THE ASSESSMENT OF MODULATORY EFFECTS OF BLACKCURRANT (RIBES NIGRUM L.) AND CHOKEBERRY (ARONIA MELANOCARP A L.) ON OVARIAN CELL FUNCTIONS IN VITRO

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ABSTRACT

Blackcurrant fruits (Ribes nigrum L.) or black chokeberry fruits (Aronia melanocarpa L.) rich in phytochemicals that may act as potent antioxidant and anticancer agents have recently gained increasing attention. A beneficial health effect of blackcurrants and chokeberries is mostly attributed to their phenolic and anthocyanin contents. We investigated the influence of blackcurrant and chokeberry extracts at the concentration range 10-100 µg/mL (24h) on human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3. All parameters were evaluated spectrophotometrically. Cell viability was measured by AlamarBlue™ assay, and the release of steroid hormones (17β-estradiol and progesterone) was assayed by ELISA. The results showed that the viability of non-cancer cells HGL5 significantly increased by blackcurrant extract at a concentration of 10 µg/mL and by chokeberry extract at 10, 20, 50, and 100 µg/mL concentrations. The number of viable cancer cells OVCAR-3 was significantly reduced by blackcurrant extract at the concentrations of 20, 50 and by chokeberry extract at 100 µg/mL and at 100 µg/mL. Chokeberry extract slightly stimulated 17β-estradiol release by HGL5 cells and blackcurrant extract exhibited stimulatory effect on 17β-estradiol release at a concentration of 10 µg/mL. On the other hand, both extracts failed to affect progesterone release. Extracts did not cause any significant changes in total superoxide dismutase (SOD) level determined in HGL5 cells. On the other hand, a significant decrease of SOD level was noted in cancer cells OVCAR-3 treated by blackcurrant extract at the concentrations 20, 50 and 100 µg/mL and in cancer cells treated by chokeberry extract at a concentration of 100 µg/mL. In conclusion, this in vitro study suggests the action of both, blackcurrant and chokeberry extracts on human ovarian cell functions including viability and secretory activity, and possible use for the benefit of human health.

Keywords: blackcurrant, chokeberry, ovarian cells, viability, steroidogenesis, cancer, SOD

INTRODUCTION

Phytonutrients, especially polyphenols and anthocyanins have recently gained increasing research interest due to their effects on biological systems, including cytotoxic and anti-cancer properties, as well as their possible use for the benefit of human health (Lourenço et al., 2019; Gill et al., 2020). Many studies have showed that polyphenolic compounds may have a potential to ameliorate alterations of the reproductive system (Michalova et al., 2019; Baldovská et al., 2019, 2020; Sirotkin and Kolesarova, 2022). The research of novel regulators of reproductive processes is important for finding solutions of various problems of modern society, such as infertility and increasing risk of reproductive disorders (Canipari et al., 2020; Sirotkin and Kolesarova, 2022). Moreover, gynecological cancers are the key cause of mortality in women (Akkol et al., 2020) and ovarian cancer is one of the most common malignant tumours (Siegel et al., 2018). The majority of reproductive dysfunctions have similar causes and mechanisms such as the accumulation of reactive oxygen species (ROS), which lead to oxidative stress in the cells. On the other hand, oxidative stress is can be prevented by antioxidants (Wojsiat et al., 2017). Some phytosubstances can positively impact reproductive processes and prevent numerous reproductive disorders (Akkol et al., 2020; Sirotkin and Kolesarova, 2022).

Blackcurrants (Ribes nigrum L.) and black chokeberries (Aronia melanocarpa L.) represent fruits species utilized mainly as jams, jellies, juices, wine, and as important food colorants or nutritional supplements (Jurikova et al., 2017) and both present a rich source of valuable phytonutrients (Bernier et al., 2021). They are polyphenols, especially cyanidins, that are responsible for health benefits (Olechno et al., 2022). Anthocyanins are the most important polyphenolic compounds contained in blackcurrant fruits responsible for their health benefits. Modern laboratories have demonstrated the anti-inflammatory, antioxidant, antiviral and anti-cancer effects of blackcurrant constituents (Bishayee et al., 2010; Gopalan et al., 2012). Anthocyanins derived from anthocyanidins cyanidin and delphinidin are typical for blackcurrant berries, especially their rutinoside and glucoside forms. Major components, concretely delphinidin-3-rutinoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and cyanidin-3-glucoside usually represent about 97-98% of total anthocyanins in blackcurrant fruits (Simerdová et al., 2021) and together with flavonols, phenolic acids and polysaturated fatty acids are found to protect cells from oxidative stress (Gopalan et al., 2012; Hui et al., 2021). Black chokeberry is one of the richest sources in polyphenols such as phenolic acids (neochlorogenic and chlorogenic acids) and flavonoids (anthocyanins, proanthocyanidins, flavanols and flavonol glycosides), particularly cyanidin-3-galactoside and cyanidin-3-arabinoside, as well as (−)-epicatechin units (Rubiná et al., 2012; Jurikova et al., 2017). Black chokeberry fruits exhibit a wide range of therapeutical properties, among which the most frequently mentioned are antioxidant, anti-inflammatory, anti-proliferative, and anti-cancer effects (Kędzierska et al., 2012; Budá et al., 2020, Gilt et al., 2020).

Nevertheless, little is known about the effect of blackcurrant and chokeberry on female reproductive processes. The objective of the present in vitro study was to compare the effects of blackcurrant and chokeberry extracts prepared from berries on human ovarian cells and to assess cell viability, release of steroid hormones and antioxidant defence against oxidative stress associated with superoxide dismutase (SOD) levels in cells treated by extracts ranging from 10-100 µg/mL using human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 as cellular models.

MATERIAL AND METHODS

Extract preparation

Blackcurrant fruits and chokeberry fruits were obtained from the Botanical Garden of the Slovak University of Agriculture in Nitra. Ethanol blackcurrant extract and ethanol chokeberry extract from lyophilized fruits were prepared prior to cell culture experiments. Processing, preparation, and subsequent extraction of plant material by solid-liquid extraction was performed. The extraction agent was non-denatured ethanol (80% v/v). Freeze-dried berries were thoroughly crushed and weighed. An amount of 2 g (accurate to 4 decimal places) of samples was extracted in 20 mL of non-denatured ethanol (80%; Centralchem, Bratislava, Slovak Republic) for four hours at room temperature and in the dark to prevent degradation...
of bioactive substances. Prepared suspensions were filtered using Whatman No. 1 filter paper, and until the time of use, the prepared stock solutions were placed in the refrigerator at 4 °C (Árvay et al., 2018).

Ovarian culture and treatment

Immortalized human ovarian granulosa cell line HGL5 (ABM®, BC, Canada) was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, MO, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA) and incubated in a 5 % CO2 incubator at 37 °C. Human ovarian adenocarcinoma cell line OVCAR-3 (ATCC®, VA, USA) was cultured in RPMI 1640 medium (Gibco-BRL, MD, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, MO, USA), 1 % antibiotics/antimycotics (Invitrogen, CA, USA) and incubated in a 5 % CO2 incubator at 37 °C. Initial concentrations of cells were set up the culture ranged from 10^4 to 10^5 per mL. The cells were grown in a standard T75 cell culture flask (Corning Life Sciences, NY, USA) to 75 % confluence (Baldovska et al., 2020). Prior to the experiments, berries’ extracts were diluted to the desired concentrations in culture media. Depending on the treatment, the cells were cultured in plates without (control group) or with blackcurrant or chokeberry extract at the concentrations 10, 20, 50 and 100 µg/mL for short-term application (24 h). For the experiments, cells treated with ethanol in an amount corresponding to the highest used concentration of the extract were used as positive controls (+Control).

Cell culture experiments

AlamarBlue assay

AlamarBlue™ (BioSource International, Nivelles, Belgium) cell viability assay was used to evaluate the cell viability and cellular health. Briefly, 100 µL of cell suspension per well (1.5 x 10^4 cells per mL) was seeded to a 96-well microplate (Grainer, Germany) and incubated (37 °C and 5% CO2) overnight. After treatment, 10 µL of AlamarBlue reagent was added to each well at the indicated time of 4 hours before the endpoint and incubated at 37 °C. Absorbance was measured at 560 nm and 590 nm by a microplate reader (Multiskan FC, ThermoFisher Scientific, Finland). AlamarBlue reduction as a result of multiple metabolic reactions was reported as relative cell viability values. For each experiment, wells without cells containing only the AlamarBlue solution were prepared and incubated. The fluorescence measured in those was used as a background and subtracted. The results were expressed as the percentage of viable cells (Baldovska et al., 2019).

ELISA (enzyme-linked immunosorbent assay)

Concentrations of steroid hormones (17β-estradiol and progesterone) and total superoxide dismutase (SOD) were determined spectrophotometrically by using ELISA kit according to the manufacturer’s instructions (17 β-Estradiol, DNOV003, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany; Progesterone, DNOV006, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany; Human total SOD ELISA Kit, EH4706-1, Fine Biotech, Wuhan, China). Cells were re-seeded in a 6-well cell culture plate (Grauer, Germany) at a density of 2 x 10^4 cells per well. After treatment, the levels of 17β-estradiol and progesterone were determined from the culture medium using an ELISA kit (NOVATEC, Dietzenbach, Germany). The intra-and inter-assay coefficients for 17β-estradiol was set at ≤9% and ≤10%, for progesterone it was set at ≤4% and ≤9.3%, respectively. The sensitivity was 8.68 pg/mL for 17β-estradiol and 0.05 ng/mL for progesterone. Supernatants of the cell lysates were subjected to the quantitative determination of SOD concentrations by using a total SOD ELISA kit (FineTest, Wuhan, China) as described by the manufacturer. The sensitivity was 0.469 ng/mL for total SOD. Briefly, ELISA microplates were pre-coated with an antibody. Standards and samples were pipetted into the wells. After the removal of any unbound substance, a biotin-conjugated antibody was added to the wells. After washing, streptavidin-conjugated horseshadish peroxidase was added to the wells. After incubation, microplates were washed and substrate solution was added to the wells. Color development was stopped and the intensity of the color was measured using a microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland). The results were averaged over 3 different independent experiments with replicates per experiment (Baldovska et al., 2020).

Statistical analysis

All data were expressed as mean ± SEM from at least three independent experiments. All analyses were performed three times and represent data from three individual experiments. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by an appropriate Dunnett’s test to evaluate the statistical significance of differences of the data. All of the statistical analyses were performed with GraphPad Prism 5 program (version 3.02 for Windows; GraphPad Software, CA, USA). The statistical significance was set at probability values of P<0.05.

RESULTS AND DISCUSSION

Effect on ovarian cell viability

To investigate the effects of blackcurrant and chokeberry on cell viability, granulosa cells HGL5 were treated with blackcurrant extract and chokeberry extract for 24 h. We observed a significant (P≤0.05) increase in the viability of HGL5 cells after blackcurrant extract treatment at the concentration of 10 µg/mL. Interestingly, treatment with chokeberry extract significantly (P≤0.001) increased the number of viable HGL5 cells at all the concentrations used in the study (10, 20, 50 and 100 µg/mL). The results are shown in Figure 1.

Effect on steroidogenesis

ELISA assay was performed to evaluate steroid hormone release, particularly the secretions of 17β-estradiol (Figure 3) and progesterone (Figure 4) by granulosa cells HGL5. The results exhibited a tendency of increase in 17β-estradiol secretion at all used concentrations of chokeberry extract. Interestingly, the level of 17β-estradiol secreted by the cells treated with blackcurrant extract was significantly (P≤0.05) increased at the concentration of 10 µg/mL. Notably, the current study suggests that both extracts could have an impact on the steroidogenesis in human granulosa cells in vitro.

Figure 1 Viability of human ovarian granulosa cells HGL5 after treatment with blackcurrant (A) and chokeberry (B) extracts. Control represents a medium without treatment. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as mean ± SEM. AlamarBlue.

Figure 2 Viability of human ovarian carcinoma cells OVCAR-3 after treatment with blackcurrant (A) and chokeberry (B) extracts. Control represents a culture medium without treatment. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as mean ± SEM. AlamarBlue.
revealed a significant (P<0.05) decrease of SOD concentrations in OVCAR-3 cells treated by blackcurrant extract at the concentrations 20, 50 and 100 µg/mL and a significant (P<0.05) decrease of SOD level in OVCAR-3 cells treated by chokeberry extract at a concentration 100 µg/mL.

![Graph A: Release of 17β-estradiol by HGL5 cells after treatment with blackcurrant (A) and chokeberry (B) extracts. Control represents a culture medium without treatment.](image1.png)

![Graph B: Chokeberry extract (µg/mL)](image2.png)

Figure 3 Release of 17β-estradiol by HGL5 cells after treatment with blackcurrant (A) and chokeberry (B) extracts. Control represents a culture medium without treatment. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as mean ± SEM. ELISA.

To further evaluate the effects of blackcurrant and chokeberry on human ovarian cells in vitro, we measured the release of progesterone by treated HGL5. However, no concentration of blackcurrant extract, neither chokeberry extract used in this study significantly affected (P>0.05) the progesterone secretion in comparison to control.

![Graph A: Progesterone levels in control (µg/mL)](image3.png)

![Graph B: Control (µg/mL)](image4.png)

Figure 4 Release of progesterone by HGL5 cells after treatment with blackcurrant (A) and chokeberry (B) extracts. Control represents a culture medium without treatment. +Control with ethanol in an amount corresponding to the highest used concentration of extract. The significance of differences between the groups was evaluated by One-way ANOVA followed by Dunnett’s multiple comparison test. The data are expressed as mean ± SEM. ELISA.

Natural phytoneutrients with anti-cancer activity can suppress the occurrence and development of tumours, by inhibiting telomerase activity, triggering DNA damage, and activating or inactivating oncogenes (Liu et al., 2020; Sirodotin and Košerová, 2022). Moreover, they have been receiving attention for their beneficial contribution to reproductive functions (Roychoudhury et al., 2017, 2018, 2021; Michalcová et al., 2019; Baldovská et al., 2019, 2020, 2021; Košerová et al., 2021). Various coloured berries, especially blackcurrant, chokeberry or blueberry, contain a large number of polyphenols, such as phenolic acids, flavonoids and anthocyanins (Olas, 2018). The scope of the modulating effects of blackcurrant and chokeberry fruits can be greatly expanded, given the wide spectrum of beneficial effects that their phytosubstances demonstrate, including antioxidant, anti-inflammatory and anti-microbial properties (Gopalan et al., 2012). Several studies have investigated the anti-cancer potential of blackcurrants and chokeberries using several cancer cells of human origin (Gopalan et al., 2012; Bishayee et al., 2010; Wenzel et al., 2020; Bagchi et al., 2004; Zhao et al., 2004). In this study, blackcurrant extract and chokeberry extract have been studied in ovarian cell cultures for assessment of their possible use as protective regulators in the female reproduction system.

In order to prove the cytotoxic and anti-cancer effect of berries’ extracts, we evaluated cell viability. The extracts beneficially impacted granulosa cells HGL5 leading to increase of the number of viable cells. Interestingly, extracts exhibited the cell-specific cytotoxic effect on ovarian cancer cells OVCAR-3, whereas blackcurrant extract was effective even at lower doses compared to chokeberry extract. In accordance with our results, a previous study showed the antiproliferative effects of blackcurrant fruit skin extract on liver cancer cells HepG2, possibly due to synergistic effects of polyphenolic chemicals. In comparison, cytotoxic effect of the extract on HepG2 cells was more pronounced than that of delphinidin and cyanidin, two major aglycones of anthocyanins present in blackcurrant fruits (Bishayee et al., 2010). Additionally, an antiproliferative potential of anthocyanin-rich fractions obtained from commercially available blackcurrant juice was reported using murine melanoma cells B16F10, ovarian cancer cell A2780 and cervical cancer cells HeLa (Diaconeasa et al., 2015).

Another study confirmed the anti-cancer effect of blackcurrant extract against colon cancer cells HT29 and at the same time revealed the suppression of the p21WAF1 signaling pathway as the underlying mechanism (Gopalan et al., 2012). Our results are also in line with recent study, which demonstrated the anti-cancer effect of three extracts derived from Aronia arbutifolia, Aronia prunifolia, and Aronia melanocarpa. Inhibitory effect of extracts on cell growth correlated also with total phenolic content, antioxidant activity, and levels of caffeic and chlorogenic acids (Gill et al., 2020). By contrast, commercially prepared chokeberry (Aronia melanocarpa E.) anthocyanin-rich extracts exhibited strong chemopreventive effect against colon cancer cells HT-29. The study described approximately 50% inhibition of cancer cell growth after 48 hours of exposure to 25 µg/mL chokeberry extract. Variation in anti-cancer activity could be explained by difference in chokeberry extraction techniques (Zhao et al., 2004). Chokeberries’ antioxidant activity is among the highest of all berries, though chokeberry extraction techniques frequently employ environmentally unfavorable solvents or are time-inefficient (Wenzel et al., 2020). Moreover, berries can differ in exerting health beneficial effects due to factors such as cultivar, fertilization, maturation or climatic conditions, as well as the date of harvest, which can affect the content of minerals, vitamins, carbohydrates, amino acids, organic acids, fats, aromatic compounds and especially polyphenols and other substances (Jurendić et al., 2021). On the other hand, in accordance with our findings, the growth of nontumorigenic colonic cells NCYM460 was not inhibited at lower concentrations.
of extract, illustrating greater growth inhibition of colon cancer, as compared to nontumorogenic colon cells (Zhao et al., 2004). The proliferative or anti-proliferative effects can be associated with 17β-estradiol release in cells and the molecular complexity of the 17β-estradiol-induced intracellular signaling pathway triggered by the estrogen receptors (Aconcia and Marino, 2011). Steroid hormones play an important role in the control and regulation of biological responses and can strongly affect physiological processes and risk factors for the initiation and progression of hormone-related cancers (Deroo and Korach, 2006). Granulosa cells are involved in the process of ovarian steroidogenesis, as well as play a crucial role during oocyte development. This cell type mainly secretes progesterone and estradiol, among various other factors (Ai et al., 2019). This study was also carried out to reveal the stimulatory effect on steroidogenesis, if any. To evaluate the effect of berries’ extracts on the release of steroid hormones, human ovarian granulosa cells HGL5 were treated with both studied berry extracts, and the effect of 17β-estradiol release, with significant increase of 17β-estradiol level at 10 µg/mL of blackcurrant extract. Our previous studies indicated that polyphenols present in pomegranate extract or elderberry extract can modulate the secretion of steroid hormone 17β-estradiol in human granulosa cells HGL5 (Baldovšková et al., 2019, 2020, 2021). There is evidence in studies in humans, animal models, and cell lines that some dietary phytonutrients may exert estrogenic potency, which can play important beneficial roles in reproductive processes, hormone-dependent cancers, prevention of menopausal symptoms and osteoporosis, as well as the risk reduction of heart diseases (Desmawati and Sulastri, 2019). Progesterone is essential for normal ovarian cycles and contributes to the regulation of ovarian follicular development and steroidogenesis. Based on the findings, we suggest that both extracts...


