

## SUPPLEMENTARY MATERIAL

### Anticancer effect of ethanol *Lycium barbarum* (Goji berry) extract on human breast cancer T47D cell line

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The anticancer activity of ethanol extract isolated from Goji berry (EEGB) on T47D human breast cancer cell line has been reported. Cell viability and cell proliferation were examined with the use of BrdU, MTT and NR methods. Induction of apoptosis was assessed by propidium iodide and Hoechst 33342 staining. Expression of genes involved in cell proliferation, apoptosis, cell cycle control and regulation of transcription was estimated using Western blotting analysis. EEGB inhibited the proliferation of breast cancer cells in a time-, and dose-dependent manner. The study confirmed the lack of EEGB cytotoxic activity to normal human skin fibroblasts. Western blot analysis demonstrated an increase of pro-apoptotic, and decrease of anti-apoptotic proteins' expression in cells treated with the extract. Anticancer activity, and lack of toxicity against normal cells indicate a chemopreventive potential of Goji berries in breast cancer treatment.

Keywords: Goji berry; apoptosis; T47D cells; breast cancer

## 1. Experimental

### 1.1. Reagents

The ethanol extract from Goji berries (EEGB) (*Lycium barbarum*, Solanaceae) was prepared at the Department of Virology and Immunology at Maria Curie-Skłodowska University in Lublin. Fruit of Goji berries (RADIX-BIS) were dried at room temperature and mechanically shredded. A mixture of 25g of dry weight and 150 ml of

96% ethanol was shaken for one week. The solution was then filtered and the ethanolic filtrate was vaporized in a rotary vacuum. The residue was again fixed with 150 ml of 96% ethanol, vortexed for seven days, and then the distillate was evaporated. The extract was dissolved in a culture medium immediately before use. The stock solution was 10 mg/ml in free-serum DMEM/F12 Ham (1:1) culture medium. The primary antibodies against  $\beta$ -actin, Bax, BclxL, p27, p21, p53 cyclin D1, Cdk6, I $\kappa$ B- $\alpha$ , NF $\kappa$ B were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The secondary antibodies were obtained from Amersham Pharmacia Biosciences (Buckinghamshire, UK).

### ***1.2. Cells***

Human breast carcinoma cell line T47D was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Human skin fibroblasts (HSF) were obtained by the outgrowth technique from skin explants from young volunteers. The cells were cultured in DMEM/F12 Ham (1:1) (T47D) or DMEM/RPMI (1:1) (HSF) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin (Sigma-Aldrich, St Louis, USA) at 37°C in a humidified atmosphere of 95% air, and 5% CO<sub>2</sub>.

### ***1.3. Cell proliferation (MTT assay)***

The metabolic activity of living cells was assessed by the activity of dehydrogenases measurement. T47D and HSF cells were plated on flat-bottom 96-well microplates at a density of  $3 \times 10^4$  cells/well in 100  $\mu$ l of a complete growth medium. After 24 hours incubation, T47D and HSF cells were exposed to the EEGB at a concentration ranging from 0.001 to 1 mg/ml. After 24, 48, and 96 hours incubation the antiproliferative effect of the extract was estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay as described previously (Rzeski et al. 2009). The product of reaction was quantified by measuring absorbance at 570 nm wavelength using Emax Microplate Reader (Menlo Park, CA, USA).

### ***1.4. Bromodeoxyuridine (BrdU) cell proliferation assay***

DNA synthesis in proliferating cells was evaluated by measuring bromodeoxyuridine (BrdU) incorporation using a commercial Cell Proliferation ELISA System (Roche Molecular Biochemicals, Mannheim, Germany). The T47D cells were seeded into 96-

well microplates at a density of  $3 \times 10^4$  cells/ml. After 24-h incubation, the cells were exposed to 0.001-1 mg/ml of the EEGB for 48 hours, followed by 2 hours incubation with BrdU according to the manufacturer's protocol. The absorbance values were measured at 450 nm using an Emax Microplate Reader.

### ***1.5. Neutral Red (NR) cell viability assay***

Neutral red assay determines the accumulation of neutral red dye in the lysosomes of viable, uninjured cells. The T47D cells were plated on 96-well microplates at a density of  $3 \times 10^4$  cells/well in a complete growth medium. After 24 hours incubation the growth medium was replaced with a fresh medium (DMEM/F-12Ham+ 2% FBS), and the cells were exposed to 0.001-1 mg/ml of the EEGB for 24 hours. The cells were then washed with pre-warmed PBS, followed by incubation with the NR reagent for 3 hours, fixed with NR fixative solution (1% CaCl<sub>2</sub> in 0.5% formalin) for 3 min in room temperature (RT), and solubilized in 1% acetic acid in 50% ethanol under shaking for 20 min in RT. Absorbance was measured at 550 nm using an Emax Microplate Reader.

### ***1.6. Propidium iodide and Hoechst solution staining***

The staining mixture of propidium iodide (PI) (Sigma) and Hoechst 33342 (Sigma), while PI stains cells with damaged cytoplasmic membrane (necrosis), Hoechst 33342 stains more brightly the condensed chromatin of apoptotic cells than the non-apoptotic cells. Necrotic cells are stained in red, and late apoptotic cells in bright blue. T47D cells with a density of  $1 \times 10^5$  cells/ml were exposed to 0.1, 0.5, 1 mg/ml of the EEGB for 24 h (37°C; 5% CO<sub>2</sub>). Next, the medium was removed, and the cells were washed with 0.5 ml of PBS, followed by incubation with staining solution [propidium iodide (0.15 mg/ml), and Hoechst 33342 (0.24 mg/ml)] in darkness for 5 minutes. Percentage of apoptotic cells was assessed with Confocal Microscopy using a Nikon Elipse Ti microscope and the NIS-Elements software (Nikon).

### ***1.7. Western blotting analyses***

The T47D cells were seeded on 6-well plates at  $2 \times 10^5$  cells/well, treated with the EEGB at the concentrations of 0.05, 0.1, 0.5, 1 or 2 mg/ml for 48 hours, and lysed in RIPA buffer (Sigma) on ice for 60 min. Next, the lysates were centrifuged at  $1.400 \times g$  for 10 min at 4°C. The protein concentration in supernatant was quantified using a BCA

protein assay kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). The supernatants of RIPA cell lysates were mixed with Laemmli buffer (40% glycerol, 0.24M Tris-HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) and boiled for 5 min. Equal amounts of protein extract were loaded on 10% polyacrylamide gel (SDS-PAGE). The separated proteins were transferred onto PVDF membranes (Millipore Corporation), blocked with (5% nonfat dried milk in TBST (TBS/0.1% Tween 20) for 1 hour at RT, then probed with dilutions of primary antibodies according to the manufacturer specifications [ $\beta$ -actin, cyclin D1, Cdk6 (mouse primary antibodies); Bax, BclxL, p27, p21, p53, I $\kappa$ B $\alpha$ , NF $\kappa$ B (rabbits primary antibodies)] overnight at 4°C. The membranes were then washed 3 times for 5 minutes with TBST, incubated with dilutions of respective secondary antibodies (1:2000 in 1% BSA/TBS/0.1% Tween 20, Amersham Pharmacia Biosciences, Buckinghamshire, UK) coupled with horseradish peroxidase for 1 hour at RT, and visualized with ECM Western Blot Chemiluminescence Reagent (Amersham Biosciences, Germany) using a Kodak Biomax film (Sigma). The blots were stripped according to the manufacturer's protocol (Stripping Buffer, Thermo Scientific, Waltham, MA, USA), and reprobed with antibodies against  $\beta$ -actin to ensure equal loading and transfer of proteins.

### ***1.8. Statistical Analysis***

The data were expressed as the means  $\pm$  standard deviation. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). The data were analyzed by one-way ANOVA test, Tukey's Multiple Comparison Post-test; \*p values <0.05, \*\*p values <0.01, \*\*\*p values <0.001, were considered to be significant.

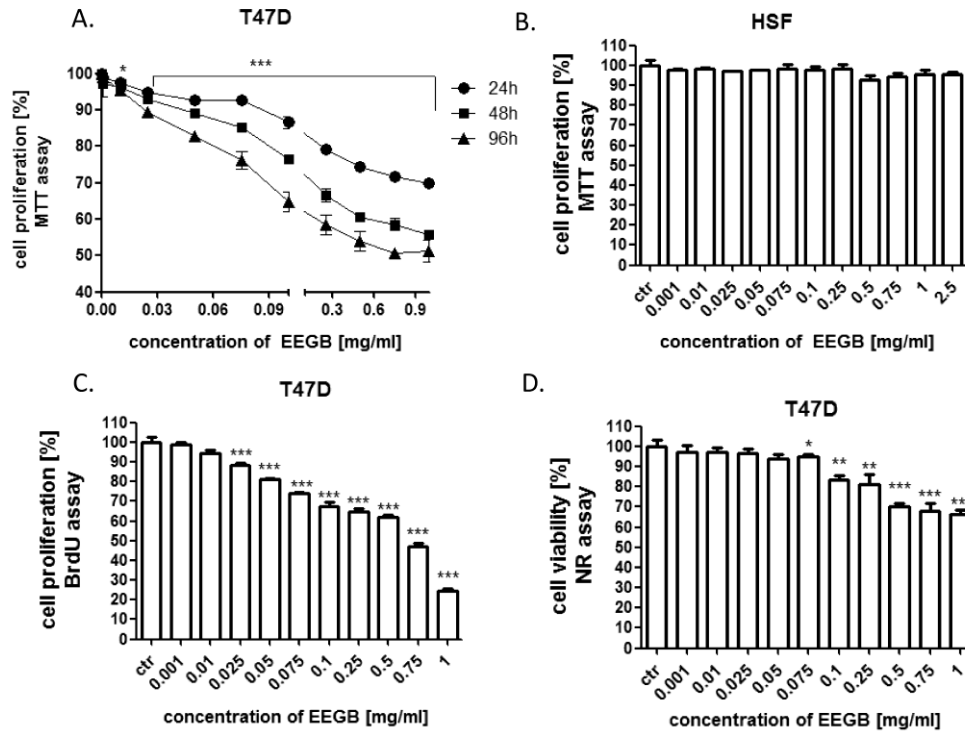


Figure S1. The influence of EEGB on the proliferation of T47D human breast carcinoma cells and HSF human skin fibroblast. The T47D cells were treated with the extract at various concentrations for 24, 48, and 96 hours by means of MTT (A), BrdU (C) and NR (D) assays. The HSF cells were treated with the extract at various concentrations for 96h using MTT assay (B). The proliferation of untreated control cell (ctr) was calculated as 100%. The results represent the mean  $\pm$  SD, and were analyzed by one-way ANOVA test, Tukey's Multiple Comparison Post-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  were considered as statistically significant), 2 independent experiment.

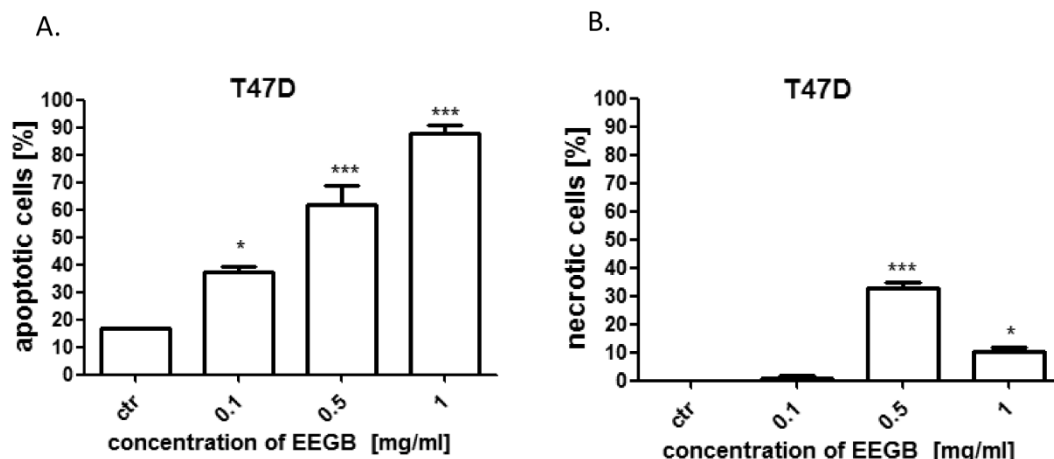


Figure S2. EEGB induces apoptosis (A) and necrosis (B) in T47D human breast carcinoma cells. The cancer cells exposed to the EEGB at concentrations 0.1, 0.5, and 1 mg/ml for 24h. The results represent the mean  $\pm$  SD, and were analyzed by one-way ANOVA test, Tukey's Multiple Comparison Post-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) were considered as statistically significant; 2 separate experiments.

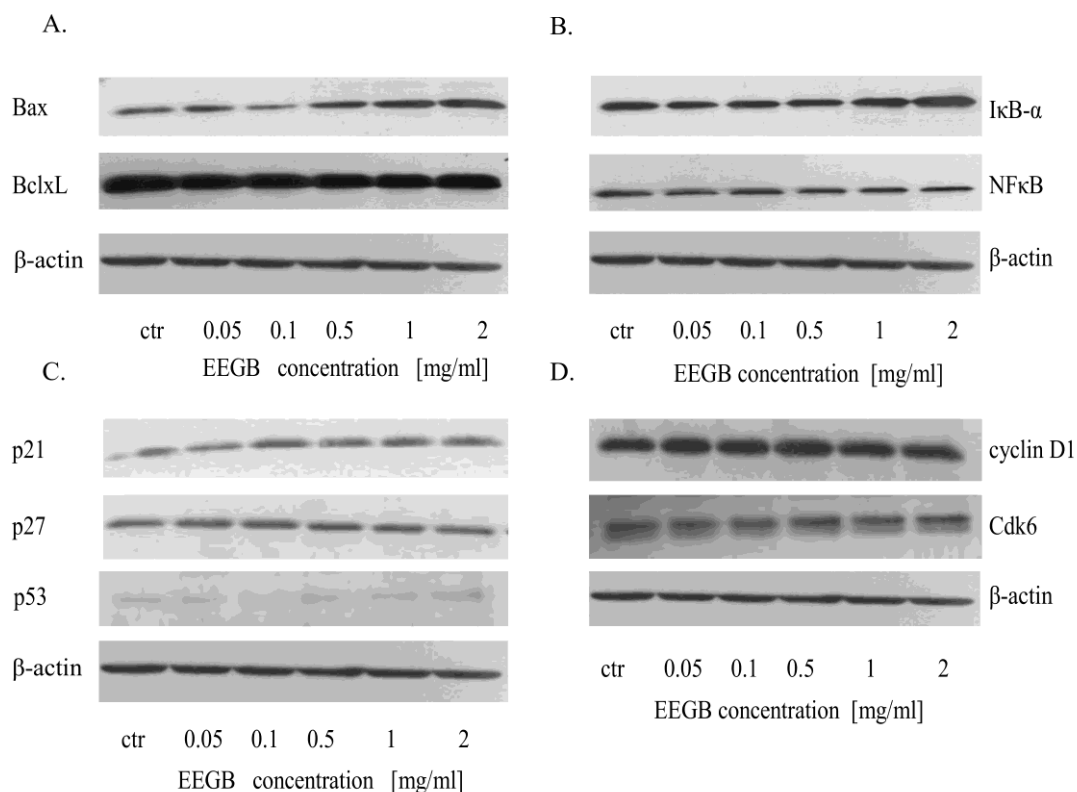


Figure S3. EEGB influences expression of Bax, BclxL (A), I $\kappa$ B- $\alpha$ , NF $\kappa$ B (B), p21, p27, p53 (C), cyclin D1, and Cdk6 (D) proteins. The T47D cells were incubated in the

culture media alone (ctr) or with indicated concentrations of the EEBG for 48h. Extracted proteins were electrophoresed, transferred onto PVDF membranes and probed with respective antibodies. Representative blots are shown.