

ORIGINAL ARTICLE

TITLE

Ovarian Steroid Hormone Secretion by Human Granulosa Cells after Supplementation of *Sambucus nigra* L. Extract

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SHORT TITLE

Effect of black elder on ovarian cells *in vitro*

SUMMARY

Beneficial effects of *Sambucus nigra* L. (black elder) as a traditional medicine have been associated with the phytoconstituents including polyphenols, terpenes and lectins. Various

antioxidant rich natural products have also been implicated with improvement of reproductive health and fertility, however, the effect of *Sambucus nigra* on the ovarian cell functions has not been investigated yet. The objectives of the present study were to screen the polyphenols in the elderflower and elderberry extracts, and to examine the secretion activity of steroid hormones 17 β -estradiol and progesterone by human ovarian granulosa cells HGL5 after supplementation of the extracts at a concentration range of 12.5 to 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Qualitative as well as quantitative screening of polyphenols by high-performance liquid chromatography with diode-array detector (HPLC-DAD) analysis revealed rutin to be the most abundant polyphenol in both elderflower and elderberry extracts. In culture, neither elderflower nor elderberry extract caused any significant impact ($p>0.05$) in cell viability as studied by AlamarBlue assay in comparison to control. However, a dose-dependent stimulation of 17 β -estradiol release was detected by ELISA after supplementation of elderflower (at 50 $\mu\text{g}\cdot\text{ml}^{-1}$; $p<0.01$) and elderberry (at 100 $\mu\text{g}\cdot\text{ml}^{-1}$; $p<0.05$) extracts at higher doses used in the study. On the other hand, both elderflower and elderberry extracts stimulated the secretion of progesterone by HGL5 cells at a lower dose (12.5 $\mu\text{g}\cdot\text{ml}^{-1}$; $p<0.05$), as compared to control. Therefore, elderflower and elderberry extracts may have the potential to regulate steroidogenesis in ovarian cells.

KEY WORDS

Black elder • HGL5 • Ovarian steroidogenesis • 17 β -estradiol • Progesterone

Introduction

Sambucus nigra L. (black elder) is used as a traditional herbal medicine across various regions of Europe. In recent times, it has found widespread use in dietary supplements as natural health products including extracts, juices or syrups to boost immunity. *Sambucus nigra* L. represents a possible dietary adjunct for the treatment of a number of diseases including *diabetes mellitus* (Ciocoiu *et al.* 2009), upper respiratory tract infections (Knudsen and Kaack

2015), human pathogenic bacteria (Gram-positive *Streptococcus pyogenes* and group C and G *Streptococci*, and Gram-negative *Branhamella catarrhalis*) and human pathogenic influenza virus infection (Krawitz *et al.* 2011), cold, flu (Mahboubi 2020), other chronic metabolic and cardiovascular illnesses (Ciocoiu *et al.* 2009). Beneficial effects on blood pressure, glycemia reduction, lipemia reduction, immune system stimulation, antitumour potential (Ciocoiu *et al.* 2009; Sidor and Gramza-Michałowska 2015) have been associated its antioxidant phytoconstituents such as polyphenols (anthocyanins, flavonols, phenolic acids and proanthocyanidins), terpenes and lectins (Sidor and Gramza-Michałowska 2015). Various antioxidant rich natural products have also been implicated with improvement reproductive health and fertility. Phytoestrogens are biologically active substances known to exert hormonal effects. Due to the similarity of chemical structure with steroid hormone 17 β -estradiol, many of them can manipulate steroidogenesis and endogenous hormone levels by interfering with the enzymes needed for steroid biosynthesis (Patisaul and Jefferson 2010). Studies in humans, animal models, and cell lines suggest that dietary phytoestrogens with biological effects including estrogenic potency can play important beneficial roles in reproductive processes, hormone-dependent cancers, prevention of menopausal symptoms and osteoporosis, as well as the risk of heart diseases (Desmawati and Sulastri, 2019). However, the effect of *Sambucus nigra* L. on the ovarian cell functions has not been investigated yet.

The elderflower and elderberry extracts are natural mixtures of substances, each of which can have different biological effects. The effects observed in the study are the results of the mixture action. The objectives of the present study were to screen the polyphenols in the elderflower and elderberry extracts, and to examine the secretion activity of steroid hormones 17 β -estradiol and progesterone by human ovarian granulosa cells HGL5 after addition of the concentration range 12.5 to 100 $\mu\text{g}.\text{ml}^{-1}$ of elderflower and elderberry extracts.

Methods

Preparation of extracts and HPLC-DAD screening of polyphenols

Elderflowers and elderberries were collected from the Botanical Garden of the Slovak University of Agriculture in Nitra, Nitra, Slovak Republic (Figure 1). Extraction of 2 g dried and grounded plant material was done in 20 ml 80% ethanol (v/v) at the room temperature for 4 hours by horizontal shaker Unimax 2010 (Heidolph Instruments, GmbH, Germany). Prior to HPLC analyses, the extracts were filtered through syringe PTFE filters (0.45 μm , 25 mm) (Agilent Technologies, Waldbronn, Germany) and stored in a refrigerator at 4 °C. All phenolic compounds were determined by high-performance liquid chromatography system with diode array detector (HPLC-DAD) instrumentation Agilent Infinity 1260 (Agilent Technologies, Waldbronn, Germany) in the crude extracts. All analytical standards (chlorogenic acid, 4-OH-benzoic acid, trans-caffeic acid, trans-p-coumaric acid, trans-ferulic acid, rutin, myricetin, resveratrol, apigenin, genistein, and kaempferol), acetonitrile (HPLC gradient grade), methanol (HPLC grade), and phosphoric acid (ACS grade) were purchased from Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Double deionized water (ddH₂O) was treated (18.2 M Ω cm⁻¹) in a Simplicity 185 purification system (Millipore SAS, Molsheim, France). All analyses were performed on a Cortecs column (4.6 mm x 150 mm x 2.7 μm) (Waters, Massachusetts, USA). The mobile phases consisted of 0.1% H₃PO₄ in ddH₂O (v/v) (A) and acetonitrile (B). The mobile phase flow was 0.6 mL min⁻¹ and the sample injection was 5 μL . The column thermostat was set to 30 °C and the samples were kept at 6 °C in the sampler manager. The detection wavelength was set at 265 nm, 320 nm, and 372 nm. The compounds were identified by comparing with standards of each identified compound using retention time, the absorbance spectrum profile, and also by running the samples after the addition of pure standards (Gabriele *et al.* 2018).

Cell culture and treatment

Elderflower and elderberry extracts were dissolved in a culture medium and diluted to the desired concentrations prior to experiments. 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/antimycotic solution (Invitrogen, CA, USA). The initial concentrations of cells before setting up the culture ranged from 10^4 to 10^5 cells per ml. Cells were cultured in plates without (control group) or with elderflower or elderberry extracts at concentrations of 12.5, 25, 50, and $100 \mu\text{g}\cdot\text{ml}^{-1}$ of extracts for 24 hours. As a positive control 80% ethanol in an amount corresponding to the highest used concentration of the respective extracts was used and the final ethanol concentration in well was less than 0.1%. All the procedures followed were in accordance with institutional guidelines (Baldovská *et al.* 2020).

Cell viability

Cell viability was examined using AlamarBlue (BioSource International, Nivelles, Belgium) assay (Michalcova *et al.* 2019). Cells (1.5×10^4 cells per ml) were re-seeded in a 96-well plate (Grainer, Germany) and grown in culture for 24 hours without (control group) or with elderflower/elderberry extracts (12.5, 25, 50, and $100 \mu\text{g}\cdot\text{ml}^{-1}$), or with 80% ethanol in an amount corresponding to the highest used concentration of the respective extracts (as positive control). Resazurin reduction (oxidized indigo blue state into the reduced pink state) was measured by recording the absorbance at 560 and 590 nm using a microplate reader (Multiskan FC, ThermoFisher Scientific, Finland) and expressed as percentage.

ELISA

Concentrations of secreted 17β -estradiol and progesterone were determined in duplicate in the incubation medium by ELISA as described previously (Roychoudhury *et al.* 2018; Kolesarova *et al.*, 2019; Baldovská *et al.* 2020) spectrophotometrically using ELISA kit (NOVATEC, Dietzenbach, Germany) according to the manufacturer's instructions. All ELISA assays were validated for use in samples of culture medium. Cells were re-seeded in a 24-well culture plate (Grainer, Germany) at a density of 1×10^5 cells per ml. For 17β -estradiol,

intra- and inter-assay coefficients of variation did not exceed 9 and 10%, respectively. For progesterone, intra- and inter-assay coefficients of variation did not exceed 4 and 9.3%, respectively. The sensitiveness was 8.68 pg.ml⁻¹ for 17 β -estradiol and 0.05 ng.ml⁻¹ for progesterone.

Statistical analysis

Three samples in one group in one experiment was used. Analyses were performed in at least three independent experiments with replicates per experiment. All data were expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was carried out using the GraphPad Prism 5 program (version 3.02 for Windows; GraphPad Software, CA, USA). One-way analysis of variance (ANOVA) along with Dunnett's test was performed as appropriate to determine the statistical significance of differences of the data. The statistical significance was set at probability values of $p < 0.05$.

Results

HPLC-DAD screening of polyphenolic compounds revealed rutin (12913.00 \pm 22.35 versus 4599.00 \pm 81.63 mg.kg⁻¹) and chlorogenic acid (5555.00 \pm 20.08 versus 810.60 \pm 14.20 mg.kg⁻¹) to be the most abundant polyphenols in both elderflower and elderberry extracts, respectively (Table 1). Polyphenolic constituents were notably higher in elderflower extract in comparison to that of elderberry extract and the concentrations of individual polyphenols also varied greatly between elderflower and elderberry extracts. Apigenin (1352.00 \pm 3.01 mg.kg⁻¹), resveratrol (768.30 \pm 2.32 mg.kg⁻¹), kaempferol (452.60 \pm 163.60 mg.kg⁻¹), trans-ferulic acid (314.30 \pm 1.40 mg.kg⁻¹), myricetin (109.30 \pm 1.88 mg.kg⁻¹), 4-OH-benzoic acid (69.44 \pm 0.37 mg.kg⁻¹), trans-caffeic acid (62.75 \pm 0.28 mg.kg⁻¹), trans-p-coumaric acid (49.70 \pm 1.16 mg.kg⁻¹) and genistein (17.48 \pm 0.088 mg.kg⁻¹) were the other polyphenolic compounds present in elderflower extract whereas elderberry extract contained trans-ferulic acid (245.20 \pm 5.21 mg.kg⁻¹), 4-OH-benzoic acid (35.70 \pm 0.94 mg.kg⁻¹) and genistein (29.12 \pm 0.11 mg.kg⁻¹).

In culture, HGL5 cells did not lose viability after supplementation of either elderflower extract or elderberry extract at all the concentrations used in the study, as compared with control (Figure 2). However, supplementation of *Sambucus nigra* L. extract stimulated the release of steroid hormones 17 β -estradiol and progesterone depending on the concentrations used. At higher concentrations used in the present study, a significantly higher secretion of 17 β -estradiol was observed after the addition of both elderflower (at 50 $\mu\text{g}\cdot\text{ml}^{-1}$; $p<0.01$) and elderberry (at 100 $\mu\text{g}\cdot\text{ml}^{-1}$; $p<0.05$) extracts, as compared to control (Figure 3). On the other hand, an increase in progesterone release was noted after administration of a lower dose of 12.5 $\mu\text{g}\cdot\text{ml}^{-1}$ elderflower as well as elderberry extracts ($p<0.05$), as compared to control (Figure 4).

Discussion

Granulosa cells forms the main cell type that is involved in the process of ovarian steroidogenesis and folliculogenesis (Ai *et al.* 2019). Their principal secretory products include steroid hormones 17 β -estradiol and progesterone which are involved in multiple physiological processes by acting on various tissues and they play an important role in the modulation of ovarian functions and female fertility (Kolesárová *et al.* 2015). Decreased gonadal steroid production causes several physiological changes that result in menopausal symptoms and the development of osteoporosis, too (Lecomte *et al.* 2017). Estradiol is considered a marker of meiotic arrest of oocytes (Dode and Graves 2003) while progesterone is a marker of ovarian follicle luteinization (Kadasi *et al.* 2014). Estradiol and its receptors are necessary for the control and regulation of many biological responses that strongly affect several aspects of physiology, such as risk factors for the initiation and progression of hormone-related cancers including ovarian and breast cancer (Deroo and Korach 2006). Progesterone is essential for normal ovarian cycles and contributes to the regulation of ovarian follicular development and remodeling (Arnhold *et al.* 2009; Hagan *et al.* 2008; Mahajan 2008). HGL5 is an immortalized cell line derived from primary hGL cells after transformation with the E6 and E7 regions of

human papillomavirus 16, and it forms an attractive model for investigating the mechanisms relating to the steroid biosynthesis as well as other pathways of hGL function (Rainey *et al.* 1994; Havelock and Rainey 2004; Bouraki *et al.* 2012). To our knowledge, this is the first report that has looked into the impact of *Sambucus nigra* L. extract on the viability of ovarian granulosa cells, and the secretion of steroid hormones 17 β -estradiol and progesterone *in vitro*.

Elderberry species have received significant attention especially for their antioxidant capacity for food applications as a natural conservative, functional food, or food supplement (Marisa Ribeiro *et al.* 2020). Elderberry (*Sambucus nigra*) is used in the treatment of many diseases due to antioxidant, anticancer, immune stimulating, antiallergic, antiviral and antibacterial properties (Młynarczyk *et al.* 2018; Oniszczyk *et al.*, 2016). Recently, the benefits of *Sambucus nigra* L. and more particularly its bioactive compounds have been reported by a number of researchers (Sidor and Gramza-Michałowska 2015; Młynarczyk *et al.* 2018; Mota *et al.* 2020; Marisa Ribeiro *et al.* 2020).

Sambucus nigra flowers and berries are rich in polyphenolic phytochemicals with important bioactivities. HPLC-DAD screening of polyphenolic compounds showed rutin and chlorogenic acid to be the most abundant polyphenols in both elderflower and elderberry extracts used in the study. Kaltsa *et al.* (2020) reported, that the richest elderberry flowers extract was produced with a 10 min ultrasonication pretreatment and then stirred-tank extraction under optimized conditions, at 80 °C, for 150 min. Data from the study on elderberry extract from *Sambucus nigra* flowers collected from Greece (the area of Neohori) were in line with results obtained in our study, reporting dominant contents for rutin, a di-p-coumaroylquic acid and chlorogenic acid by using liquid chromatography–mass spectrometry analysis (Kaltsa *et al.* 2020). The elderflower and elderberry extracts are natural mixtures of substances and can possess different biological effects. Therefore, the effects of the mixture action were observed in this study. As examined by AlamarBlue assay, supplementation of *Sambucus nigra* L. extract did not cause cytotoxicity and the viability of HGL5 cells was found intact at all the

concentrations of elderflower and elderberry extracts used in the study. On the other hand, Chen *et al.* (2013) reported a negative correlation between elderflower and elderberry extracts (at 50-1600 $\mu\text{g}\cdot\text{ml}^{-1}$ for baby hamster kidney fibroblast cells BHK-21; and at 50-800 $\mu\text{g}\cdot\text{ml}^{-1}$ for kidney epithelial cells VERO), by using the Alamar Blue assay (Castillo-Maldonado *et al.* 2017). However, dwarf elder (*Sambucus ebulus*) extract at a concentration range of 5-1500 $\mu\text{g}\cdot\text{ml}^{-1}$ was able to reduce the viability of cancer cell lines HepG2 and CT26 when examined by MTT assay (Saravi *et al.* 2013). Isoflavone rutin (the most abundant polyphenol in elderflower as well as elderberry extracts as detected in the present study) also inhibited the viability of human neuroblastoma cells LAN-5 (Chen *et al.* 2013). Rutin supplementation at 10 $\mu\text{g}\cdot\text{ml}^{-1}$ was also reported to decrease the viability of cultured porcine ovarian granulosa cells (Sirotkin *et al.* 2021).

Schroder and colleagues (2016) studied the effect of elderflower extract at 5, 50 and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ doses on chorion carcinoma cell lines JEG-3 and BeWo as well as the breast carcinoma cell line MCF7. Estradiol production was found to be inhibited in all cells, however, in JEG-3 cells an upregulation of estrogen receptor α (ER α) was seen whereas in MCF7 cells ER α was downregulated and progesterone receptor was upregulated after supplementation of elderflower extract (Schroder *et al.* 2016). A previous study reported, that *Sambucus nigra* agglutinin can reduce viability and mitochondrial activity and induce apoptosis in ovarian cancer cells. *Sambucus nigra* agglutinin activate intracellular signaling pathways of AKT and ERK1/2, which promotes dephosphorylation of dynamin-related protein-1 (Drp-1) inducing fragmentation of mitochondrial membrane resulted in mitochondrial outer membrane permeabilization. Following generation of reactive oxygen species (ROS) and cytochrome-c release into the cytosol and may result in apoptosis and cell cycle arrest before the G2/M phase (Chowdhury *et al.* 2017).

Another study examined the *in vitro* assessment of the elderberry (*Sambucus nigra*) extract on the motility, viability, and reactive oxygen species (ROS) production of bovine spermatozoa. The results showed, that lower concentrations of the elderberry extract (5 and

1 µg/mL) led to an increase in mitochondrial activity in comparison to the control group. The findings of the study indicate that *Sambucus nigra* extract can possess activity-promoting properties on bovine spermatozoa (Abdramanov et al. 2017).

There is some evidence that phytoestrogens can modulate physiological functions and may exert either a positive or a negative impact on reproductive health (Wocławek-Potocka et al. 2013). For example, genistein has been shown to induce the release of estradiol, progesterone and cAMP by the ovaries, maturation of oocyte, as well as the development of zygote in the preimplantation stage (Jefferson and Williams 2011). On the contrary, phytoestrogens present in the green and Indian turmeric has been reported to inhibit proliferation, induce apoptosis and affect steroid hormone release by animal ovarian cells. In addition, isoflavones can change animal sexual development, disrupt the estrous cycle and ovarian functions (Cederroth et al. 2012; Desmawati and Sulastri, 2019). Genistein (also present in elderberry extracts used in our study) at high doses (50–100 µM) inhibits the growth of human breast cancer cells *in vitro*, whereas it induces proliferation at lower doses (0.01–10µM), effects that were explained by their estrogenic properties at low doses and cytotoxicity at higher doses (Hsieh et al. 1998). The suppressive effect of rutin on cell viability as reported by a number of previous authors as discussed above, could be attributed to the ability of this phytoestrogen to regulate the release of steroid hormones 17β-estradiol and progesterone including by the ovarian cells (Sirotkin et al. 2020). In another recent study, Sirotkin et al. (2021) reported a stimulation in 17β-estradiol, progesterone as well as testosterone production by cultured porcine ovarian granulosa cells by 10 µg.ml⁻¹ rutin. However, it was accompanied by a decrease in cell proliferation and apoptosis (Sirotkin et al. 2021). Chlorogenic acid present also in the dominant amount in extracts may play several biological roles. It was showed, that it may affect oocyte maturation and inhibit the progression of meiosis and consequently the entire embryo development *in vitro*. On the contrary, no effect on the progesterone production by bovine granulosa cells was observed (Nunes et al. 2018). Phytonutrients and herbal supplements such as *Sambucus nigra* L. extracts might be a natural way to boost steroid

hormones during menopause, when female sex hormones drop drastically. Although most studies have focused on the antiviral properties of *Sambucus nigra* L. particularly owing to the presence of beneficial polyphenolic compounds (Młynarczyk *et al.* 2018), the potential use of elderflower extract in breast cancer prevention and/or treatment has previously been advocated (Schroder *et al.* 2016). In the present study we observed, that the secretion of 17 β -estradiol was significantly higher after the addition of higher doses of extracts (elderflower extract at 50 $\mu\text{g}\cdot\text{ml}^{-1}$ and elderberry extract at 100 $\mu\text{g}\cdot\text{ml}^{-1}$). On the other hand, a significant increase in progesterone secretion was observed after addition of a lower dose (12.5 $\mu\text{g}\cdot\text{ml}^{-1}$) of both, elderflower and elderberry extracts. Similarly, Kadasi *et al.* (2014) reported increased progesterone secretion by granulosa cells after addition of epigallocatechin gallate at a low dose (10 $\mu\text{g}\cdot\text{ml}^{-1}$ EGCG) but not at higher doses. Our study showed, that elderberry can promote steroid hormone release, especially estradiol, which is considered a promoter of ovarian cell proliferation and viability and ovarian folliculogenesis. Therefore, the stimulatory action of black elder extract on healthy ovarian cells may be suggested. Thus, phytochemicals present in *Sambucus nigra* L. may potentially become effective and less toxic therapeutic agents due to their property of up-regulation of steroid hormones secretion. However, the data obtained from *in vitro* cell culture system need not fully correspond to the situation *in vivo*.

In conclusion, black elderberry seems to possess benefits effects on reproductive health. The results of the present study on the effects of elderflower and elderberry extracts on the release of 17 β -estradiol and progesterone indicate towards the involvement of the phytoconstituents of *Sambucus nigra* L. with ovarian steroidogenesis.

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Figure legends

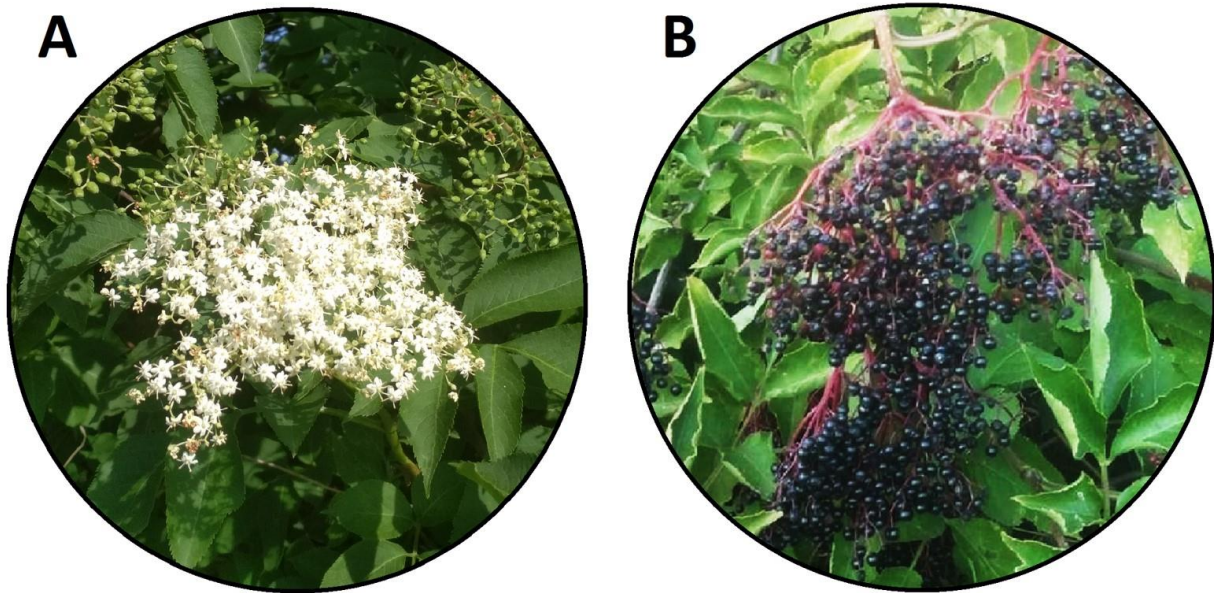


Figure 1. Elderflowers (A) and elderberries (B) harvested for the preparation of ethanolic extracts.

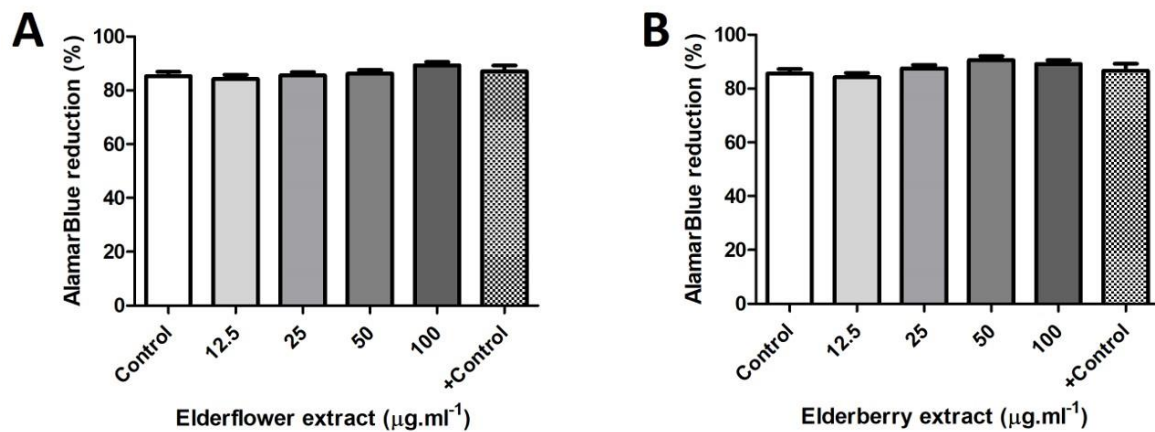


Figure 2. The effect of elderberry extract from flowers (A) and berries (B) on the viability of human ovarian granulosa cells HGL5. Control represents cells without treatment, in experimental groups elderflower and elderberry extracts were administered at

12.5, 25, 50, and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ for 24 hours. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control). The significance of differences between the groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test. The data are expressed as means \pm SEM. AlamarBlue assay.

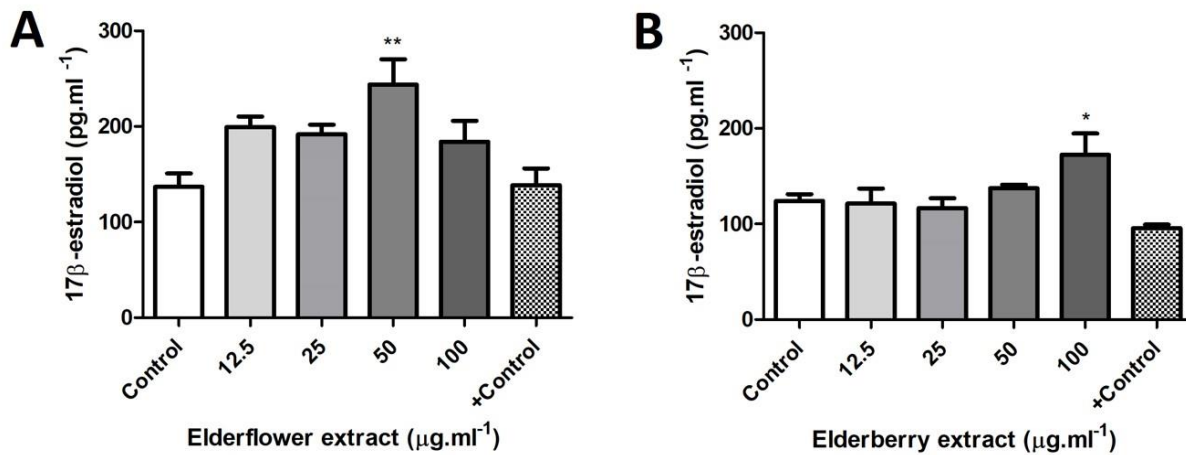


Figure 3. The effect of elderberry extract from flowers (A) and berries (B) on the release of 17β-estradiol by human ovarian granulosa cells HGL5. Control represents culture medium without treatment, in experimental groups elderflower and elderberry extracts were administered at 12.5, 25, 50, and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ for 24 hours. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control). The significance of differences *($p < 0.05$), **($p < 0.01$) between the groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test. The data are expressed as means \pm SEM. ELISA.

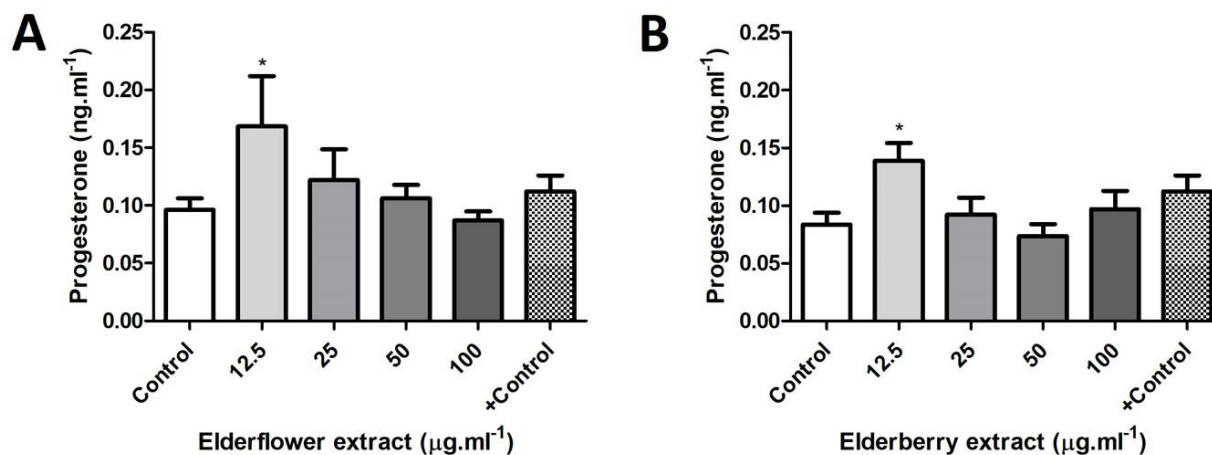


Figure 4. The effect of elderberry extract from flowers (A) and berries (B) on the release of progesterone by human ovarian granulosa cells HGL5. Control represents culture medium without treatment, in experimental groups elderflower and elderberry extracts were administered at 12.5, 25, 50, and 100 µg.ml⁻¹ for 24 hours. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control). The significance of differences *(p<0.01) between the groups were evaluated by one-way ANOVA followed by Dunnett's multiple comparison test. The data are expressed as means ± SEM. ELISA.

Table 1. Screening of the polyphenolic contents in elderberry extracts.

Polyphenols (average amount in mg.kg ⁻¹)	Elderberry (<i>Sambucus nigra</i> L.)	
	Flowers	Berries
Chlorogenic acid	5555.00±20.08	810.60±14.20
4-OH-benzoic acid	69.44±0.37	35.70±0.94
<i>trans</i> -Caffeic acid	62.75±0.28	≤LOD
<i>trans</i> -p-Coumaric acid	49.70±1.16	≤LOD
<i>trans</i> -Ferulic acid	314.30±1.40	245.20±5.21
Rutin	12913.00±22.35	4599.00±81.63
Myricetin	109.30±1.88	≤LOD
Resveratrol	768.30±2.32	≤LOD

Apigenin	1352.00±3.01	≤LOD
Genistein	17.48±0.088	29.12±0.11
Kaempferol	452.60±163.60	≤LOD

*LOD – limit of detection, ± standard error