, respectively. Statistical analysis was performed using the Welch test for the comparison of means. A P value of <0.05 was considered as being statistically significant and was denoted by an asterisk (*) in the diagrams.

Results

The extraction of elm bark yielded approximately 6.5 mg/g (residue on drying of the ethyl acetate fraction). The Py-FI mass spectra revealed 595 individual *m/z*-signals in the range of 15–700 (Fig. 1). Signals between *m/z* 350 and *m/z* 550 were most abundant, predominated by *m/z* 426 (16.9% TII), *m/z* 424 (16.4%), *m/z* 425 (6.9%), *m/z* 427 (6.2%). The tentative signal assignment to 14 compound classes indicated the dominance of triterpenes and sterols (63.6%) which molecular masses partly coincided and saturated long-chain fatty acids (C_{16}–C_{34} fatty acyls) (15.8%). Furthermore, suberins (2.4%), low-mass signals (2.0%), dilignols (1.7%) and lipids (1.3%) were assigned. All other compound classes contributed less than 1% to the TII (total ion intensity).

Figure 2 shows that the vitality of Jeg3 and BeWo cells (MTT test) decreased in a concentration-dependent manner, after application of elm bark extract, relative to the negative control 2 (C2) (100%) (Fig. 2a, b). Generally, the negative controls 1 and 2 did not differ in absorbance values, indicating that 1% ethanol did not inhibit cell growth (data not shown). The cell vitality reduction was significant after the addition of all tested concentrations of elm bark extract in the Jeg3 culture (Fig. 2a). In the BeWo culture, this effect was only partly significant (Fig. 2b). The strongest inhibition of cell vitality was measured after the addition of 500 µg/mL of the extract to the BeWo culture (Fig. 2a). The addition of 17-β-estradiol did not affect the vitality of Jeg3 and BeWo cells, but the application of tamoxifen significantly inhibited the uptake of MTT by the Jeg3 and BeWo culture by 73.6 and 59.6%, respectively (Fig. 2a, b). Addition of elm bark extract at a concentration of 10 µg/mL resulted in a weak or no inhibitory effect on the vitality of Jeg3 and BeWo cells, respectively, in comparison to the positive control tamoxifen (Fig. 2a, b). When higher concentrations of elm bark extract (250 µg/mL) were applicated, the inhibition effects in BeWo cells were similar to that of 10 µg/mL tamoxifen (Fig. 2b). However, in the Jeg3 cells these inhibition effects were weaker than that of tamoxifen (Fig. 2a). The vitality of the primary trophoblast cells was increased by all concentrations of elm bark extract and 17-β-estradiol (Fig. 2c). This was confirmed by the results of the

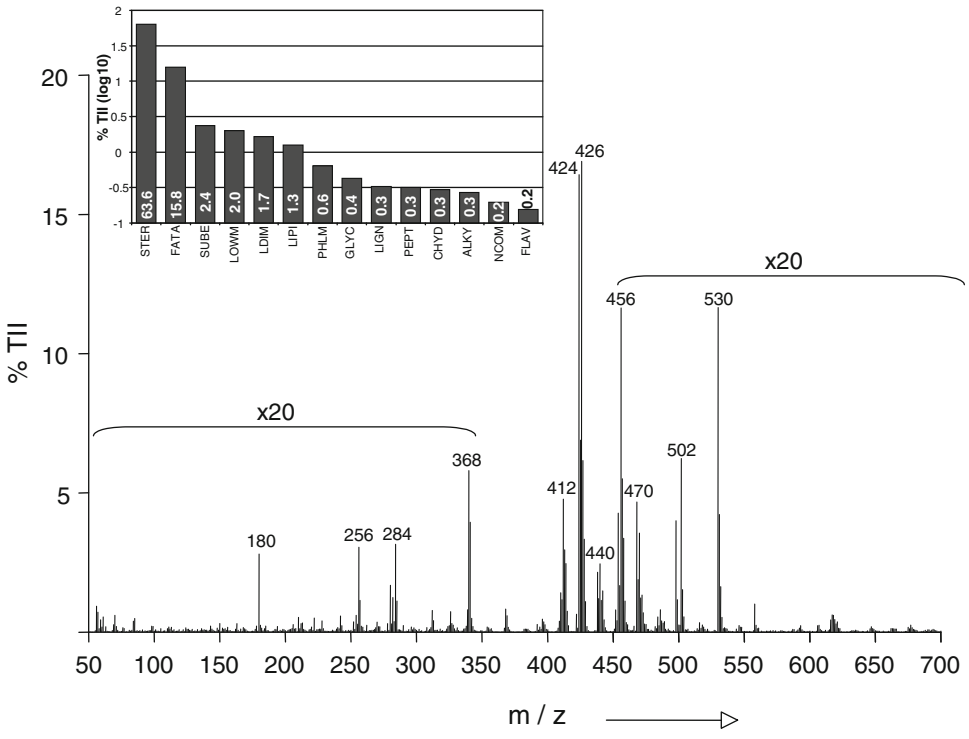

Fig. 1 Summed and averaged pyrolysis-field ionisation mass spectra with relative abundance of *m/z*-signals (% of total ion intensity) of the elm bark extract and main classes of organic compounds as derived from the pyrolysis-field ionisation mass spectra; *TII* total ion intensity in %, *STER* sterols and triterpenes, *FATA* free fatty acids, *SUBE* suberin, *LOWM* low molecular mass signals (*m/z* 15…56), *LDIM* dilignols, *LIPI* lipids, *PHLM* phenols + monolignols, *GLYC* di- + triglycerides, *LIGN* lignans, *PEPT* peptides, *CHYD* carbohydrates, *ALKY* alkyl aromatics, *NCOM* heterocyclic nitrogen containing compounds, *FLAV* flavonoids

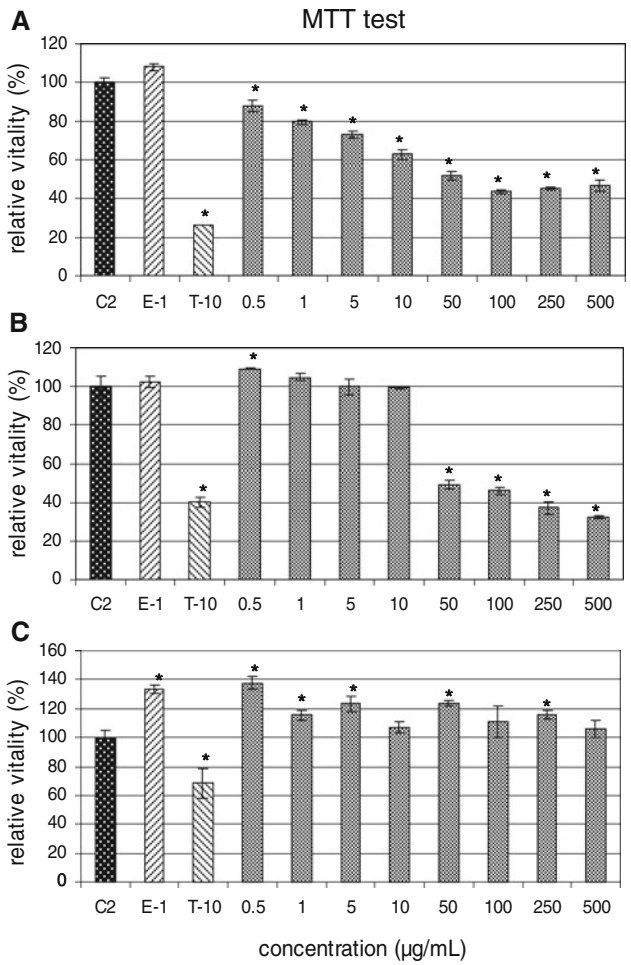

Fig. 2 Effect of different concentrations of the elm bark extract on cell vitality (MTT test) of Jeg3 (**a**), BeWo (**b**) and primary trophoblast cells (**c**). Data (mean ± SD) (standard deviation) represent relative formation of formazan from MTT in % in comparison with negative control 2 (100%) obtained in at least 3 experiments. *Asterisks* (*) indicate significant differences between treated cells and the negative control 2 ($P < 0.05$). *C2* control 2, *E-1* 1 µg/mL 17-β-estradiol, *T-10* 10 µg/mL tamoxifen

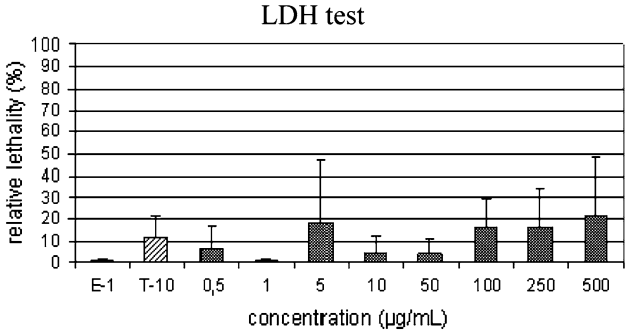

Fig. 3 Effects of different concentrations of the elm bark extract on cytotoxicity (LDH test) of primary trophoblast cells. Data (mean ± SD) (standard deviation) represent relative cytotoxicity in % in comparison with negative control 2 (0%) (data not shown) obtained in at least 3 experiments. *E-1* 1 µg/mL 17-β-estradiol, *T-10* 10 µg/mL tamoxifen obtained in at least three experiments

cytotoxicity test. Over the whole range of all elm bark extract concentrations, no significant cytotoxic effects were observed on primary trophoblast cells (Fig. 3). By contrast, tamoxifen significantly reduced their vitality by 31.7% (Fig. 2c).

Discussion

A lot of the identified substance classes are described as potential anticancer products in different tumour cell lines. The triterpenes and sterols as being the main constituents of the bark extract and identified by other research groups in elm bark extracts [18] may exert anti-cancerogenic effects in breast cancer cells [19]. Wang et al. [1, 20] have isolated the sesquiterpenes manson E and F and some new triterpenoids from *Ulmus pumilus* and reported anti-proliferative and cytotoxic effects on the human tumour cell lines A375-S2, HeLa, MCF-7 and U937. In a recent publication we demonstrated the inhibitory effects of bark extracts from *Ulmus laevis* on endometrial carcinoma cell lines [21]. Anticancer effects of free fatty acids such as palmitic acid and octadecenoic acid as well as oleic acid resulted in apoptosis-inducing activity in colon tumour cells [22, 23]. It has been suggested that plant cell walls containing significant amounts of phenolic components like suberins and lignins may be most likely to protect against cancer (dietary fibre hypothesis) [24].

Cell vitality can be enhanced by estrogens (17-β-estradiol) and reduced by anti-estrogens (tamoxifen) via the activity of the estrogen–receptor (ER). Primary trophoblast cells and the Jeg3 and BeWo carcinoma cell lines have been found to be positive for ERα and ERβ [25–27]. The positive ER mediation becomes obvious by the significantly contrasting estradiol and tamoxifen effect on the vitality of primary trophoblast cells (Fig. 2c). Tamoxifen concentrations were chosen according to the data of Szewczyk [25] showing that tamoxifen at a concentration of 10 µg/mL induced significant inhibitory effects on the growth of Jeg3 cells. The inhibitory activity of tamoxifen at 10 µg/mL was much stronger as for the same concentration of elm bark extract applicated to the Jeg3 and BeWo cells (Fig. 2a, b). These differences could be explained by two suggestions: (i) no individual substances were tested but an elm bark extract comprising of single compounds in combination, (ii) the compounds in elm bark extract probably can affect the growth of Jeg3 and BeWo carcinoma cell lines through ER mediated as well ER independent mechanisms of action. In the primary trophoblast cell culture, tamoxifen inhibited cell vitality but this was not observed for the elm bark extracts. This indicates a

higher sensitivity for the elm bark extracts in the human carcinoma cell lines than in the primary trophoblast cell cultures (see Fig. 2a, b and compare with Fig. 2c).

To the best of our knowledge, this is the first study showing significant effects of extracts from *U. laevis* bark on cell vitality of Jeg3 and BeWo cell lines. We assume that co-operating single substances in the elm bark extract exert the biological activity in our in vitro cell experiments. Our results are in agreement with an in vitro study showing that the elm-bark-containing herbal tea FlorEssence significantly inhibits the proliferation of human breast cancer (MCF-7, MDA-MB-468) and leucaemia cells (Jurkat, K562) [28]. Thus, elm bark extracts may also provide resources for anticancer drug recovery. Forthcoming should be directed to identifying the individual compounds present in the bark extract of *U. laevis*, using an HPLC or LC–MS analysis, as well as testing their effects with other in vitro cell study assays and other hormone-dependent carcinoma cell lines to disclose intracellular processes.

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Conflict of interest None.

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