© by PSP Volume 28 – No. 5/2019 pages 3718-3723 Fresenius Environmental Bulletin

ROSA CANINA L. ETHANOLIC EXTRACT INDUCES THE ANTI-PROLIFERATIVE AND APOPTOSIS POTENTIAL IN MCF-7 AND MDA-MB-468 CELL LINES

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ABSTRACT

Rosa canina L. (rose hip) fruits have been used for their diuretic, laxative, anti-gout, anti-rheumatism properties in traditional medicine. Rose hip berries contain a variety of components such as flavonoid. The previous studies showed that flavonoid has anti-cancer properties. Tha aim of this study is to evaluate and screen the effect of apoptosis and the anticancer potential of rose hip ethanolic extract on human breast cancer cell lines; MCF-7 and MDA-MB-468. The anti-proliferative activity of rose hip extract was evaluated using MTT, flowcytometry by annexin V/PI double staining, and caspase-3 activity. The results of MTT showed that the ED_{50} of both human breast cancer cell lines was 25µg/mL of rose hip extract, 48 hours after treatment. Flowcytometry by annexin V/PI showed that rose hip extract induced late apoptosis in MCF-7 and early apoptosis in MDA-MB-468. In addition, the caspase-3 colorimetric method showed that caspase-3 increased in the MDA-MB-468 after treatment with rose hip extract. As a result, the ethanol of rose hip ethanolic extract induced apoptosis in both human breast carcinoma cell lines.

KEYWORDS:
Rosa canina L., rose hip, MCF-7, MDA-MB-468, anti-proliferation, apoptosis

INTRODUCTION

In normal conditions, the body has a balance between cell death and cell proliferation. This balance is known as hemostasis and is necessary for normal cell growth. If this balance is disturbed, it leads to cancer. Cancer is a serious concern all over the world and the most common cause of mortality and morbidity after cardiovascular diseases [1]. Breast cancer is the most common cancer among women; each year nearly 400,000 women suffering from this disease lose their lives, and nearly 234,000 new cases of breast cancer are reported every year [2]. The known causes of breast cancer are not so similar to those of other cancers; however, studies indicate various factors that can increase the risk of getting breast cancer, such as genetic predisposition, obesity, pregnancy after the age of 35, exposure to radiation dangers such as UV, a history of cancer in first-degree relatives, a history of breast cancer in one breast, and continuous use of birth control pills overa long period [1].

Currently, the treatment for breast cancer is dominated by modern medicine, which relies more on surgery, radiotherapy, chemotherapy, hormone therapy, immunotherapy, and so on [1]. Unfortunately, in most cases, treatment is not effective or leads to unpleasant side effects, hence researchers are trying to use compounds with fewer side effects and to induce apoptosis in cancer cells [3]. Natural products can be used as medicines to treat cancer. Since the 1950s, 60% of cancer drugs have been made from natural products or their derivatives. Plants and herbs are low-cost natural products with few side effects that can be used for cancer treatment. In this way, not only are cancer cells controlled, but also healthy cells are not damaged [4].

Rosa canina (rose hip) is the fruit of the rose plant that ranges in color from red to orange; however, there are some species whose color ranges from dark purple to black. Rose hip extracts have been used as chemopreventive agents due to their mechanistic actions on cancer cells [5]. The elevated levels of lycopene found in rose hip extracts are associated with increased apoptosis in prostate cancer. Rose hip fruits have shown potent anti-proliferative activity against colon, breast, and cervical cancer cells in vitro. Rose hip is high in vitamin C causing this pseudo fruit to be rich in ascorbic acid, phenolic components, and carotenoids [6,7]. These high antioxidant contents cause rose hip to have high antioxidant activities. Studies have shown that it is useful in treating arthritis due to its anti-inflammatory and anti-oxidant effects.

Studies have shown that the Vitamin C and flavonoids found in rose hip is responsible for the antioxidant activity associated with rose hip products [5]. However, it is the polyphenols found within this plant that is responsible for the antiproliferative activity. The anti-proliferative effects on cell proliferation in estrogen receptor positive (ER^+) breast cancer cell line (MCF-7) and estrogen receptor negative (ER^-) breast cancer cell line (MDA-MB-468) have not yet been tested. The current study was conducted to evaluate and screen the effect of apoptosis and the anticancer potential of rose hip extract on MCF-7 and MDA-MB-468 cell lines.

METHODS AND MATERIALS

Plant materials. Berries of rose hip was collected from the Bekiralanı village (on Toros Mountains) of Turkey's Mersin province (GPS coordinates 36° 59' 03.6'' N, 34° 31' 24.1'' E). Plant collection was done in May 2017. Prof. Dr. Ali Aslan confirmed the taxonomic determination of *Rosa canina* L.

Preparation of hydroalcoholic extract of rose hip. After the collection, rose hip berries were dried in indirect light and a clean environment. The freeze-dried rose hip berries were grounded to powder and then 70% ethanol solution was extracted using a Soxhlet extractor. The solution was filtered and evaporated under a rotary evaporator in order to obtain a hydroalcoholic extract; the solid extract was stored in a freezer at -20 °C.

Cell culture. Cancer cell lines; MCF-7 and MDA-MB-468 were obtained from from American Type Culture Collection (ATCC). The cell lines were grown adherently as a monolayer in 75 mL plastic flasks in RPMI 1640 medium supplemented with 10% FBS (heat inactivated 30 minutes, 56 °C before use), 100 U/mL penicillin, and 100 µg/mL streptomycin in incubator under standard cultured condition (37 °C and 5% CO_2). For enumeration, 30µL of trypan blue (0.2%) stained the same volume (30 µL) of cell concentration, and neobar lam was used for counting and viability (more than 95% for adhering cell lines before testing) of the cells.

Cell treatments. For treatment with rose hip extract, first rose hip extract as powder was dissolved in DMSO and kept in a freezer at -20 °C. The cell lines were seeded into sterile 6 or 96 well plates; the cell number was almost equal in all the wells for adhering cell lines to button plates. Incubation was done over night. The medium was aspirated, and different concentrations that were prepared with medium and different concentrations of rose hip extract (0-12.5-25-50-100 µg/mL) were added. The number of cell lines in seeding was different in different tests as indicated therein.

Cell viability assay. Cell viability and antiproliferation of rose hip extract were carried out through an MTT reduction assay [6]. The measurement was repeated in triplicate to confirm the results. The results were calculated by dividing the percentage of absorbance in the treated cells by the percentage of absorbance in the untreated (control) cells, defined as the viability percentage. ED_{50} was 50%, the concentration range that inhibits the growth of cell lines.

Apoptosis assay. The apoptotic effect of rose hip extract on the cell lines was analyzed by means of a flowcytometry assay, using an annexin V/Propidium iodide (PI) double staining Kit (Biovision, San Francisco, California, USA), according to the manufacturer's protocol. Partec PAS II flowcytometry was used for analysis of the samples. The results consist of four sections: living cells (were not stained with PI or annexin V), early apoptosis (stained with annexin V connected to phosphatidylserine in outer layer of cell membrane), late apoptosis (stained with both annexin V and PI to fragmented DNA), and necrosis cells (stained with PI) [9].

Caspase-3 activity assay. A colorimetric assay kit (R&D System Co., Minneapolis, Minnesota, USA) was used for measuring caspase-3 activity according to the manufacturer's protocol.

Statistical analysis. The data were analyzed using SPSS 15.0 software package. The values are expressed as mean ± SD. Analysis of variance (ANOVA) was conducted, followed by Tukey correction, to test for differences in mean values between groups. The results were considered significant at $p<0.05$.

RESULTS

The morphology of the MCF-7 cell line changed in a dose- and time-dependent manner; in low concentrations, the cells were deformed, and with increasing dose and time, granulated cellular contents, dropsy, and shrinkage were increased, even, after 48 hours (at a 100 µg/mL concentration) and 72 hours (at 50µg/mL and 100µg/mL concentrations), the rupture of membranes and the release content of cytosol were clearly observed. The MDA-MB-468 cell line had approximately the same condition (Figure 1).

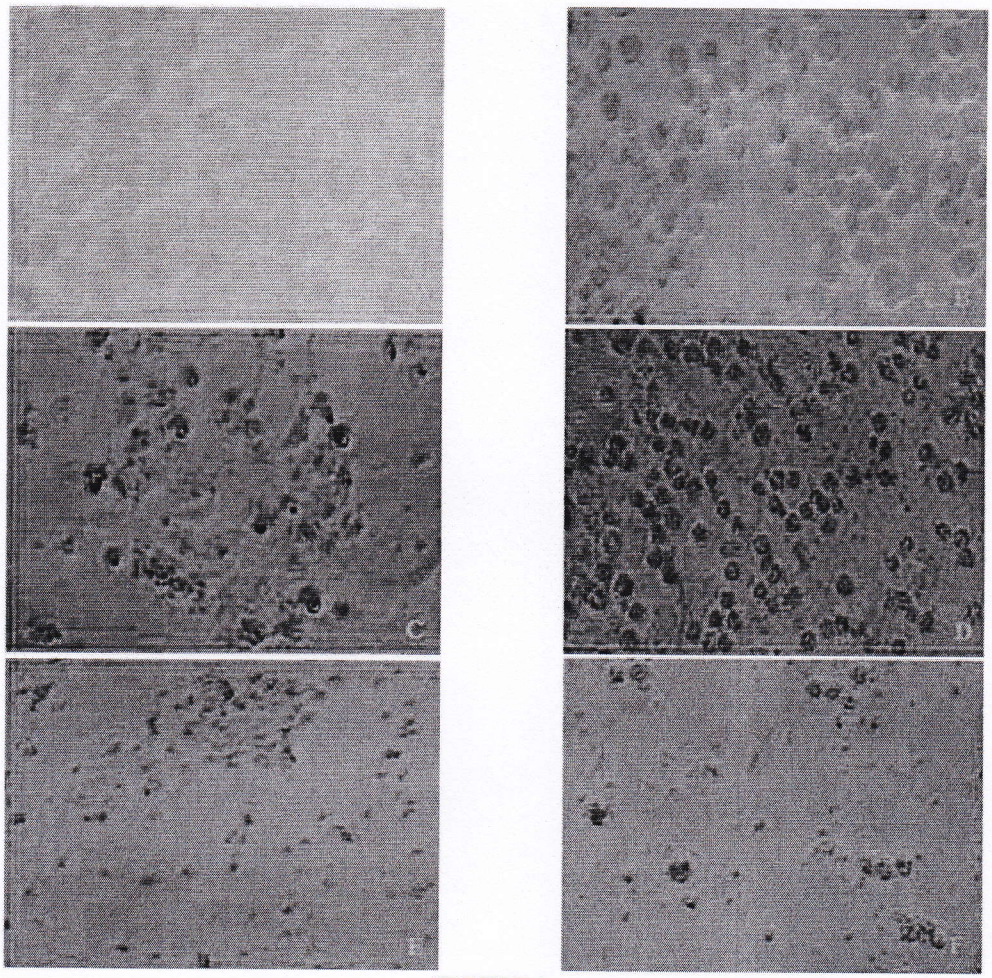

FIGURE 1

Effects of rose hip extract on the morphological alterations of MCF-7 and MDA-MB-468 cells were monitored for 72 hours at two different doses of rose hip extract.

Control MCF-7 cells (Figure 1A) and control MDA-MB-468 cells (Figure 1B) dispersed homogeneously with distinct boundaries after over night incubation. Figure 1C; MCF-7 cells exposed to 50 µg/mL rose hip extract for 72 hours, Figure 1D; MDA-MB-468 cells exposed to 50 µg/mL rose hip extract for 72 hours, Figure 1E; MCF-7 exposed to 100µg/mL rose hip extract for 72 hours, and Figure 1F; MDA-MB-468 exposed to 100µg/mL rose hip extract for 72 hours.

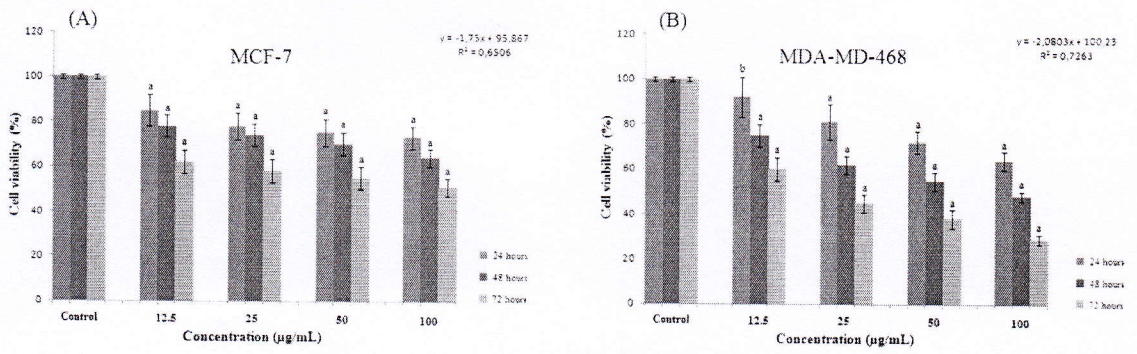

FIGURE 2

Effect of rose hip extract in inhibition of cell growth of the breast cancer MCF-7 (shown in Figure 2A) and MDA-MB-468 (shown in Figure 2B) cell lines.

Cells were treated with different concentrations of rose hip extract for 24, 48 and 72 hours, and proliferation was measured withan MTT assay. Rose hip extract reduced cell proliferation in MCF-7 (25µg/mL) and MDA-MB-468 (25µg/mL) breast cells in a time- and dose-dependent manner. Each value is presented as a mean ± SD of three experiments (each triplicate).[a]p <0.01; [b]p<0.05 compared to untreated control groups.

The effects of rose hip extract on both human breast carcinoma cell lines were examined. The cells were exposed to different concentrations of rose hip extract for 24, 48, and 72 hours. After these periods, the cell lines' viability was measured by an MTT assay. Concentration 25 µg/mL of rose hip extract had significant inhibitory effects on both cell lines: 51.67±1.527 for MCF-7 and 53,48±1.577 for MDA-MB-468 after treatment for 48 hours. Exposed to 25 µg/mL concentration of rose hip extract during 48 hours can inhibit growth by 50% in both cell lines. Figure 2 shows the results of MTT in both cell lines, showing a significant difference in the dose-time manner ($p<0.001$).

The cell lines were stained with annexin V/PI and analyzed using flowcytometry to explore whether rose hip extract shows cytotoxicity through induction of apoptosis. To do so, the cell lines were treated in appropriate doses (0-12.5-25-50-100 µg/mL) for 48 hours. The cell population shifted from viable (annexin V^-/PI^-) to early apoptosis (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+) in high doses and in the MDA-MB-468 cell line more towards early apoptosis (annexin V^+/PI^-) through an increase in the dose, based on the results of annexinV/PI double staining also in the MCF-7 cell line through induction of late apoptosis (annexin V^+/PI^+). These shifts were significantly different in both cell lines ($p<0.001$) (Figure 3).

Caspase enzymes play an important role in apoptotic responses; therefore, the effect of rose hip extract in inducing apoptosis in the cell lines was investigated by measuring the activity of caspase-3. The treatment of the MDA-MB-468 cell line of breast cancer in a 25 µg/mL concentration of rose hip extract was observed. Also, a significant increase in caspase-3 activity of MDA-MB-468 was observed after treatment with rose hip extract in ED_{50} (approximately 25 µg/mL) at various times (6, 12, and 24 h), and the amount of caspase-3 differed significantly between treated and untreated cell lines ($p<0.001$); however, there was no such increase in the MCF-7 cell line (Figure 4).

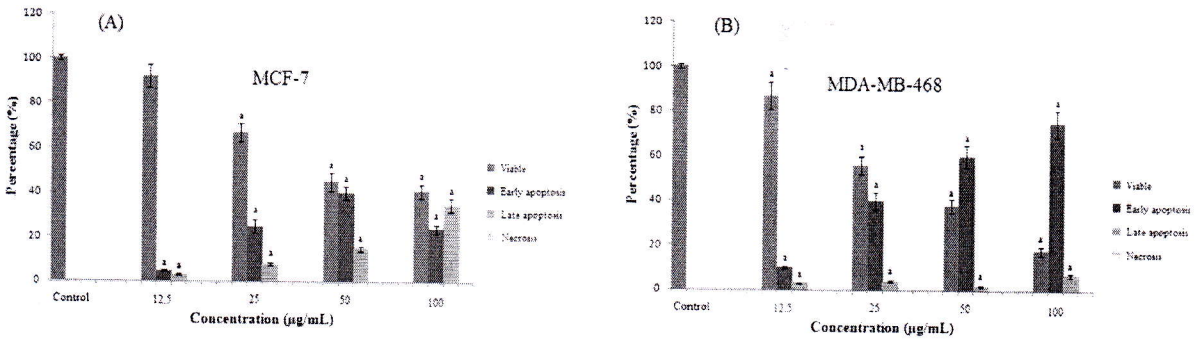

FIGURE 3

Flowcytometric evaluation of apoptosis in MCF-7 cells (Figure 3A) and in MDA-MB-468 cells (Figure 3B) using annexin-V/PI double staining.

After 48 hours treatment, rose hip extract resulted in a significant increase in late apoptotic cells and a moderate increase in early apoptotic cells, in a dose-dependent manner. Results, mean ± SD three independent experiments. [a]$p<0.01$ compared to untreated control groups.

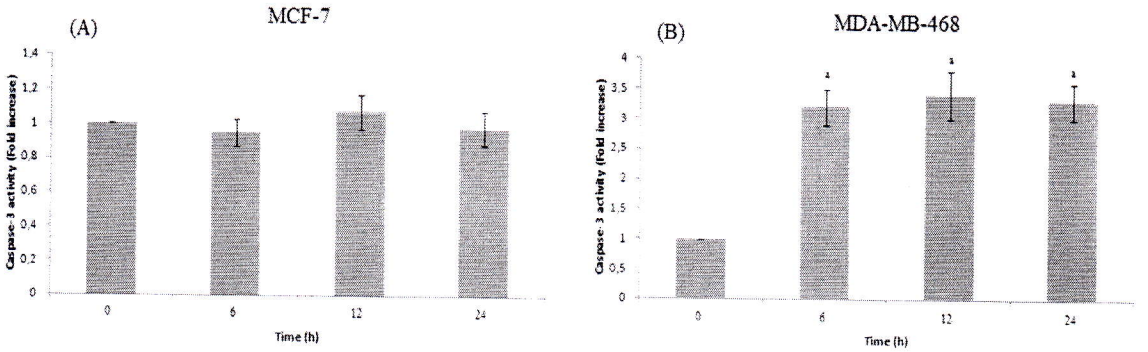

FIGURE 4

The effect of rose hip extract on caspase-3 activity in MCF-7 (Figure 4A) and MDA-MB-468 (Figure 4B) cells.

Cells were incubated to a concentration of rose hip extract (25µg/mL) in a time-dependent manner (6, 12, and 24 hours). [a]$p<0.01$ compared to untreated control groups.

DISCUSSION

Breast cancer has the highest prevalence among women compared to other cancers, and surgery is the main therapy for this disease, with chemotherapy, radiotherapy, hormone therapy, and gene therapy used as minor therapies. However, these therapies have several problems. For example, in surgery, in addition to tumor cells, healthy cells are also removed; the radiation used in minor therapy harms normal cells [10, 11].

Moreover, these treatments are expensive. Thus, many researchers have focused on lowpriced new drugs with a natural origin that induce apoptosis in tumor cells, but not in healthy cells, and are especially prepared using local and traditional medicines [3, 4].

Plants as natural resources have played a vital role as a source of effective agents against cancer [3]. Natural products such as *Ginkgo biloba* have been shown to have anti- proliferative properties in breast cancer cells [12]. Park et al. [13] observed the inhibitory effects of cell proliferation using *Ginkgo biloba* extract in MDA-MB-231 breast cancer cell line. Pomegranate extracts and genistein have been observed to have cytotoxic and anti-proliferative effects in MCF-7 cancer cells [14]. Tanih et al. [15] observed the crude acetone extract of *Sclerocarya birrea* inhibited proliferation of the MCF-7 cell line via an apoptotic programmed cell death. Yang and colleagues [16] observed the anti-migratory and anti-invasion effects of chrysin in MDA-MB-231 and BT-549 cell lines.

Rose hip is commonly known as "*kuşburnu, itburnu, gülburnu, gül elması*" in Turkish herbal medicine. Previous studies have shown that it has antioxidant properties and contains carotenoids, flavonoids, and polyphenolic compounds that are known as anticancer components. Interestingly, Rose hip extract has antioxidant properties [5]. In the current study, the anticancer and apoptotic activities of rose hip extract were investigated. The results showed that rose hip extract exhibited cytotoxicity towards the MCF-7 and MDA-MB-468 human breast cancer cell lines with an ED_{50} of 25 µg/mL for both; this was determined by means of an MTT assay. To determine the type of cell death (apoptosis or necrosis), annexinV/PI double staining was used. The findings showed that rose hip extract induced apoptosis in MCF-7 and MDA-MB-468, while the apoptotic cells of both cancer cell lines were shifting to late apoptosis and early apoptosis, respectively.

Apoptosis is a programmed cell death that regulates normal physiological processes and plays an essential role in the progress and maintenance of tissue homeostasis. Apoptosis has two main pathways, an extrinsic apoptosis pathway (death receptor dependent pathway) and an intrinsic apoptosis pathway (mitochondria-dependent apoptosis) [17]. Briefly, in the extrinsic apoptosis, the interaction between the death ligand and the death receptor leading to caspase-8 activation as a starter caspase, activated caspase-8 then activates caspase-3, while in the intrinsic apoptosis pathway, cytochrome C is released from mitochondria leading to caspase-9 activation as a starter caspase, activated caspase-9 then activates caspase-3 [18]. Thus, in this study, caspase-3 activity was measured; it was found that caspase-3 increased in the MDA-MB-468 cell line in a time dependent manner (25µg/mL of rose hip extract). Caspase activity did not change in a time-dependent manner (25µg/mL of rose hip extract) because the MCF-7 cell line did not express caspase-3 due to deletion (functional 47 bp) inside the exon 3 of capsase-3 gene. Therefore, the induction of apoptosis in the MCF-7 cell line was treated with rose hip extract, probably due to a caspase-independent or a non-caspase-3-dependent mechanism [19].

There is little data regarding the effect of rose hip extract on cell lines. The anti-oncogenic properties of rose hip extracts have been tested in HeLa, Caco-2, and HT-29 cancer cell lines and their effects have been suggested to be related to anti-oxidant and anti-proliferative effects [6, 7]. Rose hip extracts have also been reported to modulate the activity of one or more phases involved in cell cycle regulation. One of the key factors in the development and progression of cancer is the ability of cells to promote cell proliferation and inhibit apoptosis [7]. Therefore, any treatment possessing antiproliferative effects in cancer cells regaining that balance between cell proliferation and cell death are indicative of having potential anti-oncogenic properties [6, 7].

In conclusion, our study demonstrates for the first time the cytotoxic effect of the ethanol extract of rose hip against the MCF-7 and MDA-MB-468 cancer cell line depending on the dose of exposure without affecting the normal cells. We also reported that rose hip ethanol extract display cell lysis by the apoptosis pathway. These findings provide evidence that rose hip has potential as an anti-breast cancer agent.

REFERENCES

[1] Arteaga, C.L. (2013) Progress in breast cancer: overview. Clinical Cancer Research. 19, 6353-6359.

[2] Rastegar, H., Ahmadi Ashtiani, H., Anjarani, S., Bokaee, S., Khaki, A. and Javadi, L. (2013) The role of milk thistle extract in breast carcinoma cell line (MCF-7) apoptosis with doxorubicin. Acta Medica Iranica. 51, 591-598.

[3] Buyel, J.F. (2018) Plants as sources of natural and recombinant anti-cancer agents. Biotechnology Advances. 36, 506-520.

[4] Kapinova, A., Stefanicka, P., Kubatka, P., Zubor, P., Uramova, S., Kello, M., Mojzis, J., Blahutova, D., Qaradakhi, T., Zulli, A., Caprnda, M., Danko, J., Lasabova, Z., Busselberg, D. and Kruzliak, P. (2017) Are plant-based functional foods better choice against cancer than single phytochemicals? A critical review of current breast cancer research. Biomedicine and Pharmacotherapy. 96, 1465-1477.

[5] Chrubasik, C., Roufogalis, B.D., Müller-Ladner, U. and Chrubasik, S. (2008) A systematic review on the Rosa canina effect and efficacy profiles. Phytotherapy Research. 22, 725-733.

[6] Tumbas, V.T., Canadanović-Brunet, J.M., Cetojević-Simin, D.D., Cetković, G.S., Ethilas, S.M. and Gille, L. (2012) Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. Journal of the Science of Food and Agriculture. 92, 1273-1281.

[7] Jiménez, S., Gascón, S., Luquin, A., Laguna, M., Ancin-Azpilicueta, C. and Rodríguez-Yoldi, M.J. (2016) *Rosa canina* extracts have antiproliferative and antioxidant effects on Caco-2 human colon cancer. PLoS One. 11, 1-14.

[8] Hashemi, M., Karami-Tehrani, F., Ghavami, S., Maddika, S. and Los, M. (2005) Adenosine and deoxyadenosine induces apoptosis in oestrogen receptor-positive and -negative human breast cancer cells via the intrinsic pathway. Cell Proliferation. 38, 269-285.

[9] Dai, G., Zheng, D., Guo, W., Yang, J. and Cheng, A.Y. (2018) Cinobufagin induces apoptosis in osteosarcoma cells via the mitochondria-mediated apoptotic pathway. Cellular Physiology and Biochemistry. 46, 1134-1147.

[10] Patten, D.K., Sharifi, L.K. and Fazel, M. (2013) New approaches in the management of male breast cancer. Clinical Breast Cancer. 13, 309-314.

[11] Akram, M. and Siddiqui, S.A. (2012) Breast cancer management: past, present and evolving. Indian Journal of Cancer. 49, 277-282.

[12] Zhao, X.D., Dong, N., Man, H.T., Fu, Z.L., Zhang, M.H., Kou, S. and Ma, S.L. (2013) Antiproliferative effect of the Ginkgo biloba extract is associated with the enhancement of cytochrome P450 1B1 expression in estrogen receptor-negative breast cancer cells. Biomedical Reports. 1, 797-801.

[13] Park, Y.J., Kim, M.J., Kim, H.R., Yi, M.S., Chung, K.H. and Oh, S.M. (2013) Chemopreventive effects of Ginkgo biloba extract in estrogen-negative human breast cancer cells. Archives of Pharmacal Research. 36, 102-108.

[14] Jeune, M.A., Kumi-Diaka, J. and Brown, J. (2005) Anticancer activities of pomegranate extracts and genistein in human breast cancer cells. Journal of Medicinal Food. 8, 469-475.

[15] Tanih, N.F. and Ndip, R.N. (2013) The acetone extract of Sclerocarya birrea (Anacardiaceae) possesses antiproliferative and apoptotic potential against human breast cancer cell lines (MCF-7). The Scientific World Journal. 2013, 1-7.

[16] Yang, B., Huang, J., Xiang, T., Yin, X., Luo, X., Huang, J., Luo, F., Li, H., Li, H. and Ren, G. (2014) Chrysin inhibits metastatic potential of human triple-negative breast cancer cells by modulating matrix metalloproteinase-10, epithelial to mesenchymal transition, and PI3K/Akt signaling pathway. Journal of Applied Toxicology. 34, 105-112.

[17] Francis, S.H., Turko, I.V. and Corbin, J.D. (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. Progress in Nucleic Acid Research and Molecular Biology. 65, 1-52.

[18] Lugnier, C. (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacology and Therapeutics. 109, 366-398.

[19] Yu, J.H., Zheng, G.B., Liu, C.Y., Zhang, L.Y., Gao, H.M., Zhang, Y.H., Dai, C.Y., Huang, L., Meng, X.Y., Zhang, W.Y. and Yu, X.F. (2013) Dracorhodin perchlorate induced human breast cancer MCF-7 apoptosis through mitochondrial pathways. International Journal of Medical Sciences. 10, 1149-1156.

Received: **03.07.2018**
Accepted: **19.03.2019**

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