INFLUENCE OF POLYPHENOL EXTRACT FROM EVENING PRIMROSE (OENOThERA PARADOXA) SEEDS ON PROLIFERATION Of CACO-2 CELLS AND ON EXPRESSION, SYNTHESIS AND ACTIVITY OF MATRIX METALLOPROTEINASES AND THEIR INHIBITORS

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INTRODUCTION

Currently, cancer is the most frequent disease occurring in the modern world. Despite the progress in cancer research and treatment, as well as a huge amount of money given to find new, more effective drugs, it still remains a major worldwide health problem. Global statistics show that colorectal cancer ranked third place in males as the most commonly diagnosed cancer whereas in females ranked second [Jemal et al., 2011]. Moreover, in Eastern Europe the incidence rate of colorectal cancer is still increasing [Center et al., 2009].

Matrix metalloproteinases (MMPs) are endopeptidases containing Zn²⁺ in an active center and requiring Ca²⁺ for catalysis. On the basis of substrate specificity and domain organization MMPs are classified into: collagenases (MMP-1), gelatinases (also known as type IV collagenases: MMP-2, MMP-9),stromelysins (MMP-3), matrilysin (MMP-7) and membrane-type MMPs (MMP-14). Collectively they are able to degrade all protein components of the extracellular matrix (ECM) and some non-ECM proteins [Ra & Parks, 2007]. Under physiological conditions, the activities of MMPs are precisely regulated; first of all, at the transcription level, by activation of the zymogens and by their natural inhibitors (tissue inhibitors of metalloproteinases; TIMPs). Except for neutrophils, cells do not accumulate MMPs. After synthesis, MMPs are secreted into the ECM as a latent form that can be activated by either proteolytic cleavage or by conformational changes induced, among others, by reactive oxygen species (ROS) [Hadler-Olsen et al., 2013]. MMPs play a key role in physiological processes such as tissue repair and morphogenesis, as well as in many pathological processes including tumorgenesis, angiogenesis, metastasis [Kupai et al., 2010]. These enzymes are produced by fibroblasts, monocytes, lymphocytes, neutrophils, endothelial cells as well as cancer cells. The cancer cell lines are the simplified models of tumor. In vivo cancer cells might be in contact with interleukins, growth factors, and cytokines, which induce MMP synthesis [Noel et al., 2008]. Transcription factors NF-κB and activator protein-1 (AP-1) are involved in the MAPK-mediated MMP expression in response to pro-inflammatory cytokine TNF-α. AP-1 can also be induced by phorbol-ester-12-O-tetradecanoylphorbol-13-acetate (TPA) [Labrie & St-Pierre, 2013]. Strategies for the prevention of proteolytic matrix degradation have mainly focused on MMPs. Synthetic MMP inhibitors such as bati...
mastat and marimastat, were developed and tested in clinical trials, first and foremost to treat cancer. However, the results from these trials in cancer patients were disappointing [Gialeli et al., 2011].

As far as we know, highly effective anticancer drugs that could effectively inhibit MMP activities with minor side effects have not been found to date. Natural plant polyphenols are quite an interesting group of compounds that might have such properties.

In the past decade an increased interest in polyphenolic compounds occurring in our diet has been observed. Polyphenols are plant secondary metabolites widespread in the plant kingdom. They have many beneficial properties proved in in vitro and in vivo studies, namely: antibacterial [Daglia et al., 2007], antifungal [Báidez et al., 2006], neuroprotective [Kumar et al., 2007] and also anticancer, in particular: antioxidant [Robaszkiewicz et al., 2007], antimitastatic [Ogasawara et al., 2007], proapoptotic [Mertens-Talcott & Percival, 2005], antiproliferative [Letchoyoun et al., 2007], antiangiogenic [Lamy et al., 2006], anti-inflammatory [Clavin et al., 2007]. More precisely, they can influence anti-apoptotic proteins (e.g. Bcl-2, Bcl-X1), proapoptotic proteins (e.g. caspases), cell cycle proteins (cyclins, cyclin-dependent kinases), protein kinases (e.g. IKK, EGFR, MAPK), cell adhesion molecules, growth factor signaling pathways and transcription factors (e.g. AP-1, STAT3) [extensively reviewed by González-Vallinas et al., 2013]. The best-known polyphenols having well-documented anticancer activities are: epigallocatechin gallate (EGCG), resveratrol, isoflavons and derivatives of catechin. A number of reports suggest that these types of compounds not only may inhibit inflammation, which can lead to hyperproliferation and carcinogenesis, but also can inhibit angiogenesis and metastasis [González-Vallinas et al., 2013].

Many herbal plants are under investigation for their potential as chemopreventive agents. In our studies we decided to examine a less known plant having health benefits. Evening primrose (Oenothera paradoxa Hudziok) is a weed originated from North America. In Europe it is cultivated because its seeds contain a high amount of polyunsaturated fatty acids, particularly γ-linolenic acid (GLA) [Peiretti et al., 2004], which might be used as an alternative treatment of hypercholesterolemia or rheumatoid arthritis [Jaszewska et al., 2010]. After extraction of oil the remaining defatted seeds contain polyphenolic compounds that can have beneficial health effect. So far, the extracts obtained from defatted seeds from Oenothera paradoxa Hudziok caused a decrease of plasma cholesterol and low density lipoprotein cholesterol in rats [Balasinska, 1998], a dose-dependent inhibition of lipid peroxidation in mouse lymphocytic leukaemic L1210 cells [Balasinska & Trozynska, 1998] a dose-dependent inhibition of metalloproteinases activity [Kiss et al., 2008], induction of apoptosis in human skin melanoma HTB-140 cells [Jaszewska et al., 2009] and reduction of cell viability and cell invasiveness in human breast cancer MDA-MB-231 cells [Lewandowska et al., 2013].

We examined the influence of a polyphenol extract from defatted seeds of evening primrose (Oenothera paradoxa) on proliferation of human colorectal adenocarcinoma cell line (Caco-2) as well as their influence on the expression of several MMPs (especially MMP-9), their inhibitors (TIMP-1 and TIMP-2), and enzymatic activity of type IV collagenases (MMP-2 and MMP-9) synthesized by human peripheral blood mononuclear (PBMC) cells.

**MATERIALS AND METHODS**

**Plant materials**

Evening primrose (Oenothera paradoxa Hudziok) defatted seeds were obtained from Agropharm S.A./Adamed Group pharmaceutical company (Tuszyń, Poland).

**Chemicals**

All the reagents, cell culture medium and its supplements were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO), except for penicillin, streptomycin – Polfa Tarchomin (Tarchomin, Poland) and amphotericin B – Biochrom AG (Berlin, Germany). TRizol Reagent, oligo (dT) 12-18, random hexamer and SuperScript™ II Reverse Transcriptase were obtained from Life Technologies Corp. (Eugene, OR). Reagents for PCR reaction were bought from Finnzymes OY (Espoo, Finland). Cryptochlorogenic acid, procoynadin B1, procoynadin B2 and procoynadin C1 were purchased from PhytoLab GmbH & Co.KG (Westensberg, Germany). HPLC grade acetonitrile was purchased from J.T. Baker (Griesheim, Germany).

**Polyphenol extract preparation**

Evening primrose dry polyphenol extract was obtained according to the method described by Gorlach et al. [2011]. Briefly, the waste defatted seeds obtained from Agropharm S.A./Adamed Group pharmaceutical company were milled and defatted with hexane.

Then the seeds underwent triple extraction with 70% aqueous solution of ethanol. The obtained extracts were centrifuged (4000 rpm, 15 min) and then concentrated under vacuum at the temperature below 40°C. The obtained aqueous solution of the polyphenols was lyophilized. The dry extract was stored at −20°C prior to further analyses.

**Characterization of the polyphenol extract**

The extract was characterized in terms of total polyphenol content by the Folin-Ciocalteau procedure, expressed as (+) catechin equivalents [Peri & Pompei, 1971], total flavan content by the vanillin procedure, expressed as (+)catechin equivalents [Swain & Hillis, 1959], and total proanthocyanin content after acid hydrolysis in butanol environment, expressed as cyanidin [Rösch et al., 2003]. Ellagittannins and gallotannins (after acid hydrolysis in methanol environment, 20 h, 85°C) were determined by an analytical reversed-phase HPLC system with the use of a Eurospher-100 C18 column (250 mm x 4.6 mm, 5 mm) (Knauer, Berlin, Germany). A binary mobile phase and a gradient program were the same as described below. Ellagitannin content determined at 254 nm is expressed as ellagic acid and gallotannin content at 280 nm is expressed as methyl gallate [Hartfeld et al., 2002].

**Phenolics determination by HPLC-DAD**

Phenolic profile was determined using an analytical reversed-phase HPLC system (Waters, Milford, MA) with
a 2707 autosampler and a 1525 binary HPLC pump coupled to a 996 photodiode array detector (2998), controlled by Waters Breeze 2 software (Waters). A SYMMETRY C18 column (250 mm x 4.6 mm, 5 mm) (Waters) was used. According to Dyrbj et al. [2001], the binary mobile phase consisted of water and formic acid in the ratio of 90:10 (v/v), respectively (solvent A); water, acetonitrile and formic acid in the ratio of 49:50:10 (v/v/v), respectively (solvent B). The phenolic separation was performed using the following gradient program with a flow rate of 1 mL/min: 0 min, 88% A + 12% B; 26 min, 70% A + 30% B; 40–43 min, 0% A + 100% B; 48–50 min, 88% A + 12% B. Detection was performed by scanning from 200 to 550 nm. Peak identification was carried out by comparison of retention times and diode array spectral characteristics with the standards.

Cell culture

A human colorectal adenocarcinoma cell line (Caco-2) was purchased from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland). Caco-2 cells were cultured in MegaCell™ MEM culture medium supplemented with 3% heat-inactivated fetal bovine serum (FBS), 4 mM/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 1.25 μg/mL amphotericin B at 37°C in a humidified atmosphere containing 5% CO2. All experiments were carried out between passages 3 and 9. The cells were seeded on 96-well plates. After 24 h, the cells were exposed to the polyphenol extract from evening primrose or EGCG or gallic acid for the following 24, 48 and 72 h. Next, the culture medium was removed and the cells were fixed in situ with ice-cold 10% aqueous solution of trichloroacetic acid (TCA) for 1 h at 4°C. After that, TCA was removed and each well was washed with deionized water and allowed to air-dry. The cells were stained for 30 min at room temperature with 0.4% SRB solution in 1% aqueous solution of acetic acid. The dye excess was removed and each well was washed with 1% acetic acid and allowed to air-dry. The protein-bound dye was solubilized by 10 mM/L unbuffered Tris on a shaker for 10 min at room temperature. The optical density (OD) was measured by a 96-well plate reader (iMark™, BioRad, Herkules, CA, USA) at the wavelength of 490 nm.

Gelatin zymography and quantitative analysis of MMP-9 secretion

Caco-2 cells were harvested, suspended in the growth medium mentioned above and seeded on 96-well plates. After 24 h, the cells were washed twice with PBS and then suspended in the medium without FBS. Subsequently, the cells were treated with 10 ng/mL tumor necrosis factor α (TNF-α), 10 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA), the polyphenol extract from evening primrose or gallic acid or EGCG and incubated for 24 and 48 h. MMP-9 is an inducible enzyme, therefore TNF-α and TPA were added in order to stimulate Caco-2 for its synthesis. Afterwards, conditioned media were harvested and stored at −20°C for further analysis. MMP-9 secretion was determined by means of zymographic analysis. Briefly, the same volumes of the conditioned media (usually 20 μL) were mixed with a sample buffer containing 4% SDS (sodium dodecyl sulfate), 30% glycerol, 0.25 mol/L Tris-HCl (pH 6.8) and 0.01% bromophenol blue. Electrophoresis was carried out in 10% polyacrylamide gel containing 1 mg/mL gelatin. After the electrophoresis, SDS was removed from the gels by incubation in a buffer containing 2% Triton X-100 and 50 mM/L Tris-HCl (pH 7.4) and the enzyme reaction was allowed to proceed at 37°C for 21 h in a buffer containing 1% Triton X-100, 50 mM/L Tris-HCl (pH 7.4) and 5 mM/L CaCl2. Next, the gels were stained for 1 h in a solution containing 0.1% amido black, 7% acetic acid and 20% ethanol. After the staining procedure, the gels were captured using an Olympus camera (Olympus Corp., Tokyo, Japan). MMP-9 was visualized as a dark blue background of the amido black-stained gels. Densitometry analysis was carried out using Quantity One® 4.4 software (BioRad, Herkules, CA).

Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

The cells were harvested, suspended in the growth medium mentioned above and seeded on culture dishes. After 24 h, the cells were washed twice with PBS and then suspend-
ed in the medium without FBS. Subsequently, the cells were exposed to 10 ng/mL TNF-α, 10 ng/mL TPA and the evening primrose polyphenol extract for 16 h. After incubation, the cells were lysed by the TRIZol reagent (1 mL per 10^6 cells). Further, RNA isolation was performed according to the manufacturer’s protocol. The obtained RNA pellet was allowed to air-dry and then was redissolved in RNase-free water. Total RNA content was quantified using a Beckman DU650 spectrophotometer (Beckman Coulter, Inc., Brea, CA) at 260 nm. The samples were stored at –80°C for further analysis.

cDNA was synthesized using total RNA template, oligo (dT)_{18} random hexamer and SuperScript™ II Reverse Transcriptase (RT). The reaction was carried out according to the manufacturer’s protocol enclosed with SuperScript™ II Reverse Transcriptase. Then the samples were stored at –80°C for further analysis.

The cDNA amplification was carried out in 50 μL volume. The reaction mixture consisted of: 5 μL cDNA template, DyNAzyme EXT buffer (50 mmol/L Tris-HCl, pH 9.0, 1.5 mmol/L MgCl₂, 15 mmol/L (NH₄)₂SO₄, 0.1 % Triton X-100), 10 μmol/L dNTPs, 1 U DyNAzyme™ EXT DNA Polymerase and 10 pmol forward and reverse primers (presented in Table 1). The reaction was submitted to 35 cycles of denaturation (94°C for 30 sec), annealing (for 30 sec at temperatures given in Table 1) and extension (72°C for 60 sec). The amplified products were detected in 2% agarose gel containing 0.5 μg/mL ethidium bromide, documented by VersaDoc™ Imaging System (BioRad, Hercules, CA) and analyzed using Quantity One® 4.4 software (BioRad). The amount of RT-PCR product for the gene of interest was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample.

**Statistical analysis**

Statistical comparisons were made using nonparametric Kruskal-Wallis test and one-way ANOVA followed by Bonferroni test (Analyze-it software, v. 2.21, Leeds, UK). Data were presented as mean ± SEM. A p value of less than 0.05 was considered to be significant.

**RESULTS**

**Characterization of the polyphenol extract from evening primrose seeds**

The examined extract was isolated according to the procedure presented above. The characterization of the preparation is presented in Table 2. HPLC analysis revealed that the evening primrose polyphenol extract contained among others: gallic acid, procyanidin B1, procyanidin B2, (+)catechin, procyanidin C1, (+)-epicatechin and ellagic acid.

**Isolation and culture of human peripheral blood mononuclear cells (PBMC), and zymographic detection of PBMC-derived type IV collagenases after incubation with the evening primrose polyphenol extract**

Peripheral blood collected from a healthy volunteer donor was diluted with PBS in the ratio of 1:1 and layered on Histopaque-1077. After centrifugation at 400×g for 30 min at room temperature, the layer of mononuclear cells was collected, and the cells were washed twice with PBS and suspended in RPMI 1640 culture medium containing 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL fungizone and 10% FBS. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ at the initial concentration of 1×10⁶ per 1 mL. PBMC-conditioned medium was used as a source of MMP-2 and MMP-9. Zymographic detection of PBMC-derived type IV collagenases after incubation with the evening primrose seed extract was carried out according to a procedure described previously [Stręgów et al., 2007]. For the zymographic assay, the extract was dissolved in 70% ethanol aqueous solution and further diluted with the incubation buffer so that the final concentration of ethanol was <0.01% (v/v).

**Influence of the polyphenol preparation on Caco-2 proliferation (CVS, SRB assay)**

The influence of the polyphenol extract from evening primrose on the proliferation of Caco-2 was evaluated color-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5’→3’</th>
<th>Annealing temperature</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>L: AGCTCAGGATGACATTGATGG</td>
<td>50°C</td>
<td>198 bp</td>
</tr>
<tr>
<td></td>
<td>R: AGCTCACTTCCGGGTTAGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>L: TCAGGAAAGTTGATGCGGGAAC</td>
<td>50°C</td>
<td>471 bp</td>
</tr>
<tr>
<td></td>
<td>R: GCTAAATGGCGAAGGAGGACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>L: ACTTTGACGACGACAAGAAGTG</td>
<td>49°C</td>
<td>151 bp</td>
</tr>
<tr>
<td></td>
<td>R: CCCTCAGTGAGCGGATACATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>L: CATCCAGAAGAGGACCATC</td>
<td>54°C</td>
<td>150 bp</td>
</tr>
<tr>
<td></td>
<td>R: GAGAGAAGAGTGCCAAATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>L: AGCCAACGTTGAAGTCTTGG</td>
<td>49°C</td>
<td>157 bp</td>
</tr>
<tr>
<td></td>
<td>R: TACTTCCACAGTCCACAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-2</td>
<td>L: TTCTCTGTGACCAGTCCTCAC</td>
<td>49°C</td>
<td>170 bp</td>
</tr>
<tr>
<td></td>
<td>R: ACCCTCTGTGCATTCATCGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>L: TGATGACATCAAGAAGGTGGTGGAAG</td>
<td>54°C</td>
<td>240 bp</td>
</tr>
<tr>
<td></td>
<td>R: TCTTTGAGGCCATGTGGGCCAT</td>
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</tr>
</tbody>
</table>
TABLE 2. Characterization of the polyphenol extract from defatted evening primrose seeds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (mg/g of dry extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polyphenols</td>
<td>668.71±15.76</td>
</tr>
<tr>
<td>Total flavanols</td>
<td>190.02±5.32</td>
</tr>
<tr>
<td>Total proanthocyanidins</td>
<td>142.86±4.05</td>
</tr>
<tr>
<td>Total flavanols and HBA</td>
<td>n.a.</td>
</tr>
<tr>
<td>Total flavanols and HBA</td>
<td>228.05±4.65</td>
</tr>
<tr>
<td>(+)catechin</td>
<td>11.77±1.24</td>
</tr>
<tr>
<td>(-)-epicatechin</td>
<td>11.36±0.87</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>7.34±0.36</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>19.47±0.95</td>
</tr>
<tr>
<td>Procyanidin C1</td>
<td>7.84±1.24</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cryptochlorogenic acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Other HCA acids</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Quercetin 3-rhamnoside</td>
<td>n.d.</td>
</tr>
<tr>
<td>Other flavonols</td>
<td>0.78±0.05</td>
</tr>
<tr>
<td>Free ellagic acid</td>
<td>2.65±0.23</td>
</tr>
<tr>
<td>Free gallic acid</td>
<td>5.17±0.11</td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>4.12±0.19</td>
</tr>
<tr>
<td>Gallotannins</td>
<td>89.08±8.13</td>
</tr>
</tbody>
</table>

Mean ± SD, n ≥ 3. *determined by Folin-Ciocalteu reagent as (+) catechin equivalents. **determined by vanillin reagent as (+)catechin equivalents. *determined by acid hydrolysis as cyanidin equivalents. *determined by HPLC at 280 nm as (+)catechin. *determined by HPLC at 280 nm as (+)catechin. *determined by HPLC at 280 nm as acid gallic. *determined by HPLC at 320 nm as chlorogenic acid. *determined by HPLC at 360 nm as quercetin. *determined by HPLC at 254 nm by acid hydrolysis as ellagic acid. *determined by HPLC at 280 nm by acid hydrolysis as methyl gallate. HBA - hydroxybenzoic acids; HCA - hydroxycinnamic acids; n.d. – not detected; n.a. – not analyzed.

imetrically after staining the cells with crystal violet and sulforhodamine B. The cells were seeded on plates and exposed to polyphenols. After the exposure time, the number of cells was evaluated according to the protocols described above. The analysis revealed that 25 μmol/L GAE of the evening primrose polyphenol extract seemed to stimulate the proliferation of Caco-2 (especially after 24 and 48 h incubation) while this effect was not observed at higher concentrations (Figure 1A). However, after 72 h one can observe a decline in the cell number at 100 μmol/L GAE. IC₅₀ was not reached in the experiment. EGCG, the main green tea polyphenol with well-documented antiproliferative effect, at the concentration of 25 μmol/L stimulates the cell proliferation but higher concentrations (50, 75 and 100 μmol/L) suppress their division (Figure 1B). It is worth mentioning is the fact that gallic acid, one of the major constituents of polyphenol extract, stimulates Caco-2 proliferation at each concentration tested (Figure 1C). The lack of the antiproliferative effect of the polyphenol extract form evening primrose on Caco-2 might be caused by the high content of gallic acid in this preparation. The same tendency was observed when SRB was used for proliferation assay (data not shown).

Influence of the evening primrose polyphenol extract on expression of selected MMPs and their inhibitors

In the present work we assessed the influence of the polyphenol preparation from evening primrose on the expression of MMP-1, MMP-7, MMP-9, MMP-14, TIMP-1 and TIMP-2. In our experiment a low level of constitutive synthesis of MMP-9 was detectable in untreated Caco-2 cells but the expression of this gene can be stimulated with TNF-α and TPA (Figure 2). The expression of MMP-1 was not detected in cells not treated with TNF-α and TPA, but the addition of these two compounds induced the expression of this protease (Figure 3). In our experimental conditions, TNF-α and TPA stimulation of Caco-2 did not influence the expression levels of MMP-7, MMP-14, TIMP-1 and TIMP-2.

Our studies revealed that the polyphenol extract from evening primrose inhibited the expression of MMP-1, MMP-7, MMP-9 and MMP-14 in a dose-dependent manner. Among the MMPs investigated, the polyphenol extract seems to be the most potent inhibitor for MMP-7, MMP-9 and MMP-14.
The preparation did not significantly influence the expression of TIMP-1 and TIMP-2 (data not shown).

**Gelatin zymography and quantitative analysis of MMP-9 secretion**

In our study, MMP-9 expression was induced in Caco-2 cells by TNF-α and TPA as non-stimulated cells do not secrete this enzyme to the medium (Figure 4, lane 2). Therefore, to determine the influence of the polyphenol extract of defatted evening primrose seeds on MMP-9 synthesis and secretion, the cells were stimulated with inducers. Gelatin zymography can also be used to detect the presence of MMP-2 (the enzyme produced mainly by fibroblasts in tumor environment which is responsible for tissue remodelling and tumor progression). However, MMP-2 was not detected in Caco-2.

Caco-2 cells were incubated with the polyphenol preparation at different concentrations. After experiment, the conditioned media were collected and subjected to zymographic analysis. The results revealed that the polyphenol extract inhibited the synthesis and secretion of MMP-9 in a time- and dose-dependent manner (Figure 4A). The polyphenol extract was a more potent inhibitor of MMP-9 secretion than EGCG as it is presented in Figure 4C. One of the major constituents of the examined extract – gallic acid – did not influence the MMP-9 secretion (Figure 4C).

**Inhibition of MMP-2 and MMP-9 activities by the evening primrose polyphenol extract**

PBMC secrete both type IV collagenases in approximately equal amounts, as shown in Figure 5 and by Stręc et al. [2007]. We therefore decided to use PBMC-conditioned medium as a source of MMP-2 and MMP-9 for our study on the influence of the *O. paradoxa* defatted seed extract on the activities of those enzymes. The extract inhibited the activities of both type IV collagenases in a concentration-depen-

![FIGURE 2. The inhibitory effect of the polyphenol extract from evening primrose on the expression of MMP-9 determined by RT-PCR. The concentrations of the extract are expressed as gallic acid equivalents (GAE). Data represent the mean ± SEM of three independent experiments. Statistical significance of differences between means: *p < 0.05, **p < 0.01, ***p < 0.001 vs. control.](image)

![FIGURE 3. The influence of the examined polyphenol extract on the expression of: A) MMP-1, B) MMP-7, C) MMP-14 in Caco-2 cell line determined by RT-PCR. The concentrations of the extract are expressed as gallic acid equivalents (GAE). Data represent the mean ± SEM of three independent experiments. Statistical significance of differences between means: *p < 0.05, **p < 0.01, ***p < 0.001 vs. control.](image)
et al. [2011] using MALDI-TOF MS confirmed the presence of (+)catechin, (-)epicatechin, B-type dimeric procyanidin digallate and/or B-type dimer of (epi)catechin gallate, A-type procyanidin dimer, B-type procyanidin trimer, procyanidin tetramer and hydrolysable tannins (mono- and trigalloylglucose). Kiss et al. [2008] analyzed the composition of a polyphenol extract prepared from evening primrose defatted seeds originated from the same source as in our study (Agropharm S.A./Adamed Group, Tuszyn, Poland) and were also able to identify: (-)epicatechin gallate, procyanidin B3 (dimer), oenothein B (macrocyclic ellagitannin dimer) and penta-O-galloyl-β-D-glucose (PGG), which indicates that the polyphenol extract from evening primrose is a reservoir of versatile biological compounds.

The aim of our study was to evaluate the effect of the polyphenol extract from defatted seeds of evening primrose on human colorectal adenocarcinoma cell line (Caco-2).

We investigated the effect of the polyphenol extract on cell growth by means of crystal violet staining and sulforhodamine B assay. We intentionally did not use 3-(4,5-dimethyl-
thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in our experiments, as Bernhard et al. [2003] revealed that resveratrol increased MTT-reducing activity without an observed increase in cell number showing that this test is ineffective in the evaluation of cell proliferation after treatment with polyphenolic compounds. Therefore, to determine cell growth we have chosen such reagents that do not interfere with phenolic compounds. In our studies, the proliferation assays (CVS and SRB) revealed that the preparation from evening primrose exhibited a stimulating effect on Caco-2 proliferation at low concentrations whereas at higher concentrations it had rather no influence on cell division. Gallic acid, one of the components of this preparation, exhibited a similar effect on Caco-2 cells. Other studies showed that the influence of the polyphenol extract on proliferation of cancer cells might be a cell-specific phenomenon. For instance, Li et al. [2010] testing a polyphenol extract from Devil’s Club (Olopanax horridus) on different colorectal cancer cell lines observed that this preparation did not influence HT-29 proliferation at all whereas significantly reduced the growth of HCT-116 cell line. Moreover, in our studies, we observed a strong inhibition of MDA-MB-231 human breast cancer cell division by the polyphenol extract from evening primrose [Lewadowska et al., 2013].

It is well documented that MMPs, especially type IV collagenases, play a key role in the development and progression of cancer [Kessenbrock et al., 2010]. Therefore, we decided to investigate whether this preparation might influence the expression of matrix metalloproteinase 9. Augmented MMP-9 expression frequently correlates with cancer progression and metastasis [Kessenbrock et al., 2010], as the enzyme is able to degrade components of ECM, especially collagen type IV, the main component of the basement membranes. High expression of MMP-9 was observed in colorectal cancer cells as well as in stromal cells [Lubbe et al., 2006]. Moreover, in animal studies where human pancreatic cancer cells were implanted into nude mice, a rapid growth of tumor cells was observed in the mice expressing MMP-9 gene, whereas the animals lacking this gene produced significantly smaller tumors [Nakamura et al., 2007]. In our studies, we induced the expression of MMP-9 in Caco-2, as in this cancer cell line the MMP-9 expression was on the very low level. MMP-9 was both TNF-α- and TPA-induced, as applying only one of the stimuli was insufficient to obtain the satisfying results (data not shown). Probably, the up-regulation of MMP-9 expression in these cells requires binding of two activated transcription factors, namely AP-1 and NF-κB, to the transcription factor binding motifs in MMP-9 promoter region at the same time [Labrie & St-Pierre, 2013]. There is probably a synergistic effect of both transcription factors.

To evaluate the influence of the polyphenol extract on MMP-9 expression, the cells were seeded on culture plates and the extract at different concentrations was added simultaneously with TNF-α and TPA. After 16 h cells were lysed, and total RNA was extracted and used for RT-PCR assays. In parallel with this experiment the cells were cultured for 48 h and the conditioned media were subjected to zymographic analysis.

The results have shown that the polyphenol extract from evening primrose has a high potency to inhibit MMP-9 expression, as assessed by RT-PCR and gelatin zymography. Zymographic analysis revealed that the polyphenols from evening primrose were able to inhibit MMP-9 secretion even more effectively than EGCG. These data suggest that the preparation might significantly attenuate tumor progression by blocking MMP-9 synthesis. The main polyphenol from green tea, EGCG, likewise the polyphenols tested, was able to decrease MMP-9 expression in 95-D lung carcinoma cells at the concentration of 40 μmol/L [Yang et al., 2005], whereas in periodontal diseases it was effective even at the concentration of only 20 μmol/L [Yun et al., 2004].

Other matrix metalloproteinases, such as MMP-1 and MMP-7, similarly to MMP-9 exhibit proteolytic activity towards ECM, and these two enzymes also correlate with an aggressive phenotype of colorectal cancer [Hadler-Olsen et al., 2013]. Our study is the first report on the inhibition of MMP-1 expression by the evening primrose polyphenol extract in a colon cancer cell line. Similar effects were observed by Toegel et al. [2012], who examined the influence of a Caesalpinia sappan extract on human bone chondrosarcoma SW1353 cells. What is worth mentioning is that contrary to the results of Kim et al. [2007], who reported that EGCG stimulates the expression of MMP-7 in colorectal cancer cell line HT-29, our polyphenol extract proved to be an effective repressor of MMP-7 gene. The activity of MMP-7 is thought to promote tumor cell survival by shedding Fas receptor from the cell surface which is responsible for apoptosis of the cell [Li et al., 2006].

MMP-14 (membrane-type(MT)-1-MMP) is one of the six membrane-bound MMPs which also plays a significant role in tumor progression by activating other members of the MMP family participating in cancer development, especially MMP-2 [Poincloux et al., 2009]. In our studies, we observed a strong inhibition of MMP-14 expression by the polyphenol extract from evening primrose. These results indicate that the polyphenols tested effectively lowered MMP-14 mRNA level, in contrast with the results obtained by Huang et al. [2011a], for an extract from Phyllanthus urinaria which did not change the level of MMP-14 mRNA in human umbilical vein endothelial cells (HUVECs).

MMPs activity is regulated, among others, by the natural inhibitors – TIMPs (Tissue Inhibitors of Matrix Metalloproteinases). To date, four proteins belonging to this family (TIMP-1–4) have been described [Brew & Nagase, 2010]. The expression of tissue inhibitors of matrix metalloproteinases, namely TIMP-1 and TIMP-2, was unaffected by the examined extract (data not shown).

The evening primrose polyphenol extract tested in the present work not only reduced MMP-1, -7, -9 and -14 expression in Caco-2 cells (as discussed above), but also inhibited the activities of PBMC-derived MMP-2 and -9 in a concentration-dependent manner. There are literature data showing the inhibitory activity of evening primrose seed polyphenol-rich extracts obtained with the use of several solvents against two metallopeptidases, namely aminopeptidase N and neutral endopeptidase [Kiss et al., 2008, 2012]. The highest inhibition was observed for aminopeptidase N (IC₅₀ = 2.8 and 2.9 μg/mL for aqueous and 30% isopropanolic extracts, respectively) [Kiss et al., 2008]. However, to
the best of our knowledge, the present work is the first report on type IV collagenase activity inhibition by a polyphenol-rich extract derived from evening primrose seeds. The inhibition mechanism of MMPs activity is still unknown. Ellagic acid, also the constituent of investigated polyphenol extract, was reported as an MMP-2 inhibitor with zinc-chelating properties exerted in situ, i.e. in the active center [Huang et al., 2011b]. The authors suggested that a complex is formed in MMP-2 active center as a result of ellagic acid binding to the catalytic Zn-coordinated water molecule by its phenol group. According to Kolomecki [2000], blocking substrate cleavage by MMPs is one of the strategies of their inhibition. The inhibition of MMP activities observed on zymographic bands after their incubation with the evening primrose polyphenol extract may result from binding of high MW tannins to type IV collagens and, consequently, blocking the access of type IV collagenases to their substrate. The research conducted by Garbisa et al. [2001] investigating the inhibition of MMP-2 and -9 activities revealed that EGCG – a low molecular weight (MW) compound – exhibited a higher affinity towards type IV collagenases than to their substrate (gelatin). On the other hand, high MW tannins exhibited much stronger affinity to proteins having an open conformation (such as collagen and its denaturation product, gelatin) than to globular proteins [Naczk et al., 2001; Deaville et al., 2007].

CONCLUSION

To sum up, the polyphenol extract from defatted seeds of evening primrose might modulate MMP-9 expression and secretion in a dose- and time-dependent manner. The presented results also indicate that it influences the expression of other matrix metalloproteinases (MMP-1, MMP-7 and MMP-14) which are involved in cancer progression. Furthermore, the extract inhibited the activities of both type IV collagenases. Therefore, our preparation might significantly reduce the metastatic potential of colon cancer cells, however, further studies are needed.

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