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THE INFLUENCE OF *VIBURNUM OPULUS* POLYPHENOLIC COMPOUNDS ON METABOLIC ACTIVITY AND MIGRATION OF HELA AND MCF CELLS

Abstract
In recent years, research of antitumor activity of natural compounds isolated from plant material has increased. Polyphenols have gained significant attention due to their proapoptotic abilities and their involvement in migration and inhibition of metastasis processes. The anticancer effects of polyphenolic extracts of *Viburnum opulus* fruit against human breast (MCF-7) and cervical (HeLa) cancer cell lines have been confirmed in this study. It was demonstrated that the tested preparations (methanol – M and acetone – A from pomace, juice – J and juice after extraction to the solid phase SPE – PF) show cytotoxic activity and regulate the migration process of cancer cells. The degree of inhibition of cell migration was measured at two times - 24 h and 48 h after addition of the tested preparations. The highest toxicity towards both cell lines was demonstrated by the polyphenol fraction obtained after juice purification SPE (IC_{50} values at concentration of 63,541 and 19,380 µg/mL for HeLa and MCF cell lines, respectively). At the same time, the same preparation inhibited cell migration the most (nearly 70% compared to controls at both times at the concertation of 15 and 30 µg/mL). All preparations showed the antioxidant ability, but the *Viburnum opulus* juice (200 and 350 µg/mL) and the preparation after its purification (15 and 30 µg/mL) have larger ability to inhibit the intracellular oxidative stress (30-40%) than preparation obtained from pomace (nearly by 20% at concentration of 20 and 50 µg/mL of M and A). Despite the antioxidative capacity of the preparations, they simultaneously decreased cellular mitochondrial potential. The results obtained indicate the high potential of components of *Viburnum opulus* polyphenolic compounds can be used in the production of innovative dietary supplements or pharmacological preparations for people with an increased risk or inclination towards developing breast or cervical cancer.

Key words
Viburnum opulus, polyphenols, cancer cells migration, mitochondrial potential, anti-oxidant effect.

Introduction

Despite significant advances in medical technology cancer remains one of the most aggressive and debilitating diseases worldwide. Surgery, chemotherapy and radiotherapy are commonly used for cancer treatment, however due to the harmful and painful side effects of these treatments, patients usually do not cope well with them [1]. Recently, much attention has been paid to the identification of natural chemopreventive substances capable of inhibiting, delaying or reversing the process of multistage carcinogenesis. Most of these naturally occurring phytocompounds retain antioxidant and anti-inflammatory properties that seem to contribute to their chemoprevention. Antioxidants, such as vitamins and polyphenols, include many compounds that can capture reactive oxygen species. Therefore, it has been proposed that antioxidants have potential benefits for the prevention and treatment of diseases associated with increased generation of reactive oxygen species and can be effective in reduction of carcinogenesis [2].

Viburnum opulus belongs to the Adoxaceae plant family, which can be found in eastern, north-eastern, western and central Europe and Turkey. The fruits are quite small, have a red color and are very acidic. They contain a large amount of polyphenols, as well as ascorbic acid and L-malic acid. According to several authors, *Viburnum opulus* juice is a source of flavonoids that contain (+) – catechin, (-) – epicatechin and quercetin glycosides. The juice also contains a large amount of chlorogenic acid (54% of the total phenolic compound) and carotenoids, coleoic acid, epigallocatechin gallate, and quercetin [3, 4]. Due to the presence of these ingredients, it has a strong antioxidant effect and can be used in treatment of certain diseases, such as menstrual cramps, disorders of the nervous system as well as liver and biliary disorders. Recent studies have

shown that it has a high antimicrobial potential and antioxidant activity, which are considered effective in reduction of risk of cancer[2].

Global data indicates that Western European countries have the highest rate of incidence of breast and cervical cancer. For many years researchers have been trying to identify the risk factors and to develop effective methods for their prevention and treatment [5]. Due to these reports we decided to investigate the effects of polyphenolic extracts obtained from *Viburnum opulus* fruits as anti-tumor agents able to counteract metastases. As cellular models, HeLa and MCF-7 cell lines were chosen. Furthermore, we focused on determining the antioxidative capacity of preparations of such polyphenolic extracts and their impact on potential cellular mitochondrial regulation. The research aimed to determine the usefulness of *Viburnum opulus* fruits in the development of preparations and dietary supplements dedicated for people with an increased risk of breast and cervical cancer with a tendency to metastasis.

Materials and methods

In order to obtain polyphenol extracts, the fruits of *Viburnum opulus* were homogenized and then centrifuged at 5000 rpm for 10 min at 16°C. The obtained juice was divided into a fraction enriched with polyphenols by solid phase extraction (SPE) on a Waters Sep-Pak® C_{18} 35 cc Vac (10 g sorbent per cartridge) under pressure. The enriched polyphenol fraction was eluted with methanol at a volume equal to twice the bed volume. The pulp was further extracted with methanol: acetone: water (2:2:1 v/v/v) in a ratio of 1:10 w/v on the stirrer at 800 rpm for 30 min and then centrifuged at 5000 rpm for 10 min at 16°C. The residue was back-extracted with a 70% acetone solution in a ratio of 1:10 w/v and again centrifuged. The extracts obtained - polyphenols enriched, methanol and acetone - were concentrated at 40°C in a vacuum rotary evaporator (Büchi, Switzerland) and lyophilized.

The content of the total polyphenols was determined by using the Folin-Ciocalteu method. In a 96-well plate 10 µL of extract or water (control) was mixed with 40 µL of 10-times diluted Folin-Ciocalteu reagent. The reaction was initiated by adding 20 µL of 20% (w/w) Na2CO3. The volume of reaction mixture was adjusted to 200 µL with distilled water. After incubation at room temperature for 20 min the absorbance was measured at 760 nm (Synergy™ 2, BioTek Instruments Inc.). Total polyphenols content was expressed as mg of gallic acid equivalent (GAE) per g of lyophilized material.

For biological activity assays, centrifuged juice (J) and preparations of polyphenol fraction (PF), methanol (M) and acetone (A) extracts dissolved in a 50% DMSO solution were used. Samples were stored at -20°C before usage.

All cell culture reagents were obtained from Life Technologies (Carlsbad, USA). Human cervix adenocarcinoma HeLa cell line and human breast adenocarcinoma MCF-7 cell line were purchased from the American Type Cell Collection (ATCC). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and 95% air. They were grown in DMEM medium with 10% fetal bovine serum (FBS), 100 µg/mL ampicillin, 100 µg/mL streptomycin and 100 IU/mL penicillin.

To determine the maximum non-toxic concentration and the IC_{50} dose, cells were seeded into 96-well plates at 10^4 cells per well in complete medium and grown for 20 h, then incubated in the presence of the extract diluted in DMSO and culture medium for 24 h. Following incubation, 10 µl of PrestoBlue cell viability reagent (Life Technologies, Van Allen Way, CA, USA), a resazurin-based solution, was added into each well and incubated further for 30 min at 37°C with 5% CO_2 and 95% air. Cell viability was determined by measuring the fluorescent signal at F530/620 nm (Excitation/Emission) on a Synergy 2 Microplate Reader (Bio-Rad, CA, USA). The obtained fluorescence magnitudes were used to calculate cell viability expressed as a percent of the viability of the untreated control cells.

To investigate the effect of the preparations on cell migration, cells were seeded into 96-well plates containing cell seeding stoppers (ORIS™ method) at a ratio of $4 \cdot 10^4$ per well for HeLa cells and $5 \cdot 10^4$ per well for MCF-7 cells in complete medium. After 24 h of incubation the stoppers were removed and cells were incubated in serum free medium in the presence of the extract diluted in DMSO and cultured medium for 24 h. Closure of the cell-free space was measured and recorded with a Leica M205C microscope (MDG4 model) using the Leica program, both immediately and at 24 h and 48 h after removing the stoppers, and then compared to the initial

cell-free space size at 0 h. The extent of migration was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area. As a positive control, 10% FBS was used.

The mitochondrial membrane potential was assayed with a JC-1 probe. After treatments with the studied compounds, the medium was changed and JC-1 (1 μg/mL) was added for 20 min. Then, the cells were washed with serum-free medium and the fluorescent signal was measured at F530/620 nm, F485/528 nm, 485/620 nm. As a positive control, the known mitochondrial uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was used at a concentration of 50 μM. To determine the effect of the *Viburnum opulus* extracts on the intracellular generation of ROS, the DCFH-DA assay was performed. After incubating the cells for 20 h with extracts, they were washed with PBS and loaded with the DCFH-DA dye at a final concentration of 1 μM in serum-free medium for 40 min. The cells were then washed twice with PBS and the fluorescent signal at F485/528 nm was measured.

Results and discussion

There are few reports on *Viburnum opulus* composition and even fewer on its biological activity. However, it is known that guelder rose fruits contain large amounts of polyphenolic compounds corresponding to 65 mg of polyphenols per 1 g of fruit [6, 7]. Gallic acid was identified as the main polyphenolic compound in an amount of 8.29 g/kg fresh weight. Further compounds identified were chlorogenic acid, catechin, epicatechin, rutin, quercetin flavonoids, procyanidin B2, procyanidin trimer and proanthocyanidin dimer monoglycoside [8]. While discussing the results of the research we suggest the presence of the mentioned phytochemicals in the obtained preparations. Ulger et al. reported that the total number of tumor lesions were reduced in mice with colon cancer when treated with 1,2-dimethylhydrazine drinking water enriched with guelder juice at the initiation stage of tumorgenesis [9]. In this report we demonstrated that polyphenolic extracts of *Viburnum opulus* fruit exhibit anticancer activity with simultaneous decrease of cell metabolic activity and antioxidant properties.

PrestoBlue assay was used to assess the cytotoxicity of the preparations. Firstly, we determined the concentrations able to inhibit the viability of HeLa and MCF-7 cells at 50% compared to controls as well as the highest non-toxic concentrations. The obtained IC_{50} and IC_0 parameters are listed in Tab. 1. Fig. 1 demonstrates the influence of preparations on cellular viability. The highest cytotoxic activities against both cell lines were observed for the preparation obtained by extraction of solid phase (PF) from centrifuged juice. The IC_{50} parameters values are 63.541 μg/mL and 96.909 μg/mL for the HeLa and MCF-7 cells, respectively. In turn, the results indicate the lowest cytotoxicity of juice in both studied biological models. The lowest influence of a 50% decrease of cell viability was observed for centrifuged juice. The dependencies for both formulations seem to be justified. During the extraction to the solid phase we isolated certain flavonoid fractions, as well as flavonoid aglycons. We obtained a preparation free from proteins, polysaccharides and nucleic acids, which was chemically cleaner than the centrifuged juice [10]. For the other preparations (M and A), higher IC_{50} parameter values were observed, in comparison to the PF preparation, however they were lower than for the juice. This means that the most bioactive compounds affecting the viability of cells are found in the solid phase extracted preparation. The methanolic and acetone extracts were extruded from the pomace and contained higher amount of polyphenolic compounds than the centrifuged juice. Using an MTT assay, Waheed et al. showed that almost twice the dose (200 μg/mL) of methanolic extract of *Viburnum foetens* inhibited the metabolic activity of Caco-2 cells by 50%. Significantly lower inhibition (about 20%) with the same dose was obtained by Waheed against the MDA MB-468 cell line. In the presented studies all preparations showed higher toxicity to the cervical cancer cell line (HeLa) [11].

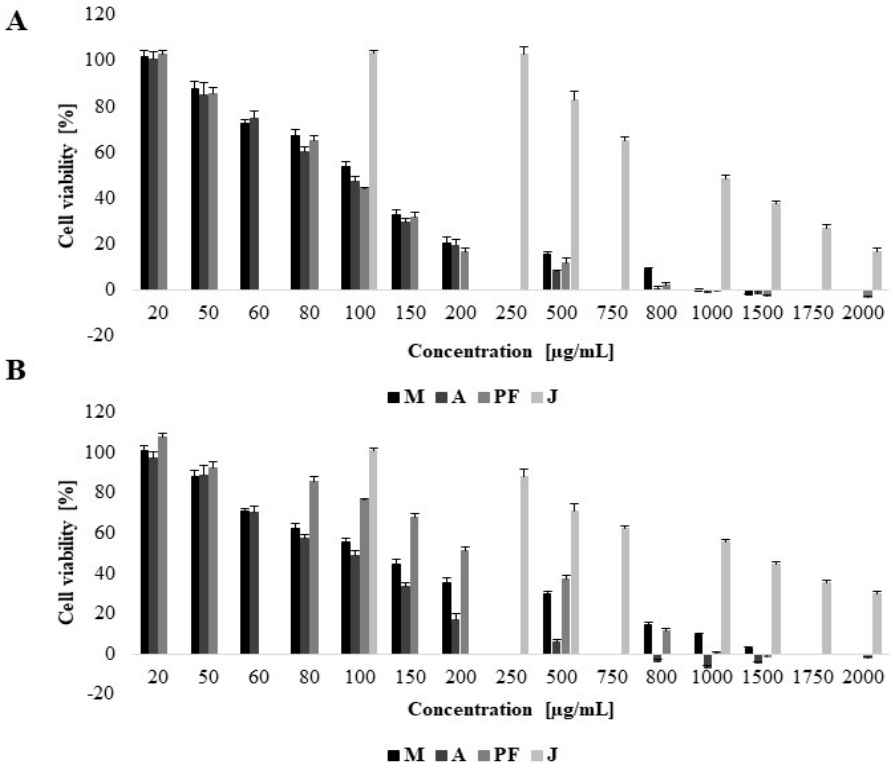

Fig. 1. Effect of *Viburnum opulus* fruit preparations on the viability of HeLa cells (A) and MCF cells (B) after 24 h incubation; values are mean ± standard deviation from at least eight independent experiments; the abbreviations used indicate the type of preparation M – methanol; A – acetone; PF – polyphenolic fraction; J – juice.
Source: Author's

Table 1. IC_{50} and IC_0 parameters of extracts obtained for HeLa and MCF-7 cells; values are means ± standard deviations from at least three independent experiments

Preparations	**HeLa**		**MCF-7**	
	IC_{50} (µg/mL)	IC_0 (µg/mL)	IC_{50} (µg/mL)	IC_0 (µg/mL)
Methanol: acetone: water	100,867 ± 0,101	20,173 ± 0,078	121,466 ± 0,121	24,293 ± 0,487
70% acetone	100,046 ±0,1	20,009 ± 0,128	243,641 ± 0,247	48,728 ± 0,307
Polyphenol fraction	63,541 ±0,162	12,781 ± 0,43	96,909 ± 0,097	19,380 ± 0,154
Juice	1031,512 ± 0,024	206,302 ± 0,362	1134,535 ± 1,135	226,907 ± 0,783

Source: Author's

The yield of inhibition of migration of the cells pre-treated with *Viburnum opulus* changed over time, which means that polyphenolic compounds contained in the preparations had a short-term effect. On the second day the number of actively migrating cells increased unambiguously, however the observed effect was stronger in HeLa cells (Fig. 2-3).

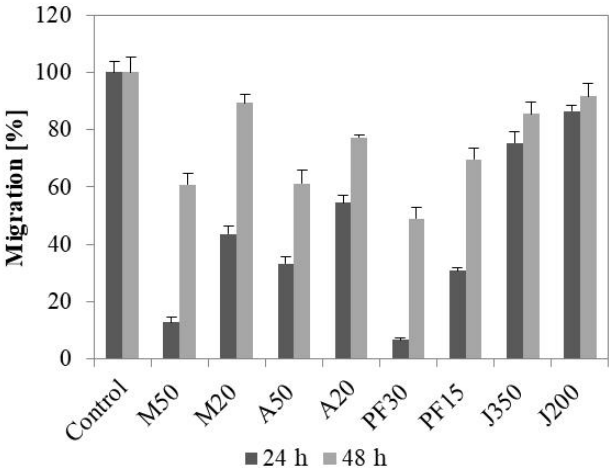

Fig. 2. Effect of *Viburnum opulus* fruit preparations on the rate of migration of the cervical cancer cell line (HeLa) for two incubation times (24 h and 48 h); values are means ± standard deviations from at least seven independent experiments; the abbreviations used indicate the type of preparation and the concentration used (for example M50 - methanol extract at concentration of 50 µg/mL)
Source: Author's

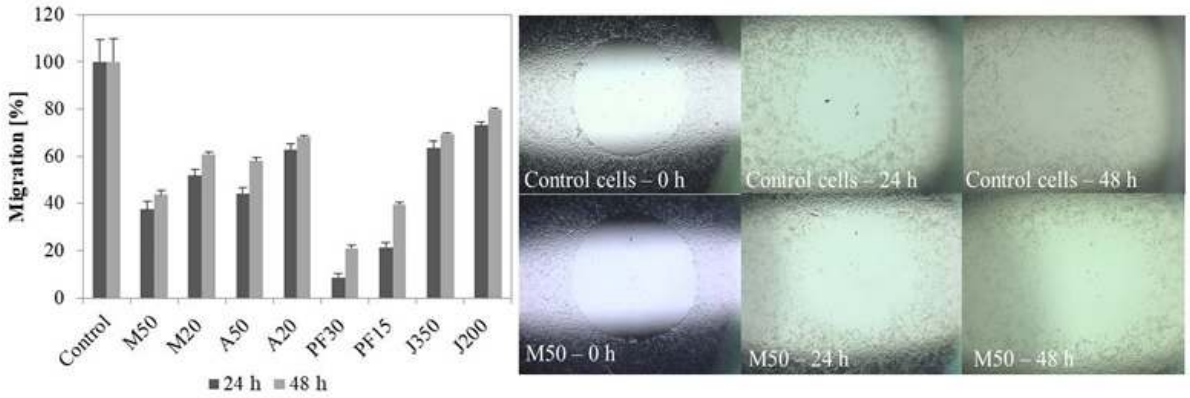

Fig. 3. Effect of *Viburnum opulus* fruit preparations on the rate of migration of the breast cancer cell line (MCF-7) for two incubation times (24 h and 48 h) and observed wound area of representative experiment; values are means ± standard deviations from at least seven independent experiments; the abbreviations used indicate the type of preparation and the concentration used (for example, M50 - methanol extract at concentration of 50 µg/mL)
Source: Author's

The anti-cancer properties of *Viburnum opulus* preparations are supported by a high content of polyphenolic compounds, like quercetin, epigallocatechin gallate (EGCG) and orgallic acid [13]. To the best of our knowledge there are no reports on the influence of extracts rich in polyphenolic compounds on the migration of tumor cells, but only reports of individual phytochemicals. Yu et al. analyzed the effect of quercetin (20 µM, 40 µM, 80 µM) on the migration of pancreatic cancer cells using the scratch test and the Transwell chamber. In all cases, after 24 h incubation the obtained results showed a correlation between the increase in the concentration of quercetin and a decrease in the number of cells that actively migrated. The inhibition of the rate of overgrowth observed was associated with the inhibition of migration, but also with a cytotoxic effect caused by quercetin [14]. In 2017, Farabegoli reported an inhibiting effect of EGCG at 25 µg/mL on MFC-7 cells migration based on the scratch assay. The concentration used did not demonstrate cytotoxicity to MCF-7 cells, but migration was inhibited by about 35% in comparison to cells cultured without polyphenols. After 48 hours the migration level was 37% lower. The effect of reduction of level of migration persisted for 72 hours and reached 45%. The study suggests that MFC-7 cells are susceptible to EGCG in the context of suppression of the migration process. This is also the basis for confirming the correctness of the results obtained in this work [15]. Another research study proved that polyphenol extract from *Phyllanthus emblica* (PEEP) tan grass leaves obtained by extraction with 70% acetone, inhibited proliferation of HeLa cells by 39% at a dose of PEEP 150 mg/mL [16]. The results of our work indicate that the preparation made with the same extraction method inhibits the migration of HeLa cells by about 85% the first day and by 40% on the second day at a non-toxic 50 µg/mL concentration.

The reduction of tumor invasiveness by lowering the rate of cell migration may have a different molecular basis. In counteracting tumor growth, modern medicine is based on reduction of the activity of small GTPases from the Rho protein family, metalloproteinases (MMP), vascular endothelial growth factor (VEGF), as well as the reduced expression of focal adhesion kinase (FAK) and c-Jun N-terminal kinases (JNK) [17, 18]. The debilitating factor of tumor cell invasiveness is mitochondrial dysfunction, which is accompanied by decrease of the mitochondrial potential, and in consequence, apoptotic cell death. Depolarization of mitochondrial membrane is caused by excessive production of reactive oxygen species (ROS), fragmentation of mitochondrial DNA (mtDNA), protein cross-linking and peroxidation of membrane phospholipids [19].

Mitochondrial dysfunction can promote progression of a cancer to an apoptosis-resistant/chemo-resistant and/or invasive phenotype by various mechanisms. During oncogenesis and tumor progression these mitochondrial alterations can activate cytosolic signaling pathways from mitochondria to the nucleus and ultimately alter nuclear gene expression for neoplastic transformation [20]. Chen et al. reported that caffeic acid phenethyl ester (CAPE), an active component isolated from honeybee propolis, induced apoptosis in human pancreatic cancer cells at 10 μg/mL, significantly decreased transmembrane potential of the mitochondrion in BxPC-3 cells and induced morphological changes of typical apoptosis (a 2-fold increase in caspase-3/caspase-7 activity in comparison to control cells) [21].

These reports have led us to attempt to determine the effect of *Viburnum opulus* on the mitochondrial potential and to link this phenomenon with a reduction in the rate of migration of tumor cell lines HeLa and MCF-7. For this purpose, we performed a test using a cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarocyanine iodide), which signals the mitochondrial membrane potential decrease. In healthy cells, the negative charge of the membrane allows the cationic dye to pass into the mitochondrial matrix where it accumulates into red aggregates with fluorescence when measured at 530/620 nm (it is excited by green light). In apoptotic cells, the mitochondrial potential decreases and the charge of the vines change, so that the cationic dye cannot accumulate in the mitochondria and accumulates in the cytoplasm in a monomeric form emitting green fluorescence, which is measured at 485/528 nm when excited by blue light. The ratio of red to green fluorescence is defined as the health of cells. The wavelength of 485/620 nm is used to measure fluorescence, which presents the degree of polarity of the mitochondrial membrane [22].

The results of the JC-1 test are shown for the HeLa cervical cancer cell line (Fig. 4) and for the human breast cancer cell line (Fig. 5). For both lines, at each concentration of preparations, a decrease of mitochondrial potential, as well as a decrease in metabolic activity, were noticed. These results were closely correlated with the applied concentration of formulations as well as the degree of migration of HeLa and MCF-7 cells. Again, the most active was a highly purified PF preparation. The preparations reduced the polarity of the mitochondrial membrane by nearly 50% at a non-toxic IC_0 concentration, and at a concentration of 30 μg/mL by nearly 60%, which was comparable to the CCCP used as positive control. It is worth noting that all tested preparations showed a higher ability to induce a decrease in potential and metabolic activity in the MCF-7 breast cancer line, which could also be noted in the long-term inhibition of the proliferation rate of these cells. An equally high depletion of mitochondrial potential was observed for centrifuged juice, however it worked more strongly against the MCF-7 than HeLaline. Significant differences in the effects on the two cell lines were observed for the M and A formulations. This time, the methanol: acetone: water (v/v/v) preparation was more active against the HeLa line than the MCF-7. However, the preparation extracted with 70% acetone reduced the mitochondrial potential and metabolic activity to the same extent for both cell lines.

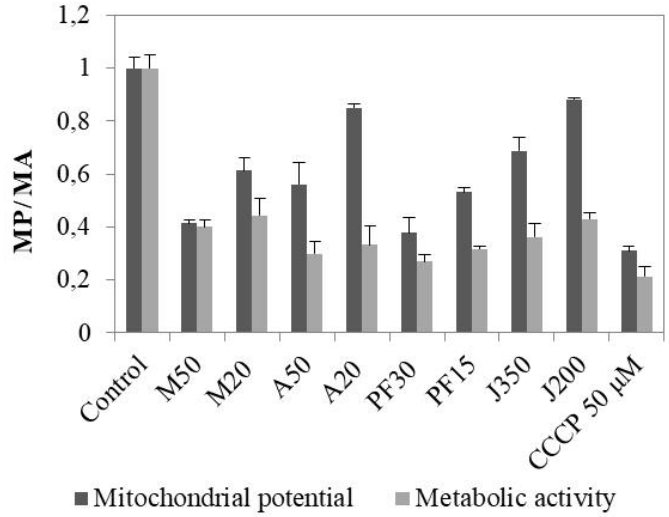

Fig. 4. Effect of *Viburnum opulus* fruit preparations on mitochondrial potential (MP) and metabolic activity (MA) of cervical carcinoma cell lines (HeLa); values are means ± standard deviations from at least three independent experiments; the abbreviations used indicate the type of preparation and the adjusted concentration (for example M50 - methanol extract in a concentration of 50 µg/mL)
Source: Author's

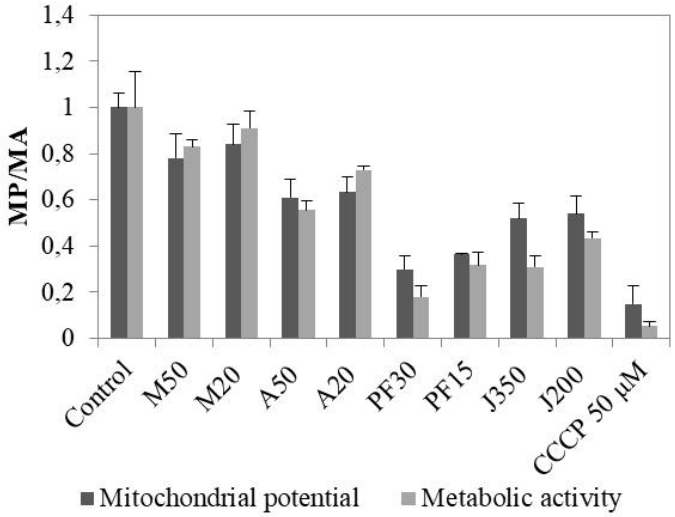

Fig. 5. Effect of *Viburnum opulus* fruit preparations on mitochondrial potential (MP) and metabolic activity (MA) of breast cancer cell lines (MCF-7); values are means ± standard deviations from at least three independent experiments; the abbreviations used indicate the type of preparation and the concentration used (for example, M50 - methanol extract at a concentration of 50 µg/mL)
Source: Author's

Drastic reduction of mitochondrial potential with simultaneous loss of cell biological activity usually lead to cellular apoptosis. Resistance to apoptosis is the main cause of cancer's insensitivity to conventional therapies. Therefore, one of the strategies employed is to look for factors that lead to cellular apoptosis induction. The latest reports showed that caffeic acid phenethyl ester (CAPE), a functional ingredient isolated from propolis, effectively reduced the number of proinflammatory cytokines and inflammatory mediators by inhibiting the transcription of the nuclear factor κ-light chain inhibitor of activated B cells (NF-κB). Several papers focused on the protective role of CAPE against general tumor models, both in vivo and in vitro for melanomas, lung and prostate cancers [23]. CAPE is a natural phenolic compound and an ester of phenethyl alcohol in the form of caffeic acid. Its presence was also found in the fruit of *Viburnum opulus* [8]. These reports lead us to conclude that the *Viburnum opulus* preparations we examined are capable of inducing mitochondrial dysfunction. This was proven by the reduction of the mitochondrial potential and the decrease in the metabolic activity of cancer cells. This resulted in the activation of caspase-3 and caspase-7, inhibition of the NF-κB factor, activation of the

Fas signal and ultimately in apoptosis induction. Analogous results for MCF-7 breast cancer were obtained by Liao et al., which were simultaneously proven in many independent studies with other cell lines [24].

The reduction of mitochondrial potential and dysfunction is accompanied by damage to the respiratory chain and overproduction of reactive oxygen species (ROS). This is observed due to the increased number of reduced forms of electron and proton transporters, such as the reduced form of nicotinamide adenine dinucleotide (NADH + H+) and flavin adenine dinucleotide (FADH2). The consequence of this process is the hyperpolarization of the internal mitochondrial membrane by increased electron flow through the respiratory chain. Finally, it stops the transformation of the respiratory chain at the complex III and the increased production of superoxide anion horn and mitochondrial dysfunction [25]. Taking this into consideration, we performed a study of the preparations influence on the intracellular oxidative stress using the DCFH-DA probe. This method consists of oxidizing the substrate (2',7'-dichlorodihydrofluorescein) introduced into the system in the form of an ester (diacetate, H2DCF-DA). The ester undergoes spontaneous hydrolysis reaction or a hydrolysis reaction which is catalyzed by hydrolases with the release of the product in the form of an oxidized DCF-DA, which can be recorded.

The results indicated the antioxidant capacity of the studied preparations of *Viburnum opulus* (Fig. 6). The highest decrease in ROS was observed for the PF preparation, as well as for the juice. At the IC_{20} concentration of PF, intracellular oxidative stress was reduced by 40% for HeLa and by 20% for MCF-7 cells. M and A formulations at IC_0 and IC_{20} resulted in the reduction of ROS concentrations by approximately 20%. It is clear that polyphenols contained in plant extracts have the ability to inhibit the production of free radicals, however, during mitochondrial dysfunction the apoptosis-induced ROS concentration in cells increase [18]. In 2016, Li et al. demonstrated that green tea preparation influenced internal oxidative stress of MCF-7 cells with an approximately 11-fold increase in ROS accumulation at 50 mg/mL dosage of preparation. Payen et al. showed that in SiHa-F3 cells EGCG at concentrations of 100 nM, 1 µM, 10 µM and 100 µM revealed a growing antioxidant effect which was correlated with the EGCG concentration [26].

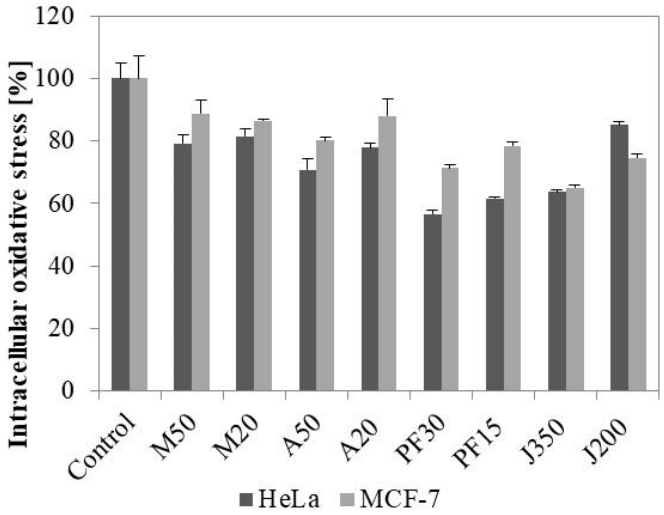

Fig. 6. Effect of *Viburnum opulus* fruit preparations on intracellular oxidative stress of cervical carcinoma cell line (HeLa) and breast cancer cell line (MCF-7); values are means ± standard deviations from at least three independent experiments the abbreviations used indicate the type of preparation and the concentration used (for example, M50 - methanol extract at a concentration of 50 µg/mL)
Source: Author's

Summary and conclusions

In summary, there is great interest in polyphenolic compounds in the context of antitumor activity, as well as in their inhibition of tumor cell migration and metastasis. In recent years a number of studies have been carried out showing a relationship between the increase of the concentration of various polyphenolic compounds (such as caffeic acid, gallic acid, quercetin, epigallocatechin gallate) and a decrease of migration process. The conducted studies showed that polyphenolic preparations obtained from *Viburnum opulus* fruits inhibit the migration of tumor cells. In some cases there is a drastic reduction in the number of migrating cells (up to 90%

- PF in the concentration of 30 μg/mL) for both of cell lines. At the same time, they show the ability to reduce the mitochondrial potential and metabolic activity of cells (up to 80% for PF for MCF cells), which was probably correlated with the degree of reduction of actively migrating cells. Despite the ability to drastically decrease the polarization of the mitochondrial membrane, the tested extracts showed antioxidant properties, which seem to also have a beneficial effect. The results obtained indicate the high potential of *Viburnum opulus* polyphenolic compounds as components that can be used in the production of innovative dietary supplements or pharmacological preparations dedicated to people with increased risk or inclination of breast or cervical cancer.

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