JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2022, 73, 1, 41-52
www.jpp.krakow.pl | DOI: 10.26402/jpp.2022.1.04

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# CHEMOPREVENTIVE EFFECTS OF JAPANESE QUINCE (*CHAENOMELES JAPONICA* L.) PHENOL LEAF EXTRACT ON COLON CANCER CELLS THROUGH THE MODULATION OF EXTRACELLULAR SIGNAL-REGULATED KINASES/AKT SIGNALING PATHWAY

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Japanese quince leaf phenol-rich extract (PRE) represents a good source of phenolic compounds, among which chlorogenic acid and naringenin hexoside are the main constituents. The aim of this research was to evaluate the chemopreventive activity of PRE in human colon cancer (SW-480 and HT-29) and human normal colon cell line (CCD 841 CoN). All cell lines were exposed to different concentrations of the extract (150–500 μg/mL for SW-480 and CCD 841 CoN; and 250–750 μg/mL for HT-29) to investigate migration and invasion, as well as the activity and secretion of metalloproteinases (MMP-2 and MMP-9) involved in these mechanisms. Moreover, the influence of PRE on the activity of ERK and AKT pathways, which are strongly involved in colon cancer development (CRC), were measured. Our results demonstrated that PRE significantly inhibited migration and invasion in SW-480, HT-29 and CCD 841 CoN cells through MMP-2 and MMP-9-dependent mechanisms. We also proved that PRE can effectively down-regulate both the activity and protein expression of MMP-2 and MMP-9 in these cell lines. The exception was the higher concentration of PRE, which up-regulated the protein expression of MMP-9 in SW-480. Additionally, we showed that significant inhibition of p-ERK/p-AKT expression in SW-480 after treatment with PRE is involved in chemopreventive effects of this extract. In case of exposure of HT-29 cells to PRE, we observed a significant upregulation of p-ERK protein expression, and suppression of p-AKT mechanism. This research of Japanese quince phenol leaf extract suggests its application in colon cancer prevention and treatment due to its ability to inhibit migration and invasion in MMP-9 and MMP-2-dependent mechanisms *via* most likely the modulation of ERK and AKT signaling pathways in colon cancer cells. Overall, our results provide an experimental foundation for further research on its potential activities and effects *in vivo*.

Key words: *human colon cancer, Chaenomeles japonica, phenolic extract, colon cells lines, metalloproteinase-2, metalloproteinase-9, invasiveness, migration, polyphenols, chlorogenic acid, anti-cancer activity*

## INTRODUCTION

Colorectal cancer (CRC) is a serious problem on a global scale, being the second most common cancer in women, and the third frequently diagnosed in men (1). The worldwide mortality of CRC ranks second among cancers, and it is expected that by 2030 the number of cases and deaths will increase by 60% in developing countries (2). The major mechanism responsible for the high mortality of CRC is the tumor's ability to metastasize. Metastasis a cascading process that occurs when cancer cells inside the primary tumor start to invade the basal membrane and enter into the blood or lymph system. Once in the blood or lymph system the cancer cells spreads to other tissues and organs and form new tumors (3). An essential step that enables migration and invasion of cancer cells is the remodeling of the extracellular matrix (ECM) and its components through enzymes such as matrix metalloproteinases (MMPs). The MMPs make up a group of over 20 multifunctional endopeptidases, which are structurally related and $Ca^{2+}$ and $Zn^{2+}$ - dependent. In general, MMPs are classified into six groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other non-classified MMPs (4). The functionality of these endopeptidases is broad, as they are involved in physiological processes such as trophoblast implantation, embryogenesis, bone growth, angiogenesis, wound repair, and tissue regeneration. Some of MMPs can also contribute to harmful pathophysiological conditions, *inter alia* cancer development, influencing its initiation, progression, or metastasis (5, 6). Among multifunctional metalloproteinases, that are both characterized to be involved in physiological processes as well as cancer development, especially in CRC, are MMP-1, MMP-2, MMP-9, MMP-7, and MMP-12 (7, 8). Specifically, the up-regulated expression and activity of gelatinases (MMP-2 and MMP-9) is often correlated with cancers aggressiveness (9-11). Additionally, previous research has shown that both MMP-2 and MMP-9 are important in the process of CRC progression,

angiogenesis, and metastasis, and the high protein expression of these enzymes is associated with a poor survival prognosis for CRC patients (12, 13). Modulation of MMP-2 and MMP-9 expression, as well as regulation of related signaling pathways such as mitogen-activated protein kinases (MAPKs) signal transduction pathways with p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK), and nuclear factor-kappaB (NF-κB), can be a target in cancer treatment (9, 14, 15).

Nowadays, the application of plants in medicine is growing in popularity, which may be the result of awareness about the toxicity of conventional drugs used in anti-cancer therapies (16, 17). Despite the fact that chemotherapy brings therapeutic benefits, it is also accompanied by numerous severe side effects. Chemotherapeutic medications do not only affect cancer cells, but also other fast-growing cells, causing damages of blood-forming cells in bone marrow, cells occurring in digestive tract or reproductive system, and also hair follicles. Moreover, some of used chemotherapies can also cause damages of kidneys, lungs or nervous system. There serval major disadvantages that notably reduced the quality of life of patients, among them nausea, vomiting, diarrhea, fatigue, infections, anemia as well as problems with concentration or focus (18). The other thing is that anti-cancer therapies are often expensive, and what is worst they do not guarantee cancer remission (18, 19). Additionally, the Diet & Cancer European Code Against Cancer in 2015 reported that a plant-based diet may have an important impact in the prevention of cancer development, especially those of the colon, rectum, stomach, esophagus and endometrium (20). The list of highly recommended dietary components include polyphenol-rich foods like fruits and vegetables, as well as herbs, seeds, spices or olive oils, and beverages such as coffee, tea, red wine (21). Previous studies have found that compounds of natural origin from the group of polyphenols, *i.e*. epigallocatechin gallate (EGCG), resveratrol, or curcumin, were indicated to have anti-cancer activity and the anti-metastatic effects (22-24). Moreover, not only single polyphenols exhibit chemopreventive potential but also plant extracts rich in these compounds (25, 26). Recently, other research has found that extracts rich in polyphenolic compounds have demonstrated anti-carcinogenic activity, also through the effective inhibition MMP-2 and MMP-9 expression or activity, as well as *via* modulation of PI3K/AKT and MAPK/ERK signaling pathways (27-30).

In this study we focused on the anti-cancer activity (particulary, anti-metastatic activity) of the extract derived from Japanese quince (*Chaenomeles japonica* L.) leaves (PRE), while in our previous work we described the chemical composition of this extract, as well as the crude extract, along with results of antioxidant capacity and cytotoxic activity of both preparations (31). Using the UPLC-Q-TOF-MS analysis, we showed that PRE contains 33 diversified phenolic compounds, including flavonols, phenolic acids, flavanols, and flavanones. Chlorogenic acid (5-*O*-caffeoylquinic acid) and naringenin hexoside were predominant phenols in the extract, accounting for 36.10% and 10.41% of the compounds in PRE (31). Zvikas and Kikowska in their studies also showed that Japanese quince (*Chaenomeles japonica* L.) leaves extracts are rich source of polyphenolic compounds, especially chlorogenic acid (32-33). Numerous research that was carried out on extracts from Japanese quince (*Chaenomeles japonica* L.) fruit presented among others anti-cancer activity. However, there have been only several studies that were carried out with the use of leaf extracts from this plant (31-35).

It is known that extracts derived from fruits and leaves of this plant can effectively modulate the expression of genes, activity of enzymes or influence cell pathways that are responsible for processes related to the development of inflammation and cancer (36-39). Our previous studies indicated that Japanese quince fruit extract can effectively regulate several genes related to apoptosis, angiogenesis, and metastasis in breast, prostate, and colon cancer cells (40, 41). Furthermore, we showed cytotoxic effect of Japanese quince leaf extract towards colon cancer cells (31), and in this study we for the first time demonstrated its anti-metastatic activity also against these cells.

## MATERIAL AND METHODS

### *Materials*

All there agents and solvents from Chempur (Piekary Slaskie, Poland) were used of analytical grade. Cell culture media, media supplements, and EGCG (>95%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (Simplicity TM Water Purification System, 68 Millipore, Marlborough, MA, USA) was used to prepare all solutions. Preparation and chemical characterization of PRE was described in the previous article (31). In this research, the same extract was used as before (31).

### *Plant materials*

The leaves of the Japanese quince (*Chaenomeles japonica* L.) were collected in the middle of October 2017 in an ecological plantation in Zwierzyniec, in the central region of Poland (GPS location: 51°29’57”N 19°22’48E”). The leaves were picked from 10 bushes. About 100 g of leaves were plucked from each bush (on all sides of the bush) and were stained green. There were no symptoms of leaf colour change and no leaf fall from the bush. All collected leaves were mixed to obtain an average sample. Then the leaves were dried for 4 hours at 60°C, ground and stored in a dark place in a sealed package at room temperature.

### *Preparation and characterization of Japanese quince leaf polyphenol-rich extract*

The preparation and characterization of PRE, which was also used in this study were described elsewhere (31). The polyphenol-rich extract was obtained by the isolation of polyphenols from a crude extract of Japanese quince leaves.

### *Cells and culture conditions*

HT-29 and SW-480 human colon cancer cells, and CCD 841 CoN human normal colon epithelial cells were purchased from the American Type Culture Collection (ATCC; ref: HTB-38, CCL-228, CRL-1790) (LGC Standards, Poland). HT-29 were cultured in the Dulbecco’s Modified Eagle Medium (DMEM) supplemented with inactivated 10% fetal bovine serum (FBS) 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. SW-480 cells were cultured in the RPMI 1640 Medium supplemented with inactivated 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. CCD 841 CoN cells were cultured in the Eagle’s minimum essential medium (EMEM) supplemented with inactivated 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. All cell lines were grown in a humidified atmosphere with 5% $CO_2$ at 37°C. Experiments were carried out between passages 3 to 8. The cells were seeded in such a quantity that the confluence at the end of the experiment did not exceed 80% in the control wells. In the work presented here, PRE was tested within the concentration range of 25 to 750

μg/mL. For all performed experiments PRE was dissolved in 70% ethanol solution in phosphate buffered saline (PBS) and then diluted to the final concentration with an adequate medium supplemented with 3% FBS. The final concentration of ethanol was lower than 0.01% (v/v). The culture media was not changed during the incubation with the tested preparation.

The experimental protocol was approved by the Ethics Committee of the Medical University of Lodz (No. RNN/382/18/KE).

*Invasion assay*

Invasion of SW-480, HT-29 and CCD 841 CoN cells was evaluated using the Matrigel BM matrix assay as described previously by our team (41). BioCoat Matrigel invasion chambers (24-well cell culture inserts containing an 8.0 μm PET membrane with a uniform layer of Matrigel) (Becton Dickinson, Bedford, MA) were used in this experiment. Cells were cultured in the upper chamber under serum-free growth medium and incubated with or without PRE (100, 150 and 250 μg/mL) or EGCG (45 or 90 μg/mL) as the control. The bottom chamber contained growth medium supplemented with 10% FBS as the chemoattractant. Cells were cultured in these conditions for 48 h (SW-480, CCD 841 CoN) or 96 h (HT-29) at 37°C in a 5% $CO_2$ atmosphere. Subsequently, the non transwell cells in the upper chamber were gently scraped away and adherent cells present on the lower surface of the insert were stained with crystal violet, photographed, and measured using NIH ImageJ analysis software.

*Migration assay*

The experiment was carried out using inserts - Falcon® Permeable Support for 24-well Plate with 8.0 μm Transparent PET Membrane (Corning, USA). The conditions of the experiment using SW-480, HT-29 and CCD 841 CoN and migration assay were same as for the invasion experiment and were described in the previous paragraph (invasion assay).

*Gelatin zymography of metalloproteinases MMP-2 and MMP-9 activity*

SW-480, HT-29, and CCD 841 CoN cells ($6\times10^3$ of CCD 841 CoN cells/well, and $1\times10^4$ of SW-480 and HT-29 cells/well) were seeded in 96-well plates in the growth medium supplemented with 10% FBS. After 24 h, the cells were suspended in the medium with 3% FBS and PRE (150–500 μg/mL) or EGCG (45 or 90 μg/mL) for 24 h. Next, culture media were collected for further analysis. Briefly, the same volumes of the conditioned media (15 μL) were mixed with an electrophoresis sample buffer (5 μL) containing 4% sodium dodecyl sulfate (SDS), 30% glycerol, 1M Tris-HCl (pH 6.8) and 0.01% bromophenol blue. Electrophoresis was conducted using a 10% polyacrylamide gel embedded with gelatin. After electrophoresis, MMP-2 and MMP-9 were renatured by incubation with 2% Triton X-100 and 50 mmol/L Tris-HCl (pH 7.4). The enzymatic reaction was allowed to proceed at 37°C for 21 h in a buffer containing 1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4) and 5 mmol/L $CaCl_2$. Subsequently, the gels were stained for 1 h in a solution containing 0.1% Amido Black, 7% acetic acid and 20% ethanol. Next, the gels were captured using an Olympus camera (Olympus Corp., Tokyo, Japan). The activity of MMP-2 and MMP-9 was visualized as a transparent band against the dark blue background of the Amido Black-stained slab gels. Densitometry analysis was carried out using GelDoc ™EQ system with Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Enzyme-linked immunosorbent assay*

Tested cells were seeded in 96-well plates ($6\times10^3$ of CCD 841 CoN cells/well, and $1\times10^4$ of SW-480 and HT-29 cells/well) in growth medium with 10% FBS for 24 h, then the medium was removed and cells were suspended in medium with 3% FBS with or without PRE (150 and 250 μg/mL for SW-480 and CCD 841 CoN, 250 and 500 μg/mL for HT-29). Positive control samples were treated with EGCG (45 μg/mL). Cells were treated with PRE for 48 h. Additionally, to determine MMP-9 protein level in HT-29, cells were stimulated with terephthalic acid (TPA; 50 ng/mL) and tumor necrosis factor-α (TNF-α; 50 ng/mL). The protein level of MMP-2 was determined using ELISA Kit for matrix metalloproteinase 2 (Clone Cloud Corp., Katy, TX, USA), and MMP-9 expression was examined with RayBio® Human MMP-9 ELISA Kit (RayBiotech Life, Inc., Peachtree Corners, GA, USA) according to the manufacturer's instructions.

*Protein preparation and Western blot analysis*

Tested cells were harvested, suspended in growth medium with 10% FBS, and seeded at the density of $1\times10^6$ cells in 25 $cm^2$ bottles. After 24 h, when the cells reached a confluence of 70–80%, they were washed twice with PBS and then suspended in medium with 3% FBS with or without PRE (50–500 μg/mL). Cells were incubated with PRE for 6 h. Positive control samples were treated with EGCG (45 or 90 μg/mL), instead of PRE. Cell lysates were prepared using a Mammalian Cell Lysis Kit (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). The protein content of the cell lysates was then determined by using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein cell lysates (50 μg protein/well) were subjected to Mini-PROTEAN® TGX™ Gels (Bio-Rad, Hercules, CA, USA). Separated proteins were transferred using a semi-dry system onto PVDF membranes (pore size: 0.45 μm; Life Technologies, Carlsbad, CA, USA) in transfer buffer containing 20% (v/v) methanol, 192 mM glycine, and 25 mM Tris, pH 8.3. The PVDF membranes were incubated at room temperature for 1 h in 5% non-fat dry milk in Tris-buffered saline with Tween 20 (PBST, 150 mM NaCl, 0.05% Tween 20, 100 mM Tris-HCl, pH 7.4) to saturate non-specific protein-binding sites. Subsequently, the membranes were incubated with specific antibodies diluted in PBST overnight at 4°C for immunodetection of the studied proteins. The mouse monoclonal antibodies conjugated to HRP were used as follows: β-actin (sc-47778), Akt1 (sc-5298), p-Akt1 (sc-293125), ERK ½ (sc-514302), and p-ERK (sc-7383). All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After wash steps (five times, 10 min) using PBST, the detection of bands was performed using the enhanced chemiluminescence blotting detection system (Super Signal West Femto Maximum Sensitivity Substrate, ThermoFisher Scientific, Rockford, USA). The intensity of the bands was quantified by densitometric analysis using the GelDoc™ EQ system with Image Lab Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Immunoblots were normalized to the β-actin content.

*Statistical analysis*

Statistical analyses were performed using PRISM 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Data is presented as mean ±SEM, as indicated in the figure legends. The number of independent experiments is given in the figure legends. The statistical significance of differences between means was determined by a one-way ANOVA followed by a *post hoc* multiple comparison Newman-Keuls test. P values of $<0.05$ were considered to be statistically significant.

## RESULTS

*Influence of Japanese quince leaf phenol-rich extract on migration, invasion, activity, and protein expression of MMP-9 and MMP-2 on SW-480 cells*

As presented on the *Fig.1* PRE caused suppression of migration and invasiveness of SW-480 colon cancer cells, as wells as down-regulation of metastasis-related enzymes MMP-9 and MMP-2. Since EGCG is a natural green tea compound with proven anti-cancer activity (19, 42, 43), it was used as a positive control at a concentration of 50 µM. Firstly, to investigate cancer cell migration we used the special assay. The results clearly demonstrated (*Fig. 1A* and *1B*) that PRE at concentrations of 150 µg/mL and 250 µg/mL after 48 h treatment significantly reduced SW-480 migration by 52.63% and 82.91% versus control. In order to measure the effectiveness of the extract on the invasiveness of SW-480 cells, we used Matrigel BM matrix assay. In this case, a 65.61% and 79.48% reduction of cell invasion was observed after 48 h incubation with PRE (*Fig. 1A* and *1B*). Zymography analysis of cell culture media was used to evaluate the impact of the extract treatment on type IV collagenases. As shown on *Fig. 1C* and *1D*, PRE similarly and markedly inhibited the activity of both MMP-9 and MMP-2 after 24 h incubation. At PRE concentration of 150 µg/mL, 250 µg/mL, and 500 µg/mL MMP-9 activity was decreased to 83.5%, 74.5%, and 42.5%, whereas reduction of MMP-2 reached 84.5%, 71%, and 40.5%, respectively. We also performed the ELISA test to investigate whether PRE affects the production of MMP-9 and MMP-2 (*Fig. 1E*). In this case, SW-480 cells were incubated with the extract concentrations of 150 and 250 µg/mL) for 48 h. The protein expression of MMP-9 was significantly decreased after cells were exposed to PRE at the concentration of 150 µg/mL to 2609.58 pg/mL, while at the higher concentration of 250 µg/mL to 3356.2 pg/mL, when compared to the level of MMP-9 (2993.83 pg/mL) in untreated cells. The protein expression of MMP-2 in SW480 cells was reduced when cells were incubated with 150 and 250 µg/mL of PRE. The lower concentration of the extract caused down-regulation to 331.46 pg/mL, and higher to 267.94 pg/mL when compared to untreated cells (333.14 pg/mL). Only, 250 µg/mL of PRE reduced MMP-2 protein expression in a statistically significant manner. Besides, the production of

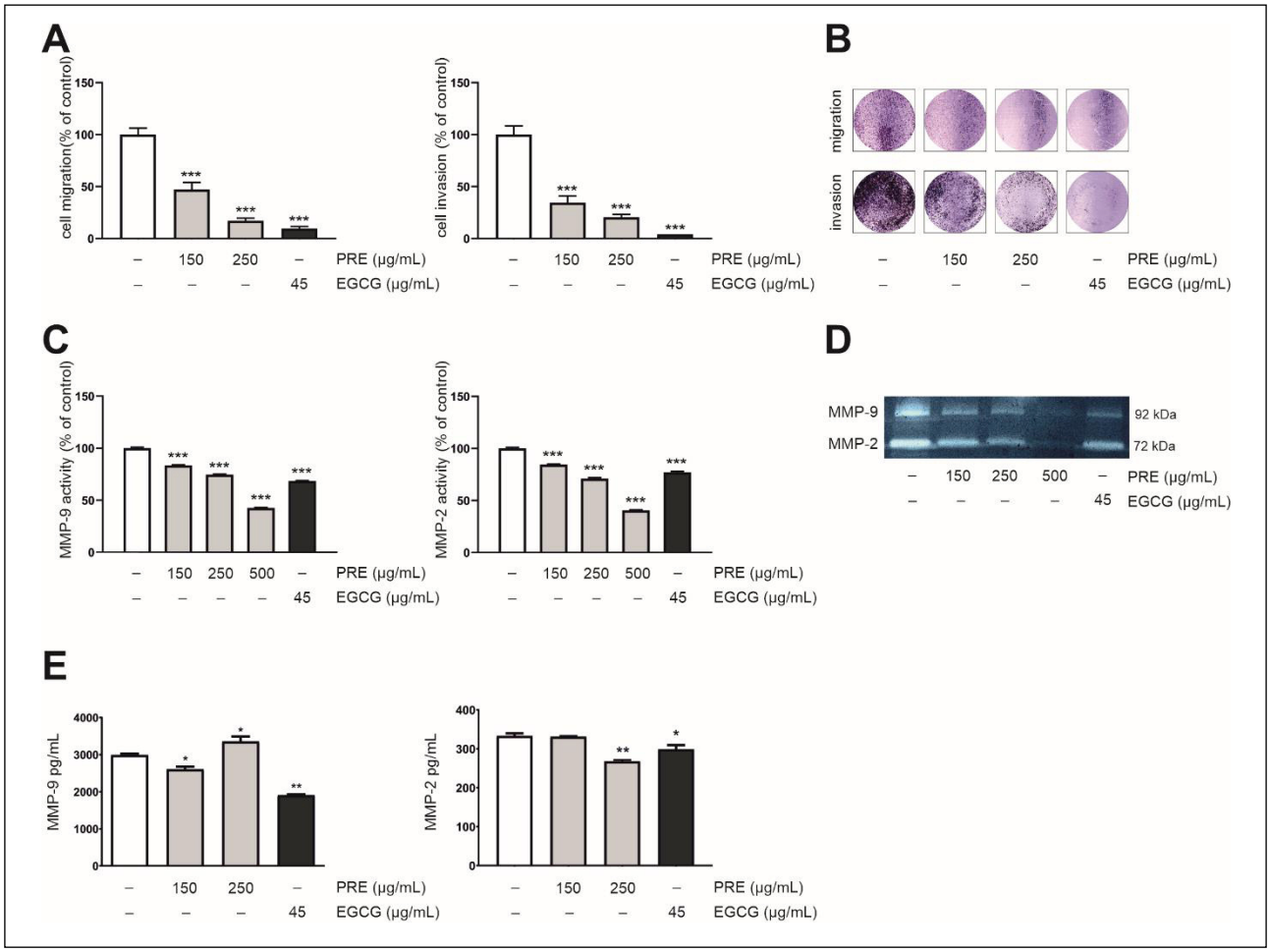


*Fig. 1*. Influence of phenolic-rich extract (PRE) treatment on migration, invasion, activity, and protein expression of MMP-9 and MMP-2 on SW-480 human colon cancer cell line. The influence of PRE (150 and 250 µg/mL) on migration and invasion (A, B) on SW-480 cells after 48 h treatment was investigated using Matrigel BM matrix assay. Zymographic analysis of MMP-9 and MMP-2 activity (C, D) in culture media of SW-480 cells incubated for 24 h with PRE (150, 250 and 500 µg/mL). Protein level of MMP-9 and MMP-2 (E) was determined in SW-480 cell supernatant after 48 h of treatment with PRE (150 and 250 µg/mL) using ELISA. EGCG (45 µg/mL) was used as a positive control. The results are expressed as the percentage of control values (untreated cells). Each value represents the mean value ±SEM, n=3 independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *P<0.05, **P<0.01, ***P<0.001 versus control cells.

MMP-2 compared to MMP-9 was lower in SW-480 cells, as it was 333.14 pg/mL (untreated cells).

*Influence of Japanese quince leaf phenol-rich extract on migration, invasion, activity, and protein expression of MMP-9 and MMP-2 on HT-29 cells*

Another colon cancer cell line HT-29 showed a lower ability to migrate and invade when compared to SW-480, therefore HT-29 were treated with PRE for 96 h. After this time, both cell migration and invasion were significantly inhibited by the extract. In case of migration, Matrigel BM matrix assay showed that it was reduced by PRE to 78.28% and 29.01% (*Fig. 2A* and *2B*). At the same concentrations of the extract, *i.e.* 100 µg/mL and 150 µg/mL, Matrigel assay demonstrated strong suppression of HT-29 invasiveness, which was decreased to 35.72% and 17.5% (*Fig. 2A* and *2B*), respectively. In the further stage of the research, the influence of 24 h incubation with PRE on the activity of MMP-9 and MMP-2 in HT-29 cells was determined. Zymography analysis (*Fig. 2C* and *2D*) revealed that the activity of MMP-9 that was secreted to the culture media of these colon cancer cells was markedly down-regulated by 38.5%, 60.5% and 66.5%, respectively, in the whole range of PRE concentrations (250, 500 and 750 µg/mL). Also, the activity of MMP-2 was significantly diminished after 24 h treatment with the extract (250, 500 and 750 µg/mL), successively by 35%, 72.5%, and 84.5%. Additionally, ELISA showed (*Fig. 2E*) that the protein expression of MMP-9 and MMP-2 was modulated by the 48 h extract treatment. PRE caused significant and concentration-dependent (250 µg/mL and 500 µg/mL) reduction of MMP-2 in HT-29 cells, from 253.16 pg/mL (untreated cells) to 128.41 pg/mL and 52.55 pg/mL. The extract showed similar effect on the MMP-9 protein expression. In this case, the production of MMP-9 was also significantly and concentration-dependly reduced by PRE treatment, from 736.72 pg/mL (untreated cells) to 652.12 pg/mL and 529.05 pg/mL. Noteworthy, in this case, HT-29 cells were stimulated with TPA (50 ng/mL) and TNF-α (50 ng/mL), since the protein expression of MMP-9 was undetectable by ELISA in unstimulated cells. Both TPA and TNF-α are known to promote cancer cells growth, proliferation, invasion, metastasis, and tumor angiogenesis,

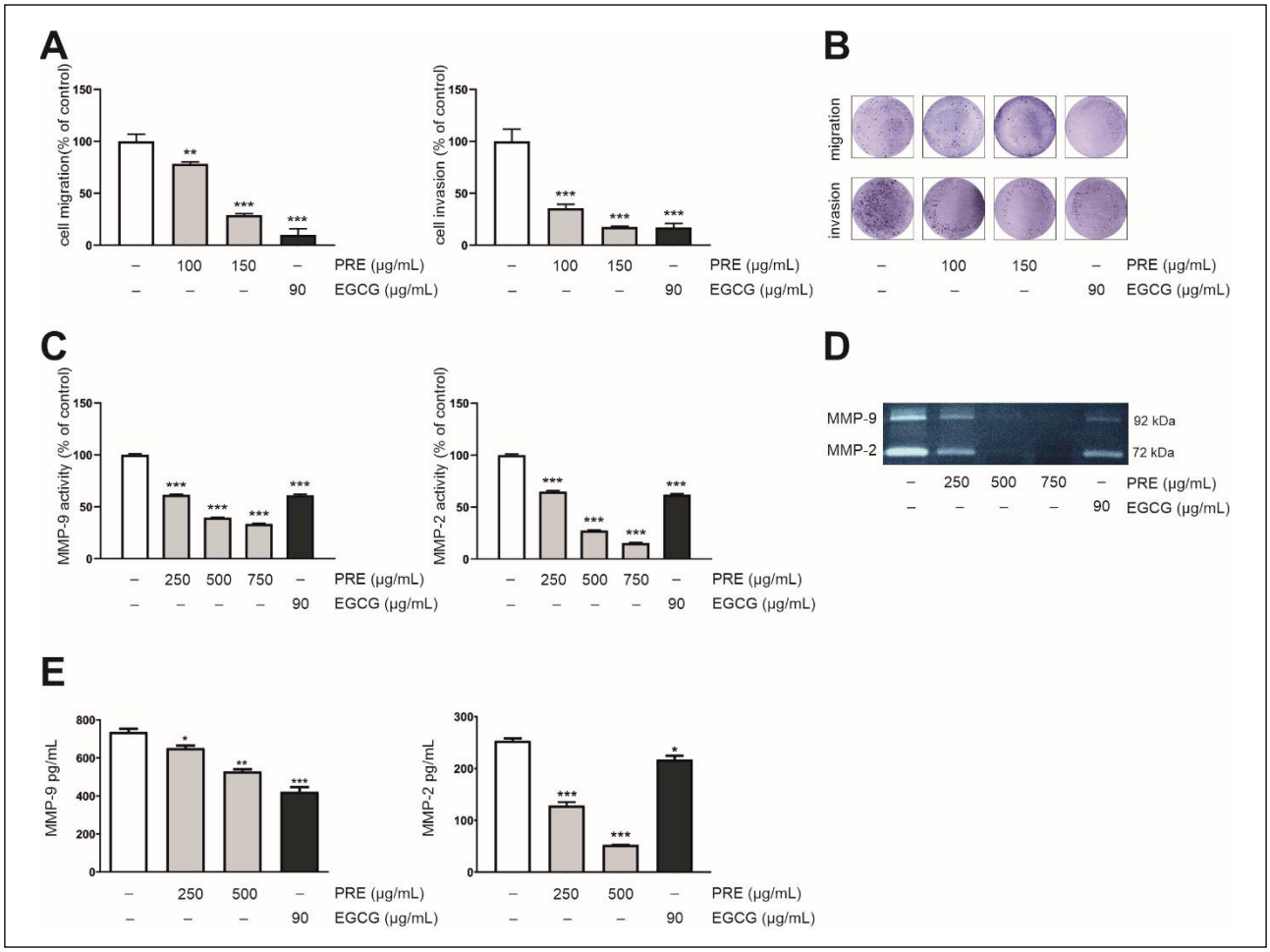


*Fig. 2*. Influence of phenolic-rich extract (PRE) treatment on migration, invasion, activity, and protein expression of MMP-9 and MMP-2 on HT-29 human colon cancer cell line. The impact of PRE (100 and 150 µg/mL) on migration and invasion (A, B) on HT-29 cells after 96 h incubation was investigated using Matrigel BM matrix assay. Activity of MMP-9 and MMP-2 (C, D) in HT-29 cell culture media was measured after 24 h with the extract treatment (250, 500 and 750 µg/mL) using zymographic analysis. Protein level of MMP-9 and MMP-2 (E) was determined in HT-29 cell supernatant after 48 h of treatment with PRE (250 and 500 µg/mL) using ELISA. EGCG (90 µg/mL) was used as a positive control. The results are expressed as the percentage of control values (untreated cells). Each value represents the mean value ±SEM, n=3 independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *P<0.05, **P<0.01, ***P<0.001 versus control cells.

*inter alia* through the activation of MAPKs (44, 45). EGCG at the concentration of 90 µg/mL was used as a positive control in all the above-mentioned research.

*Influence of Japanese quince leaf phenol-rich extract on migration, invasion, activity, and protein expression of MMP-9 and MMP-2 on CCD 841 CoN cells*

The impact of PRE was also tested on normal epithelial colon CCD 841 CoN cell line. Our research on migration and invasion showed that 48 h incubation with the extract caused a significant decrease of both parameters (*Fig. 3A* and *3B*). PRE at the concentration of 150 µg/mL reduced CCD 841 CoN migration by 52%, and at 250 µg/mL by 71.45%. The invasion was inhibited even more, and we observed suppression by 67.89% and 91.93%, respectively. Next, zymography analysis of cell culture media was used to evaluate the impact of PRE treatment on MMP-9 and MMP-2 activity. As presented on *Fig. 3C* and *3D*, the extract markedly inhibited the activity of both metalloproteinases after 24 h incubation. The activity of MMP-9 was reduced to 93.5%, 79.5% and 37% after incubation with 150 µg/mL, 250 µg/mL and 500 µg/mL of PRE, respectively. Similarly, the activity of MMP-2 was diminished to 99.5%, 87.5% and 27.5%, respectively. Also, the levels of secreted MMP-9 and MMP-2 protein in cultured media of CCD 841 CoN cells were measured after 48 h treatment with PRE (*Fig. 3E*). After incubation with 150 and 250 µg/mL of PRE we observed a significant drop of MMP-9 protein expression from 2962.95 pg/mL (untreated cells) to 2411.66 pg/mL and 2478.26 pg/mL, respectively. The protein expression of MMP-2 was also diminished after CCD 841 CoN cells were exposed to PRE. The concentration of 150 µg/mL reduced MMP-2 production from 338.19 pg/mL (untreated cells) to 308.02 pg/mL. Higher PRE concentration of 250 µg/mL significantly down-regulated MMP-2 secretion to 126.50 pg/mL. EGCG at the concentration of 45 µg/mL was used as a positive control in all the above-mentioned research.

*Influence of Japanese quince leaf phenol-rich extract on ERK/AKT signaling in human colon cancer cells*

The protein expression of p-ERK in SW-480 cells was reduced by the extract in a significant and concentration-

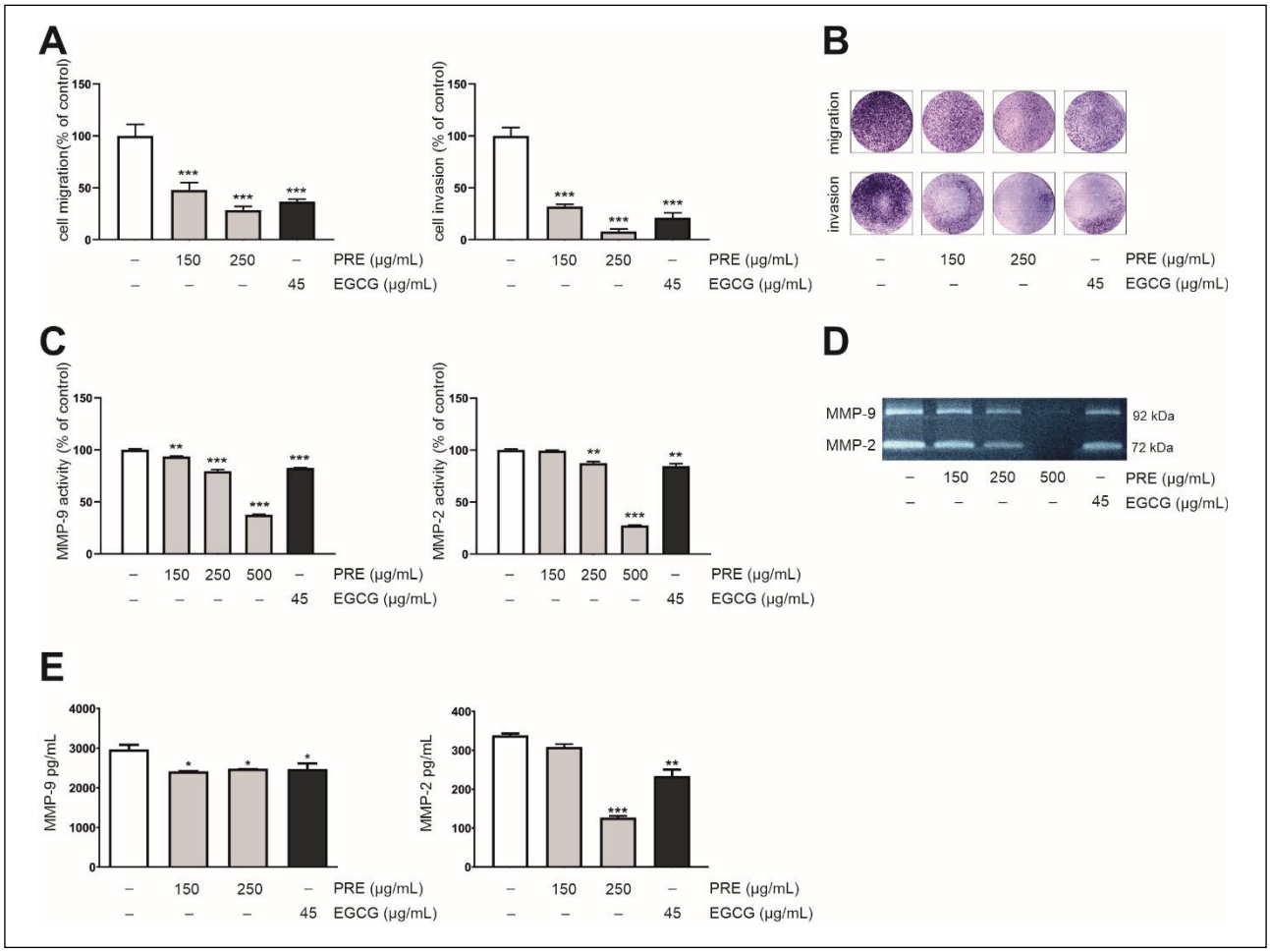


*Fig. 3*. Influence of phenolic-rich extract (PRE) treatment on migration, invasion, activity, and protein expression of MMP-9 and MMP-2on CCD 841 CoN human colon normal cell line. The influence of PRE (150 and 250 µg/mL) on migration and invasion (A, B) of CCD 841 CoN cells after 48 h treatment was investigated using Matrigel BM matrix assay. Activity of MMP-9 and MMP-2 (C, D) in CCD 841 CoN cell culture media was measured after 24 h with the extract treatment (150, 250 and 500 µg/mL) using zymographic analysis. Protein level of MMP-9 and MMP-2 (E) was determined in CCD 841 CoN cell supernatant after 48 h of treatment with PRE (150 and 250 µg/mL) using ELISA. EGCG (45 µg/mL) was used as a positive control. The results are expressed as the percentage of control values (untreated cells). Each value represents the mean value ±SEM, n=3 independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *P<0.05, **P<0.01, ***P<0.001 versus control cells.

dependent manner (*Fig. 4A*). After 6 h incubation with PRE at 150 µg/mL, 250 µg/mL, and 500 µg/mL, p-ERK levels were adequately reduced by 33%, 42%, and 51%. A similar, inhibitory effect on p-AKT protein expression was observed in this cell line after treatment with PRE (*Fig. 4B*). In this case, the extract at the concentration of 250 µg/mL and 500 µg/mL caused a significant reduction of p-AKT protein expression by 11% and 13%, respectively. EGCG, which was used as a positive control caused similar to PRE inhibition of both p-ERK and p-AKT protein expression. We also measured the protein expression of p-ERK and p-AKT in HT-29 colon cancer cells. As presented on *Fig. 4C*, 6 h treatment with the extract at 250 µg/mL, 500 µg/mL, and 750 µg/mL markedly increased the protein expression of p-ERK by 33%, 68%, and 67%. Interestingly, EGCG also up-regulated the level of p-ERK by 46%. In contrast, the protein expression of p-AKT was slightly diminished by PRE in HT-29 cells, and the extract at the concentration 750 µg/mL caused a reduction by 9% (*Fig. 4D*). The significant down-regulation (by 50%) of p-AKT expression at the protein level was only observed when HT-29 cells were treated with EGCG.

## DISCUSSION

Over the decades, polyphenols have been known as free radical scavengers, but during recent years various research has shown that these compounds do not only possess anti-oxidant activity, but have also anti-bacterial, anti-fungal, anti-inflammatory, and even anti-cancer potential (19, 46, 47). Many of these studies also provided insight about the mechanism of action of polyphenols at the cellular level (48-50). Recently, scientific attention has also focused on natural polyphenolic extracts as potential agents for cancer treatment and prevention (51-53).

The Japanese quince (*Cheanomeles japonica* L.) is plant of that has attracted attention of scientists. So far the attention has mainly been concentrated on extracts derived from fruit and leaves of this plant, and the results have demonstrated several biological activities, from antioxidant, anti-inflammatory, anti-cancer, pro-apoptotic to anti-proliferative and hypoglygemic (38-41). These properties are attributed to the chemical composition rich in polyphenols. Several scientific papers, including ours also shown that Japanese quince extracts derived from leaves are a valuable source of phytochemical composition

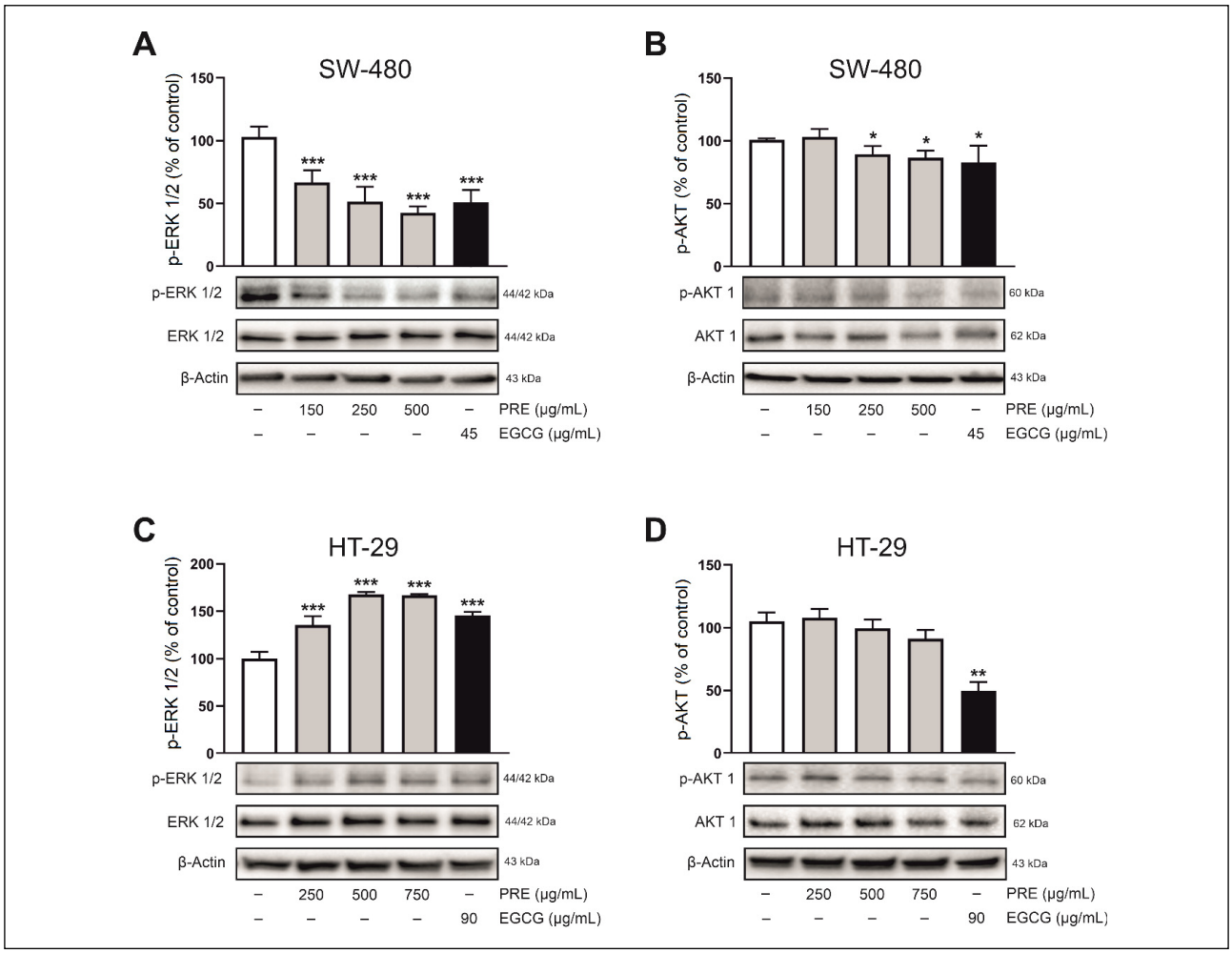


*Fig. 4*. Effects of phenolic-rich extract (PRE) on the phosphorylation of ERK 1/2 and AKT signaling pathways in SW-480 (A and B) and HT-29 (4C and 4D) human colon cancer cells. Western blot was carried out to detect the phosphorylation of ERK and AKT in SW-480 and HT-29 cells. SW-480 cells were treated with PRE at 150, 250 and 500 µg/mL, while HT-29 at 250, 500 and 750 µg/mL. Cell lines were incubated with the extract for 6 h, and EGCG (45 or 90 µg/mL) was used as a positive control. The results are expressed as the percentage of control values (untreated cells). Each value represents the mean value ±SEM, n=3 independent experiments (each experiment was carried out in triplicate). Significance of differences between means: *P<0.05, **P<0.01, ***P<0.001 versus control cells.

such as chlorogenic acid, naringenin hexoside and quercetin (31-33, 54, 55). These compounds were identified to result in many activities. Chlorogenic acid was described as polyphenol with *e.g*. anti-oxidant, anti-inflammatory and anti-cancer activity (56, 57). Similar properties are attributed to the second compound present in the extract, *i.e*. naringenin hexoside (58). Our team and others have shown evidence for the anti-oxidant activity of leaf extract from Japanese quince was not only proved by our team, additionally Teleszko, Kikowska and Urbanaviciute proved it (24, 31-33, 55). In a different study, we also showed that Japanese quince leaf extract can effectively ameliorate inflammation, reducing expression of several inflammatory mediators, such as COX-2, NO, TNF-α and IL-6, *via* NF-κB signaling pathway (34). Moreover, we examined the cytotoxic activity of extracts from Japanese quince using colon cells, showing interesting results that the extract exhibited a higher cytotoxic activity towards cancer than towards normal cells (31). Also, Zvikas and co-workers presented cytotoxic activity against glioblastoma C6 and HROG36 cells after treatment with extracts from different Japanese Quince Leaves cultivars (32). In this study, we examined the ant-invasion activity of Japanese quince leaf phenol-rich extract (PRE) using normal and cancer colon cells. Our results demonstrated that PRE possess ability to suppress cancer cells migration and invasion through the possible cellular mechanisms related to modulation of ERK/AKT phosphorylation and down-regulation of expression and activity of MMP-2 and MMP-9. These observations are similar to our previous research carried out on colon cancer cells SW-480 and Japanese quince fruit extract. The data showed the impact of the extract on MMP-2 and MMP-9 activity and expression at the protein level. In this study the mechanism of MMPs inhibition, anti-invasive as well as anti-inflammatory activity was linked with the reduced activation of NF-κB protein expression (40). Also, in 2013 we revealed that fruit extract from Japanese quince can effectively ameliorate invasion of breast and prostate cancer cells, and prostate normal cells. In this case, the expression of MMP-9 and other MMPs, that are involved in the multi-step process of cancer cells proliferation and metastasis, were down-regulated at the mRNA level. Also, TIMP-1 which is an endogenous tissue inhibitor of MMPs, was stimulated by the extract in human breast and prostate cancer cells, as well in normal prostate cells. Moreover, the activity of MMP-9 was decreased by the extract in these cancer cells (41).

Panyathep and co-workers also carried out the research on SW-480, but using polyphenolic extract derived from dried longan (*Euphoria longana* Lam.) seeds (59). This rich in gallic acid and ellagic acid extract inhibited MMP-2 and MMP-9 secretion, activity, and protein expression in less invasive cell line SW-480, but not in highly invasive cell line SW680 (59). This is not consistent with our data, since the expression of both MMP-9 and MMP-2 in more aggressive colon cancer cell line HT-29 was inhibited by PRE, while in SW-480 we noticed insignificant reduction. Another extract rich in anthocyanins isolated from *Vitis coignetiae* Pulliat attenuated both MMP-2 and MMP-9 activity and protein expression in HT-29 cells (60). Moreover, molecular docking analysis of chlorogenic acid, which makes up 30% of PRE, against matrix metalloproteinases showed an excellent binding affinity towards MMP-2, MMP-3, MMP-8, and MMP-12 (61). This may explain why PRE works so well at inhibiting the activity and the expression of MMP-2 and MMP-9. Additionally, since activity of MMP-2 and MMP-9 is crucial for the process of cancer invasion through the ability of proteolytic digestion of the extracellular matrix, we tested and demonstrated that PRE significantly suppressed migration and invasion in both colon cancer cells lines. In this study we also examined molecular mechanism of anti-metastatic activity of PRE. Numerous studies suggest that the expression of MMP-2 and MMP-9 is modulated *via* several pathways, such as MAPKs, p38 MAPK, ERK, and NF-κB (62). Our previous study that focused on fruit flavanol preparation from Japanese quince presented that the activity of these MMPs in SW-480 was reduced through the inhibition of p-NF-κB (40). Similarly, different extract rich in procyanidins from evening primrose (*Oenothera paradoxa*) also attenuated MMP-9 activity and expression in SW-480 cells inhibiting p-NF-κB signaling (63).

No less important is PI3K/AKT pathway which is involved in regulation of the intracellular signaling of cell metabolism, growth, and survival (64). This signaling is also crucial for the initiation and progression of malignancies, enhancing cell survival through activation of cell proliferation and invasiveness (65). In CRC, PI3K/AKT pathway is one of the most studied signaling cascade activated by receptor tyrosine kinases (RTKs) (64, 65). Several studies on polyphenol-rich plant extracts demonstrated the anti-cancer role through the suppression of the PI3K/AKT pathway. The extract from blueberry (*Vaccinium angustifolium*) had an influence on MDA-MB-231 cells (66). Also, the *Nelumbo nucifera* Gaertn leaves extract inhibited MMP-2 and MMP-9 activity through this mechanism. Additionally, the cell signaling of MEK/ERK was attenuated by this extract (67). Similarly, study on human prostatic carcinoma cells LNCaP, confirmed the inhibitory effect of plant extracts on PI3K/AKT signaling. In this case, *Hibiscus sabdariffa* leaf extract also suppressed NF-κB (68). In addition, in cancer cells the expression of MMP-2 and MMP-9 can be regulated *via* MAPK/ERK pathway. Also, MAPKs regulate the synthesis of chemokines, cytokines, adhesion molecules, and prostaglandins that are recruited to the site of inflammation and carcinogenesis (65). Research showed that this pathway is also highly involved in the development of CRC. Moreover, the literature suggests that there is a correlation between several genes, such as KRAS or BRAF, which mutations can also dysregulate both PI3K/AKT and MAPK/ERK signalings, affect the protein concentration of these intracellular pathway molecules, and overall have impact on prolonged survival of patients (69, 70). The exposure of MDA-MB-231 cells to black rice anthocyanins down-regulated the invasion and MMP-9 protein expression through the RAF/MAPK signaling pathway (71). The ability to attenuate MAPK/ERK transductional cascade was also demonstrated by *Nelumbo nucifera* extracts (67, 72, 73). Also, *Orostachys japonicus* extract suppressed MMP-2 and MMP-9 mRNA expression through the down-regulation of MAPK signaling pathway, including JNK, p38 MAPK and ERK1/2 (74). Deng and co-workers demonstrated that Chinese herbal formula Yi-Qi-Fu-Sheng can diminish MMP-2 and MMP-9 and ERK1/2 mRNA expression (75). In contrast to previous studies, extract from olive pomace inhibited protein expression of MMP-2 and MMP-9 through the up-regulation of the phosphorylation of p38 MAPK and ERK1/2 (76). In 2017, it was presented that chlorogenic acid (CGA) inactivated p-ERK in human colon cancer cells HCT116 and HT-29, when cells were incubated with CGA for 24 h (77). These results contradict our data that showed up-regulation of p-ERK in HT-29 cells after 6 h treatment with PRE. It might be due to the shorter time of incubation as wells as the presence of other bioactive compounds in the extract. On the other hand, p-ERK was markedly inhibited in less aggressive cancer cell line SW-480 after incubation with PRE. We also notice significant down-regulation of p-AKT in SW-480. In HT-29 the protein expression of p-AKT was slightly suppressed by PRE after 6 h treatment.

In conclusion, our results are the first to demonstrate significant inhibition of metastasis ability of human colon cancer cell lines by Japanese quince leaf phenol-rich extract. We proved that PRE can effectively ameliorate migration and invasion through the suppression of activity and protein expression of MMP-2 and MMP-9 *via* most likely the regulation of

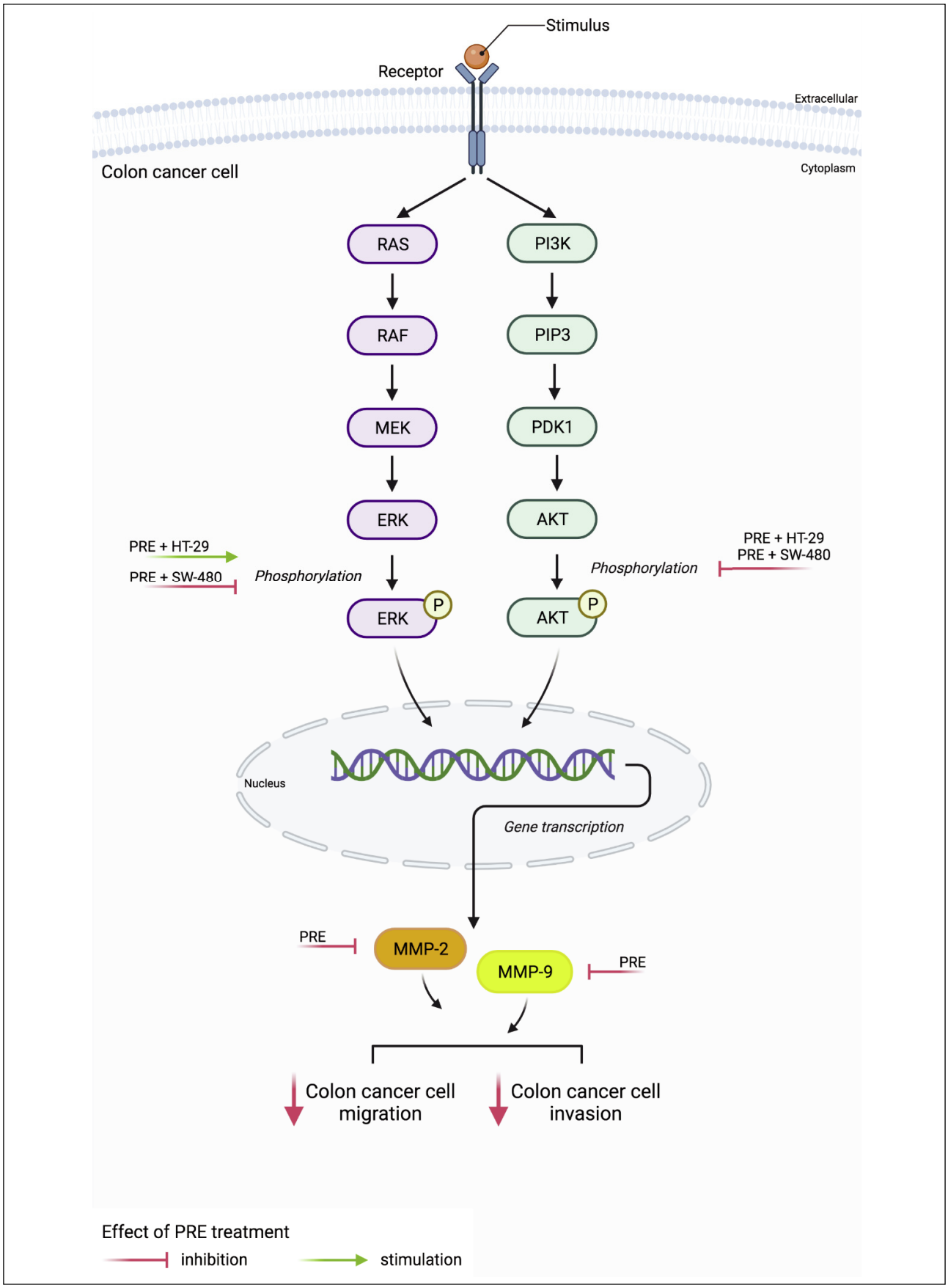


*Fig. 5.* A schematic diagram showing ERK/AKT mechanism involved in the chemopreventive effects of Japanese quince (*Chaenomeles japonica* L.) phenol leaf extract (PRE) in SW-480 and HT-29 colon cancer cells. PRE suppressed colon cancer cells migration and invasion through MMP-2 and MMP-9-dependent mechanism involving most likely the phosphorylation of ERK/AKT signaling. Created with BioRender.com.

phosphorylation of ERK/AKT signaling pathway (*Fig. 5*). Therefore, PRE could be developed as a potential antitumor candidate targeting phosphorylation of ERK/AKT mechanism for the prevention and treatment of colon cancer. Nevertheless, the extract needs in-depth study of the mechanisms involved in its chemopreventive properties. Overall, these promising results increase the knowledge in the field of chemopreventive effects of Japanese quince leaf extract and could be useful for further studies to highlight the value of phenolic compounds in PRE and the possible molecular mechanisms as well as for *in vivo* studies against colorectal cancer.

*Acknowledgements*: The study was supported by grants from the Medical University of Lodz (No. 502-03/1-156-04/502-14-362-18 and No. 503/1-156-04/503-11-001/19-00).

Conflict of interests: None declared.

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Received: January 14, 2022
Accepted: February 28, 2022

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