Full Length Research Paper

Extracts of shoots and roots of *L. angustifolius* exhibiting breast-cancer-restraining effects

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The identification of medicinal plants and derived natural products for development as anti-cancer agents is of long standing interest. We have investigated the anti-proliferative properties of *Lupinus angustifolius* on breast cancer and, in particular, whether the extracts of roots and shoots of *L. angustifolius* can be considered as candidates for primary and secondary cancer prevention. Ethanolic extracts of roots and shoots of *L. angustifolius* were analysed for their substance classes by pyrolysis-field ionization mass spectrometry. Various concentrations of these extracts (0.1, 1, 10, 25, 50, 100 and 200 µg/ml, incubation period: 24 h) were studied with respect to their effects on cell proliferation and cytotoxicity against the breast cancer cell lines MCF-7 (ER-α+, ER-β+) and BT20 (ER-α-, ER-β-). *L. angustifolius* ethanolic root and shoot extracts inhibited cell proliferation in the MCF-7 and BT20 cells; root extract: strongest inhibition MCF-7 (200 µg/ml 71.03 ± 12.62%); BT20 (200 µg/ml 99.72 ± 6.48%), IC_{50}-values: MCF-7 (52.3 ± 9.39 µg/ml); BT20 (66.86 ± 7.02 µg/ml); shoot extract: strongest inhibition MCF-7 (200 µg/ml 64.7 ± 10.07 %); BT20 (200 µg/ml 86.32 ± 9.19%), IC_{50}-values: MCF-7 (18.06 ±4.49 µg/ml); BT20 (70.27 ± 0.76 µg/ml). Thus, extracts of *L. angustifolius* roots and shoots have anti-tumour activity against receptor-positive and receptor-negative breast cancer cell lines.

Key words: Anti-tumour activity, Lupinus angustifolius, breast cancer, MCF-7, BT20

INTRODUCTION

Breast cancer is the one of the most frequently diagnosed cancer in women and ranks second as a cause of cancer deaths in the female population (after lung cancer) (American cancer Siciety, 2012; Globocan, 2013; Centers for Disease Control and Prevention, 2013). It was estimated to cause approximately 421,000 new cases and 129,000 deaths in Europe alone in 2008 (Ferlay et al., 2010). In the United States, breast cancer is one of the leading causes of cancer death of women (40,676 women, 2009) (Centers for Disease Control and Prevention, 2013). Natural product research has revealed a large variety of phytochemicals that have been shown to be successful against breast cancer in several epidemiological and *in vitro* studies. More than 60% of

currently used anti-cancer drugs were originally derived from natural sources such as plants, marine organisms and microorganisms (Aruoma et al., 2006). Various studies have suggested the potential of medicinal plants as anti-cancer drug candidates (Abarzua et al., 2012; Cragg et al., 2005; Pandey et al., 2009; Richter et al., 2013).

Lupinus angustifolius (family Fabaceae) is used in the food industry in Germany with regard to won protein and fibre (PlantProFood, 2013). Some studies have shown that lupin seeds have an immunotropic activity (Błaszczyk et al., 1994) and anti-bacterial properties (Oomah et al., 2006), whereas others indicate that lupin protein has hypocholesterolemic effects (Weisse et al., 2010), binds insulin *in vitro* and reduces plasma glucose levels (Magni et al., 2001). To date, no investigations into the possible anti-cancer effect of extracts from the root and shoot of the blue lupin *Lupinus angustifolius* have been described. Of the plants that we have examined, the blue lupin contains primarily isoprenoids, sterols, terpenes, flavonoids and polyphenols in its shoots and roots. We have used ethanolic extracts of the shoots and roots of blue lupin to determine whether they influence the cell proliferation of the breast cancer cell lines MCF-7, an estrogen receptor (ER)-positive control cell line, and BT20, an ER-negative cell line. MCF-7 and BT20 cells are useful for *in vitro* breast cancer studies, because the cell lines have retained several ideal characteristics of the mammary epithelium. Our data suggest that extracts of shoots and roots of *L. angustifolius* exhibit a breast-cancer-restraining effect in these receptor-positive and receptor-negative cell lines. Thus, roots and shoots of blue lupin might contain potential agents for the primary and secondary therapy of mammacarcinomas.

MATERIALS AND METHODS

Plants

Roots and shoots were harvested from the blue lupin, *Lupinus angustifolius (*type: Boregine), at the Saatzucht Steinbach GmbH & Co KG in Germany, during the month of July, 12 weeks after being sown. The plant was authenticated at the Herbarium unit of the Institute of Biosciences, University of Rostock, Rostock, Germany. Blue lupin roots and shoots were stored at -80°C at the University of Rostock in the University Gynaecological Clinic.

Microwave extraction

Microwave energy is a non-ionizing radiation that causes molecular motion by the migration of ions and the rotation of dipoles (Chen et al., 2008; Gu et al., 2008). Many reports have been published on the application of microwave-assisted extraction of secondary metabolites from plants (Chattip et al., 2008; Chen et al., 2007; Guo et al., 2008). The main advantages of microwave-assisted extraction are the considerable reduction in time and solvent as compared with conventional techniques. Samples of 3 g plant material (roots and shoots) from *L. angustifolius* were extracted with 15 ml ethanol (absolute) in a microwave oven (CEM Discover).

Extraction took place under the following conditions: time, 10 min; temperature, 60°C; energy level, 800 W.

Pyrolysis-field ionization mass spectrometry (screening)

An analysis of the substance classes contained within blue lupin root and shoot extracts obtained by microwave extraction was carried out by pyrolysis-field ionization mass spectrometry at the University of Rostock in the Department of Soil Science. For field ionization, an emitter was used that was activated by a pyrolytic process within a benzonitrile atmosphere, with a partial pressure of approx. 80 Pa and under high voltage (4.8kV FI Emitter), by means of a Wolfram filament heated to white heat, leading to the formation of bristle-like carbon needles. The assay material was weighed in a quartz crucible, which was introduced, via a rod, into the ionic source, and the sample was ionized. The ions were detected by a MassSpectrometry MAT 900 S, which had a double-focus sector-field analyser.

Cell culture

MCF-7 and BT20 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The breast cancer cell lines MCF-7 (ER-α +, ER-β +) and BT20 (ER-α-, ER-β-) were cultivated in the University Gynaecological Clinic at Rostock. MCF-7 cells were grown in phenol-red-free DME-medium (PAA), with 10% fetal calf serum (FCS), Amphotericin B (250 µg/ml; PAA), penicillin/streptomycin (100×; PAA). BT20 cells were grown in RPMI medium (PAA), with 10% fetal calf serum (FCS), Amphotericin B (250 µg/ml; PAA), penicillin/streptomycin (100x; PAA). Cells were cultivated at 37°C under 5% CO_2 in an incubator. Cells were passaged every 2 to 3 days by using trypsin and ethylenediaminetetraacetic acid (EDTA).

Assessment of cell proliferation and cytotoxicity

The cell proliferation and cytotoxicity of the human breast carcinoma cell lines MCF-7 and BT20 treated with various concentrations of ethanolic extracts from *L. angustifolius*, 17-β estradiol and tamoxifen were analysed by using the 5' bromodeoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (colorimetric) and lactate dehydrogenase (LDH) cytotoxicity detection kit as recommended by the manufacturer (Roche, Germany). The test conditions were optimized in preliminary experiments, with the optimal cell number being 5×10^5 cells/well. The experiments were divided into four groups: (1) negative control with 1 µl absolute ethanol, (2) 17-β estradiol and (3) tamoxifen as positive controls and various concentrations (4) of the ethanolic extracts (see above). Each group consisted of 4 replicate wells in 96-well plates. The negative control with 1 µl absolute ethanol is hereafter referred to as the control. Table 1 shows the final concentration of the ethanolic extracts and controls in the assays.

Cell proliferation (cell proliferation BrdU assay, Roche)

Proliferating cells were revealed by the BrdU cell proliferation test (Roche). The cells were grown in 96-well plates: 100 µl cell suspension (5×10^5 cells/ml) was pipetted into each well of a 96-well plate and incubated for 24 h at 37°C under 5% CO_2. Subsequently, 1 µl of the test substance was added followed by 24 h of incubation at 37°C under 5% CO_2. Every well, except for the background controls, received 10 µl marker solution (1:100 dilution with sterile medium) and the cells were further incubated for 3 h at

Table 1. Final concentrations of ethanolic extracts and controls in the assays.

Lupin root extracts (µg/ml)	Lupin shoot extracts (µg/ml)	17-β estradiol	Tamoxifen	Ethanol absolute
200	200	10^{-9} mol/l	10^{-4} mol/l	1%
100	100	0.00027 µg/ml	37.15 µg/ml	-
50	50	-	-	-
25	25	-	-	-
10	10	-	-	-
1	1	-	-	-
0.1	0.1	-	-	-

37°C under 5% CO_2. Removal of the medium from the plates by sharply knocking the inverted plates was followed by incubation of the cells in 200 µl FixDenat for 30 min. The FixDenat was removed by knocking the inverted plates and the cells were further incubated for 60 min with the antibody solution. The cells were then washed three times with 200 µl washing buffer (1:10 dilution) and then incubated in 100 µl substrate solution (tetramethylbenzidine) for 10 to 15 min. The reaction was stopped with 1 M H_2SO_4. Absorption at a wavelength of 450 nm was measured in an ELISA reader (BioRad, Hercules, CA, USA).

$$Cell\ proliferation\ (\%) = \frac{Absorbance\ sample}{Absorbance\ control} \times 100$$

Cytotoxicity assay (LDH Cytotoxicity detection kit, Roche)

Cytotoxic effects on the cells were identified with a LDH cytotoxicity detection kit (Roche). The assay for the quantification of cell death and cell lysis was based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. LDH activity is determined in a coupled enzymatic reaction; during this reaction, the tetrazolium salt INT is reduced to formazan. This formazan dye is easy to assay, since it is water-soluble and has a broad absorption maximum at approximately 500 nm. The increase in supernatant LDH activity is directly correlated to the amount of formazan formed over time. Samples taken from the cell suspension were pipetted into 96-well plates at 100 µl/well (5×10^5 cells/ml) for later treatment with each test substance and as controls. Cells were then incubated for 24 h at 37°C under 5% CO_2 in an incubator. The original medium (with 10% FCS) was exchanged for 198 µl/well fresh medium (1% FCS). Aliquots of 2 µl test substance were added to the relevant wells or 2 µl vehicle (absolute ethanol) was added to the wells as a negative control (medium plus cells plus absolute ethanol). As a positive (100% cell lysis) control, a well reaction mixture composed of 200 µl Triton X-100 and medium containing 1% FCS (at 1:46) were pipetted into four untreated wells. The cells were incubated for 24 h at 37°C under 5% CO_2 in the incubator. Subsequently, 100 µl cell suspension was removed and replaced by 100 µl well reaction mixture (at 1:46) and the reaction was stopped by the addition of 50 µl 1 M N HCl per well. The absorption of formazan was measured at a wavelength of 490 nm (reference 620 nm) in an ELISA reader and was evaluated with the program microplate manager.

$$Cytotoxicity\ (\%) = \frac{(Absorbance\ of\ the\ sample\ -\ Absorbance\ of\ the\ negative\ control)}{(Absorbance\ of\ the\ high\ control\ -\ Absorbance\ of\ the\ negative\ control)}$$

Statistical analysis

Statistical calculations were carried out with the Microsoft Office Excel 2007, Sigma Plot 11.0 and SPSS 10.0 for the Windows software package. Results are expressed as the means ± S.E.M. of three independent experiments. Student´s t-test and one way analysis of variance were used for statistical analysis; $P < 0.01$ was considered to be significant. IC_{50}-values (half-maximal inhibitory concentration) were calculated with Sigma Plot 11.0.

RESULTS

Pyrolysis-field ionization mass spectrometry (screening)

An analysis of the substance classes of the blue lupin root and of the blue lupin shoot components obtained by microwave extraction was carried out by pyrolysis-field ionization mass spectrometry (Figure 1). The substance class of LIPID (alkanes, alkenes, aldehydes, alcohols, fatty-acids, n-alkyl esters, waxes, fats) was the most strongly represented in the crude extract of lupin root (29.4%) and in that of lupin shoot (32.1%). ISOPR (isoprenoids, sterols, terpenes, carotenoids) gave complete ion strength (%) of 25.4% in the crude extract of lupin root and 25.1% in the crude extract of lupin shoot. Polyphenole (polyphenols) exhibited complete ion strength (%) of 10.4% in the crude extract of lupin root and 8.6% in the crude extract of lupin shoot.

Anti-proliferous activity of *Lupinus angustifolius* extracts (BrdU assay)

Blue lupin root and shoot extracts induced a significant
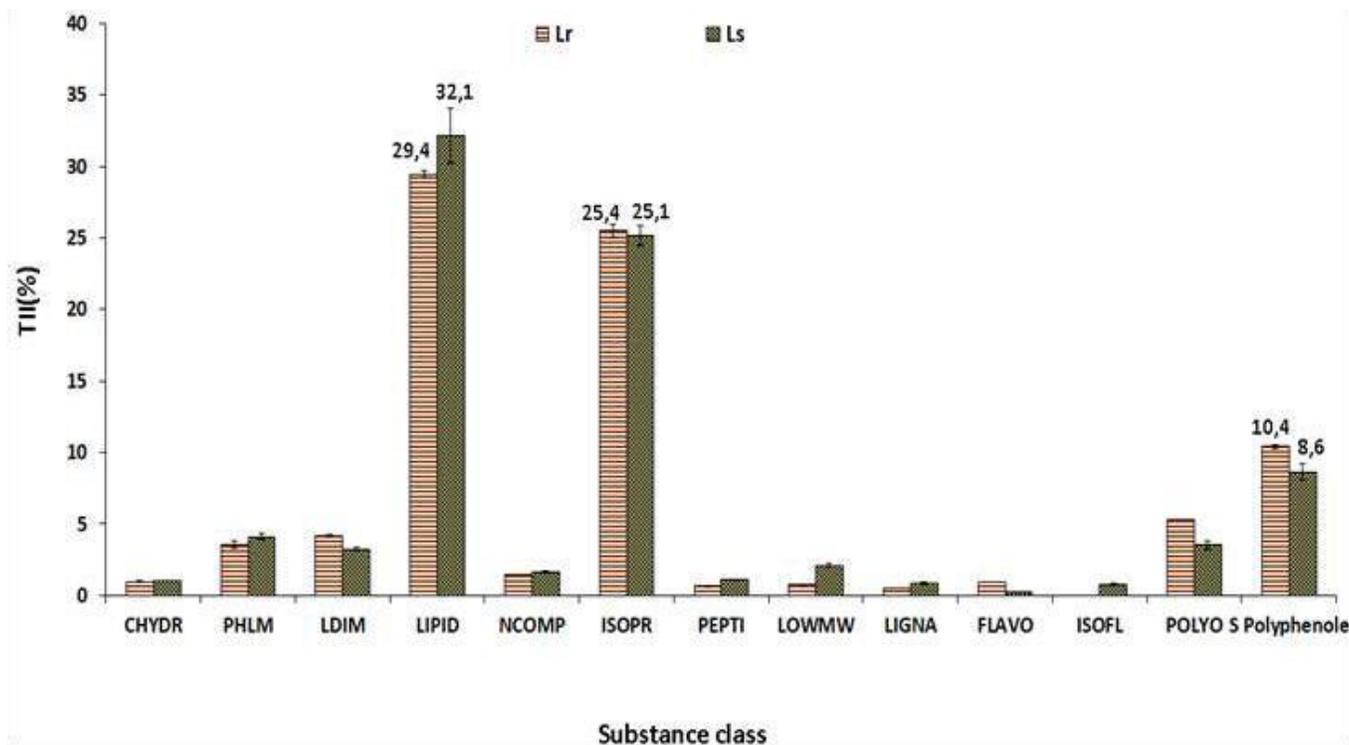
Figure 1. Pyrolysis-field ionization mass spectrometry (screening)**.** ITMS-cESI Full ms (90.00-2000.00) of ethanolic lupin extracts. Results are expressed as the mean ± SEM of 3 independent experiments. CHYDR = carbohydrates, PHLM = monolignols, LDIM = lignin dimers, LIPID = alkanes, alkenes, aldehydes, alcohols, fattyacids, n-alkyl esters, waxes, fats, NCOMP = compounds containing nitrogen, ISOPR = isoprenoids, sterols, terpenes, carotenoids, PEPTI =peptides and free amino acids, LOWMW = low-molecular compounds m/z 15-56, LIGNA = lignans, FLAVO = flavones, ISOFL = isoflavones, POLYO S = other polyphenols, Polyphenole = polyphenols. Lr = lupin root extract, Ls = lupin shoot extract.

reduction in the cell proliferation of MCF-7 and BT20 cells in a dose-dependent manner (Figure 2A and B). Strong inhibition of cell proliferation was seen at a concentration of 200 µg/ml in MCF-7 (64.7 ± 10.07%) and BT20 (86.32 ± 9.19%) cells (Figure 2A and B). The strongest inhibition was shown by the blue lupin root extract with a concentration of 200 µg/ml in MCF-7 (71.03 ± 12.62%) and BT20 (99.72 ± 6.48%) cells (Figure 2A and B). The addition of 17-β estradiol as a control at a concentration of 10^{-9} mol/l (0.00027 µg/ml) did not affect the proliferation of MCF-7 and BT20 cells. However, application of a tamoxifen control at a concentration of 10^{-4} mol/l (37.15 µg/ml) reduced growth by about 90% in MCF7 and in BT20 cells.

Cytotoxicity activity of *L. angustifolius* extracts (LDH assay)

Blue lupin root extract showed significant cytotoxicity in the MCF-7 breast cancer cell line at concentrations of 200 µg/ml (47.26 ± 6.44%), 100 µg/ml (44.98 ±10.50%) and 50 µg/ml (40.33 ± 23.44%). This extract had a lower cytotoxicity in the BT20 cells: 200 µg/ml (44.19 ± 9.78%)

and 100 µg/ml (22.44 ± 5.08%). Blue lupin shoot extract had a low significant effect with regard to cytotoxicity on MCF-7 and BT20 cells. A significant cytotoxicity on MCF-7 cells was observed at 200 µg/ml (20.39 ± 10.10%), 100 µg/ml (21.14 ± 4.84%) and 50 µg/ml (26.89 ± 14.88%). Cytotoxicity in BT20 cells was seen at 200 µg/ml (17.63 ± 9.38%) (Figure 3A and B). The control treatment with 17-β estradiol at a concentration of 10^{-9} mol/l (0.00027 µg/ml) had no cytotoxicity effect on MCF-7 and BT20 cells. On the contrary, the tamoxifen control at a concentration of 10^{-4} mol/l (37.15 µg/ml) induced a strong cytotoxicity of 90-100% in MCF-7 and BT20 cells (Figure 3A and B).

IC$_{50}$-values of *L. angustifolius* extracts

Figure 4 shows the IC$_{50}$-values of *L. angustifolius* extracts. The IC$_{50}$-values have the following order: IC$_{50}$-values for MCF-7 with shoot extract (18.06 ± 4.49 µg/ml) < IC$_{50}$-values for MCF-7 with root extract (52.30 ± 9.39 µg/ml) < IC$_{50}$-values for BT20 with root extract (66.86 ± 7.02 µg/ml) < IC$_{50}$-values for BT20 with shoot extract < (70.27 ± 0.76 µg/ml).
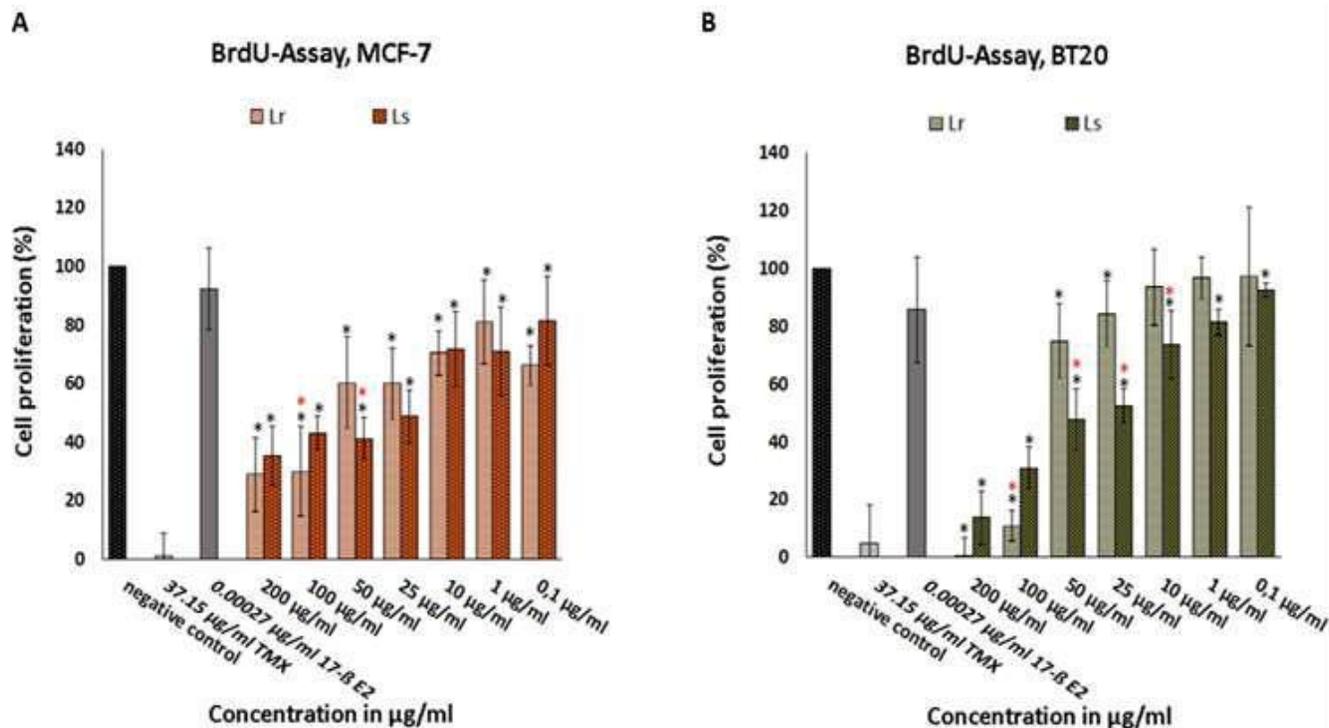
Figure 2. The effect of *Lupinus angustifolius* (Boregine) root and shoot extracts on cell proliferation (%). MCF-7 (A) and BT20 (B) cells were incubated for 24 h with root and shoot extracts (200-0.1 µg/ml). Results are expressed as the mean ± SEM of 3 independent experiments, four-fold for each experimental point. Analysis by t-test (*) and one-way analysis of variance (*) was used to compare cell proliferation in MCF-7 and BT20 cells cultured with various concentrations of lupin extracts with controls. P < 0.01 was regarded as statistically significant. Lr = lupin root extract, Ls = lupin shoot extract, TMX = Tamoxifen, 17-β E2 = 17-β estradiol.

DISCUSSION

The search is on for naturally based anti-proliferative and chemopreventive agents that can act as alternatives to chemically synthesized drugs and those are potentially less toxic and produce fewer side effects (Abrahim et al., 2012). In this study, we have tested the effects of ethanolic extracts from *L. angustifolius* on cell proliferation and cytotoxicity in the breast cancer cell lines MCF-7 and BT20. Our results demonstrate that blue lupin root and shoot extracts contain substance classes with potential anti-tumour effects (Figure 1). The substance class of LIPID (alkanes, alkenes, aldehydes, alcohols, fatty-acids, n-alkyl esters, waxes, fats) is the most strongly represented in the crude extract of the lupin root (29.4%) and in the crude extract of the lupin shoot (32.1%). Various studies have shown that mono- and poly-unsaturated fatty-acids (omega-3 fatty-acids and oleic acid (18:1) possesses anti-carcinogenic characteristics with respect to breast cancer (Carrillo et al., 2012; Mac Lennan and Ma, 2010). In addition, Menendez et al. (2006) have demonstrated that alpha-linolenic acid inhibits HER-2, which is overexpressed in breast cancer cells (Menendez et al. 2006). Our analysis

of the substance classes have shown ISOPR (isoprenoids, sterols, terpenes, carotenoids) compounds make up 25.4% of the crude extract of the lupin root and 25.1% of the crude extract of the lupin shoot. The triterpenoid saponins from soybean (*Glycine max*) and lupin (*Lupinus angustifolius*) inhibit, for example, the sialyltransferases in tumour cells (Hsu et al., 2005; Zhang and Popovich, 2010). Our results show polyphenols as being 10.4% of the crude extract of the lupin root and 8.6% of the crude extract of the lupin shoot. According to Markaverich et al. (2011) and Rahman et al. (2011), the polyphenols luteolin, quercetin, chrysin and kaempferol are able to inhibit the growth of breast cancer cells.

Approximately 70% of all breast cancer types exhibit the expression of ER. In these cases, estrogen is mainly responsible for tumour growth (Rice et al., 2006). ER-negative breast cancer accounts for approximately 20% of all newly diagnosed breast malignancies; these are ER-β-, PR- and HER-2-negative (Rakha et al., 2009; Reis-Filho et al., 2008). MCF-7 is known to express both ER subtypes (ER-α, ER-β), PR and HER-2 (Brooks et al., 1973; Brandes and Hermonat, 1983). In contrast, BT20 has been established as being receptor-negative with respect to ER-α , ER-ß, PR and HER-2 (Castles et
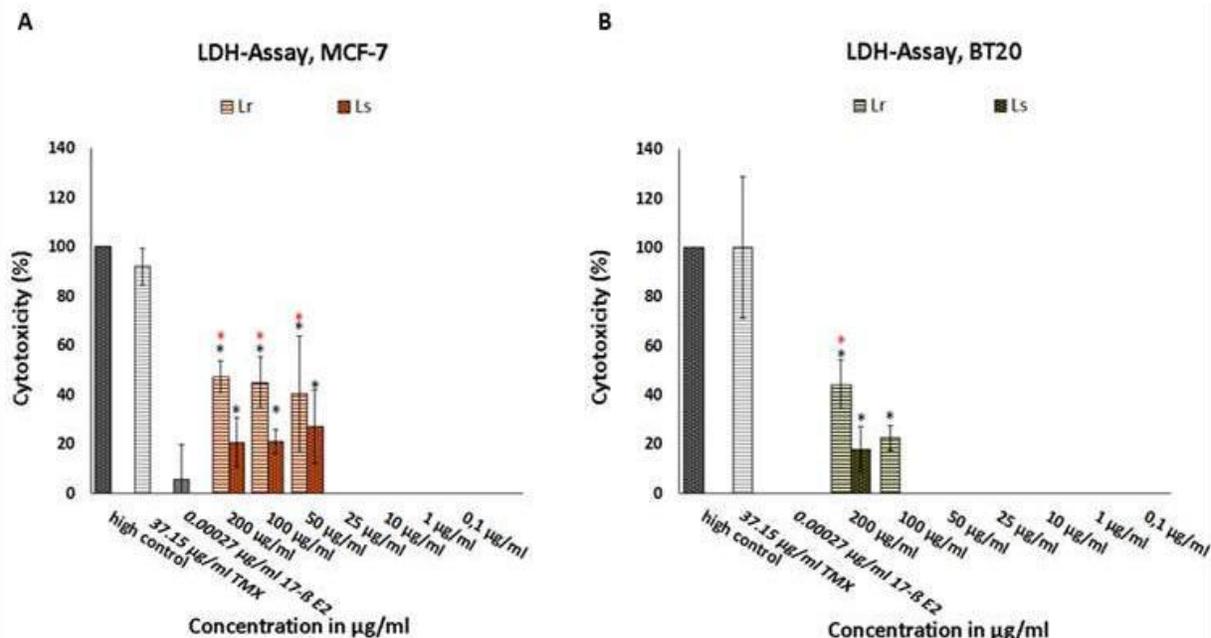
Figure 3. The effect of *Lupinus angustifolius* (Boregine) root and shoot extracts on cytotoxicity (%). MCF-7 (A) and BT20 (B) cells were incubated for 24 h with root and shoot extracts (200-0.1 µg/ml). Results are expressed as the mean ± SEM of 3 independent experiments, four-fold for each experimental point. Analysis by t-test (*) and one-way analysis of variance (*) was used to compare cell proliferation in MCF-7 and BT20 cells cultured with various concentrations of lupin extracts with controls. P < 0.01 was regarded as statistically significant. Lr = lupin root extract, Ls = lupin shoot extract, TMX = Tamoxifen, 17-β E2 = 17-β estradiol

al., 1993; Grigoriadis et al., 2012). The results of the present study have revealed that natural *L. angustifolius* extract might constitute a potential anti-tumour compound against receptor-positive and receptor-negative breast cancer. Blue lupin root and shoot extracts inhibit cell proliferation and exhibit cytotoxicity in MCF-7 and BT20 breast cancer cell lines in a concentration-dependent manner (Figures 2, 3A and B). The strongest inhibition of cell proliferation is shown by the blue lupin root and the blue lupin shoot extracts in receptor-negative BT20 cells.

The blue lupin root extract shows strong cytotoxicity in both tumour cell lines at high concentrations. Whether the measured cytotoxicity is induced by apoptosis or necrosis cannot be distinguished by the LDH assay, because this assay is based on the measurement of cytoplasmic enzyme activity released by damaged cells (Roche, 2013). LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity resulting from either apoptosis or necrosis (Caymanchem, 2013). Furthermore, whether lupin root extract reduces the cell proliferation of MCF-7 and BT20 cells by necrosis or apoptosis also cannot be determined by the LDH assay. MCF-7 and BT20 cells were incubated for 24 h with blue lupin root and shoot extracts, and the effects on both cell proliferation and cytotoxicity were studied. Compared with the inhibition of cell proliferation, blue lupin root and shoot extracts had a low significant effect with regard to cytotoxicity on MCF-7 and BT20

cells. Further study needs to be conducted with longer incubation time to examine cytotoxicity in these cells.

Tamoxifen, used as a positive control in the BrdU and LDH assays, is one of the most extensively used drugs for the treatment of breast cancer (Yang et al., 2013). It is a nonsteroidal agent (selective ER modulator) with potent anti-estrogenic properties and it competes with estrogen for binding sites in breast and other tissues (Abarzua et al., 2012). Tamoxifen is used as a hormonal therapy for patients who exhibit ER-positive breast cancer (Yang et al., 2013). However, such tamoxifen use might involve risks related to the endometrium and ovaries (Mofrad et al., 2010). In the work presented here, tamoxifen induces a strong inhibition of cell proliferation and strong cytotoxicity in MCF-7 and BT20 cells (Figures 2, 3A and B). The inhibitory effects of tamoxifen in the ER-positive and ER-negative cells supports numerous previous results showing that the greatest portion of the growth-inhibitory effect of tamoxifen *in vitro* is not ER-dependent (Zheng et al., 2007; Todorova et al., 2011). However, the addition of 17-β estradiol as a control at a concentration of 10^{-9} mol/l (0.00027 µg/ml) does not affect cell proliferation or show cytotoxicity in MCF-7 and B20 cells. However, further phytochemical studies are needed to determine the main anti-cancer molecule or molecules in these blue lupin roots and shoot extracts. Moreover, additional studies are necessary to elucidate the mechanisms that are responsible for the observed effects

Cell proliferation, IC$_{50}$-values
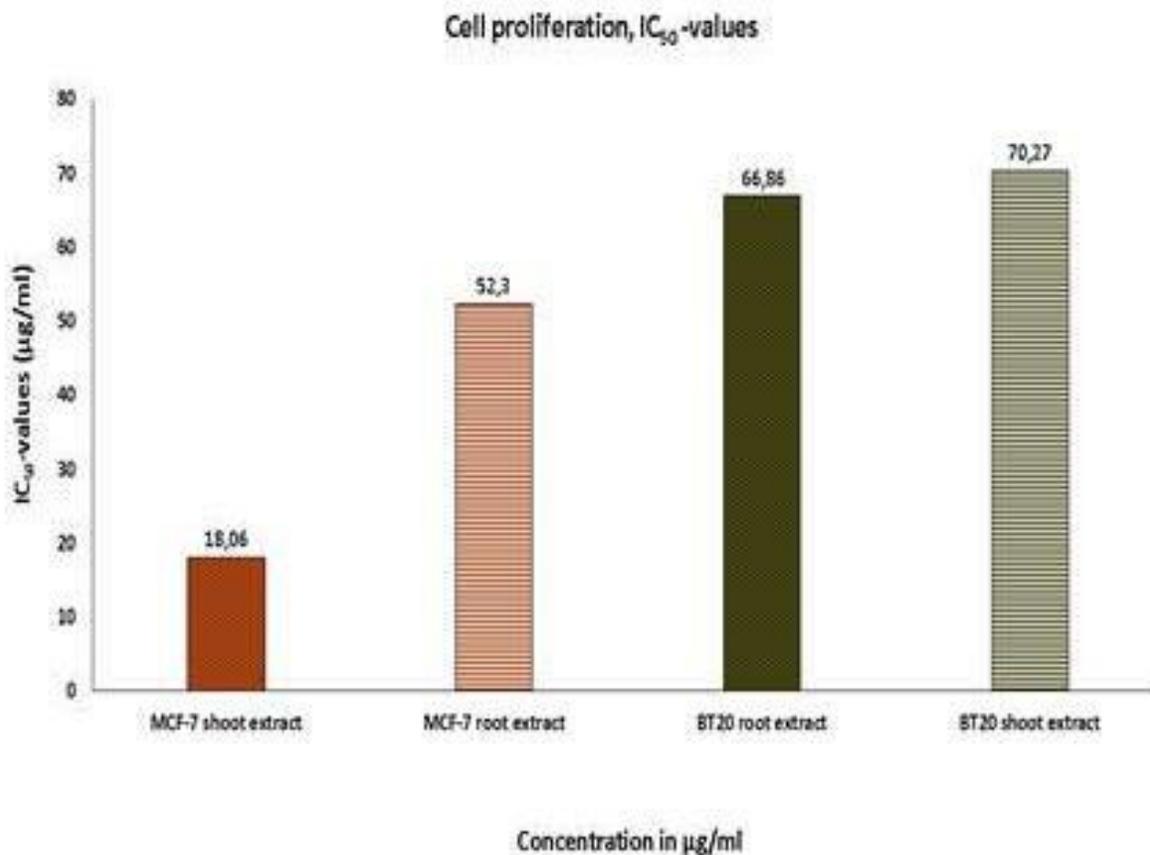
Figure 4. The IC$_{50}$-values of *Lupinus angustifolius* extracts. The IC$_{50}$-values show the half-maximal inhibitory concentration as calculated with Sigma Plot 11.0.

of the blue lupin extracts in breast cancer cells and benign cells and to determine the optimal dose for therapeutic use, possibly by using the xenograft mouse model.

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Conflicts of Interest

All authors report no conflicts of interest.

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