

A PILOT *IN VITRO* STUDY ON THE EFFECT OF BLUE HONEYSUCKLE EXTRACT ON HUMAN NON-CANCEROUS AND CANCEROUS OVARIAN CELLS

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ABSTRACT

Honeysuckle (*Lonicera caerulea* L.) is utilized in Chinese, Japanese, and Russian folk medicines for centuries. The berries referred to as blue honeysuckle are recently gaining popularity in central and southern Europe due to remarkable therapeutic potential and widespread use as an ingredient in dietary supplements. The primary objective of this investigation was to examine the impact of blue honeysuckle extract on human ovarian cells – both non-cancerous HGL5 (immortalized human granulosa cell line) and cancerous OVCAR-3 (human ovarian carcinoma cell line). Cell viability, secretion of steroid hormones (17 β -estradiol and progesterone), human transforming growth factor- β 2 (TGF β -2) and its receptor (TGFBR2), and the level of apoptosis-inducing factor (AIF) were examined in ovarian granulosa cells HGL5, and the same parameters (excepting the steroid hormones) were assessed in ovarian epithelial cancer cells OVCAR-3. Blue honeysuckle ethanolic extract was applied to cells for 24 hours. AlamarBlue™ assay was used to assess cell viability, whereas secretion of steroid hormones was evaluated by ELISA. ELISA was also used to assess the levels of TGF- β 2, its receptor TGFBR2 and AIF in HGL5 and OVCAR-3 cell lysates. Based on a range of doses ranging from 5 – 100 μ g/mL, the extract from blue honeysuckle had no adverse effects on the viability of human ovarian cells. Furthermore, it did not affect their secretory activity – be it the release of steroid hormones or that of growth factor TGF- β 2 and its receptor TGFBR2, nor did it induce apoptosis (as revealed by the AIF). The present pilot study for the first time reports the effect of blue honeysuckle extract on ovarian cell models using both non-cancerous cells HGL5 and cancerous OVCAR-3 cells. These findings provide initial insights into the safety and potential utility of blue honeysuckle extract, warranting further investigation into its therapeutic applications. In further research, it is possible to examine a longer exposure time to see if it affects the cells.

Keywords: *Lonicera caerulea* L., Ovarian steroidogenesis, 17 β -estradiol, Progesterone, TGF- β 2, TGF β 2 receptor, Apoptosis-inducing factor

INTRODUCTION

Lonicera caerulea L. known as honeyberry, blue honeysuckle, sweet berry honeysuckle, edible honeysuckle, or haskap is a plant species belonging to the family *Caprifoliaceae*. It has been utilized in the Chinese, Japanese, and Russian folk medicines for centuries. Blue honeysuckle is recently gaining popularity in central and southern Europe, particularly in Poland, Slovenia, Czech Republic, and Slovakia, due to remarkable therapeutic potential and widespread use as an ingredient in dietary supplements (Bieniek *et al.*, 2021). Berries are consumed in their natural or processed forms, including tea, ice cream, juice, soft drinks, pastries, jams, candies, snacks, gelatins, wines, and as frozen fruits (Sharma & Lee, 2021). Blue honeysuckle exhibits an antioxidant activity that is three to five times greater than that observed in more commonly consumed berries, such as blackberries or strawberries. Moreover, their ascorbic acid content is the greatest among fruits (Negreanu-Pirjol *et al.*, 2023). Berries are characterized by their elevated water content, notable presence of free sugars, predominantly fructose and glucose, substantial levels of organic acids primarily comprising citric, malic, and quinic acids, as well as the occurrence of α - and γ -tocopherols, with linoleic acid being the predominant fatty acid. These berries also encompass a diverse array of bioactive compounds, including phenolic acids such as caffeic, chlorogenic, and neochlorogenic acids, flavonols like quercetin, flavanols (dihydroflavonols), flavones, iridoids, and anthocyanins, with particular emphasis on cyanidin-3-O-glucoside as the most abundant (Kucharska *et al.*, 2017; Molina *et al.*, 2019). Blue honeysuckle extract contains a substantial content of bioactive phytochemicals known for their ability to inhibit the proliferation of tumour cells. The mechanism of action the anticancer properties of blue honeysuckle extract remains incompletely explained. In a study conducted by Zhang *et al.* (2022), it was demonstrated that blue honeysuckle extract exerts its antitumor effects by inhibiting the cyclin B1/cdc2 signalling pathways. Blue honeysuckle may offer protection against a variety of chronic diseases, such as cancer. Consequently, the use of fruits of the genus *Lonicera* as a potential source of nutritive compounds with health-promoting properties could be regarded as extremely advantageous for

consumers (Becker & Szakiel, 2019). Not only the fruits of the blue honeysuckle, but also other parts such as the seeds, leaves and branches can be a valuable benefit for health. Seeds oil made *Lonicera caerulea* is particularly rich in polyunsaturated fatty acids, especially linoleic acid. It is also interesting for its high phenol content and antioxidant capacity (Zhang *et al.*, 2023). Extracts of *Lonicera caerulea* leaves and branches may inhibit the proliferation and migration of human colorectal cancer cells (An *et al.*, 2020).

Majority of honeysuckle's bioactive compounds comprise of iridoids and anthocyanins. A substantial correlation exists between antioxidant capacity and anthocyanin content. Iridoids, which are abundant in honeysuckle blossoms, can enhance the antioxidant qualities of phenolic compounds (Kucharska *et al.*, 2017). In human ovarian carcinoma cells SKOV3, gentiopicoside induces cell cycle arrest, mitochondria-mediated apoptosis, and inhibition of cell migration, resulting in potent antitumor effects (Li *et al.*, 2019). Several *in vivo* and *in vitro* studies have provided evidence of the beneficial impacts of anthocyanins on human health (de Pascual-Teresa & Sanchez-Ballesta, 2008; Li *et al.*, 2017). These beneficial effects may be mediated by multiple signalling pathways, such as mitogen-activated protein kinase (MAPK), nuclear factor κ B (NF- κ B), AMP-activated protein kinase (AMPK), and Wnt/ β -catenin (Li *et al.*, 2017). Phytochemicals present in fruits have great potential for preventing and treating gynaecological malignancies by impacting cellular mechanisms such as the cell cycle, DNA replication, interaction with kinases and critical receptors and activating signalling pathways leading to cell death (Kunnumakkara, 2014). By concentrating on molecular targets and pathways crucial for cancer cell proliferation, tumor expansion, evasion of immune surveillance, and resistance to cell death signals, novel potential cancer drug candidates are discerned (Cortez *et al.*, 2018).

The current study examined the impact of blue honeysuckle extract at various doses ranging from 5 to 100 μ g/mL on human ovarian cells – both non-cancerous (immortalized human ovarian granulosa cell line - HGL5) and cancerous (human ovarian carcinoma cell line - OVCAR-3). Cell viability, secretion of steroid hormones (17 β -estradiol and progesterone), human transforming growth factor β -

2 (TGF β -2) and its receptor (TGFBR2), and the level of apoptosis-inducing factor (AIF) were measured in ovarian granulosa cells HGL5, and the same parameters (excepting the steroid hormones) were assessed in ovarian epithelial cancer cells OVCAR-3.

MATERIAL AND METHODS

Cell lines and reagents

HGL5 (ABM®, BC, Canada); NIH: OVCAR-3 (ATCC®, VA, USA); Dulbecco's modified Eagle medium, fetal bovine serum (Sigma-Aldrich, MO, USA); antibiotics/antimycotic solution (Invitrogen, CA, USA); RPMI1640 (Gibco-BRL, MD, USA); non-essential amino acids (Sigma Aldrich, UK); standard cell culture flask (Corning Life Sciences, NY, USA); AlamarBlue (BioSource International, Nivelles, Belgium); 6 and 96-well plate (Grainer, Germany); microplate reader (Multiskan FC, ThermoFisher Scientific, Finland); 17 β -estradiol and progesterone ELISA kit (NOVATEC, Dietzenbach, Germany); TGF- β 2, TGF- β 2R, and AIF ELISA kit (FineTest, Wuhan, China).

Cell culture and treatment

The immortalized human ovarian granulosa cell line HGL5 was grown in DMEM containing 10% fetal bovine serum and 1% antibiotics/antimycotic solution. For the human ovarian cancer cell line OVCAR-3, RPMI1640 culture medium supplemented with 10% fetal bovine serum, 1% antibiotics/antimycotics, and 1% non-essential amino acids was used. Both cell cultures were then placed in standard T75 cell culture flasks and maintained until they reached 80-90% confluence in a 5% CO₂ incubator at 37°C. Homogenized lyophilized berries of blue honeysuckle (obtained from the Botanical Garden of the Slovak University of Agriculture in Nitra, Slovak Republic) for were used for extraction for 4 hours at ambient temperature at a ratio of 1.000 g per 10 mL of 80% (v/v) ethanol. The extraction process was conducted in the dark with continuous agitation. The suspension was subsequently filtered, and the stock extract was stored at 4°C. Before conducting experiments, ethanolic extract of blue honeysuckle was dissolved in a culture medium and diluted to desirable doses (Baldovská et al., 2021). Prior to establishing the culture, the initial cell concentrations ranged 10⁴ – 10⁵ cells/mL. Cells were placed on plates and allowed to incubate for 24 hours, with one group serving as the control (no extract). The remaining groups were exposed to blue honeysuckle extract at concentrations of 5, 10, 20, 50, and 100 μ g/mL. Additionally, a positive control consisting of 80% ethanol, matching the highest extract concentration, was included, ensuring that the final ethanol concentration in the well remained below 0.1% (Baldovská et al., 2020).

Cell Viability Assessment

The AlamarBlue assay was used to determine cell viability. Initially, cells were cultivated in a 96-well plate at a density of 1 – 2.10⁴ cell per well) and grown for 24 hours (5% CO₂, 37 °C). The cells were then cultured for 24 hours without or with blue honeysuckle extract at varying concentrations (5, 10, 25, 50 and 100 μ g/mL), or with 80% ethanol (Baldovská, et al., 2019). The reduction of Resazurin (from an oxidized indigo blue state to a reduced pink state) was determined by spectrophotometric measurement of absorbance at 560 and 590 nm using a microplate reader, and the results were expressed as a percentage.

ELISA

Levels of 17 β -estradiol, progesterone, TGF- β 2, TGFBR2, and AIF were quantified using an ELISA kit following the manufacturer's guidelines. At a density of 2.5 – 5.10⁵ cells per well, cells were reseeded in a 6-well culture plate. After 24 hours, the culture medium was aspirated and replaced with fresh medium (control) and/or blue honeysuckle extract (5, 10, 20, 50 and 100 μ g/mL). Subsequently, the incubation medium was used to measure steroid hormones (17 β -estradiol and progesterone), while TGF- β 2, TGFBR2, and AIF levels were quantified in cell lysates through ELISA.

Statistical analysis

Statistical analyses were performed using a minimum of three independent experiments, each with multiple replicates. All data were expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, California, United States). Differences in statistical significance were assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test. The threshold for statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Earlier research has consolidated the *in vitro* and *in vivo* findings regarding the health-enhancing attributes of blue honeysuckle including the anti-inflammatory, anti-obesity, antioxidant, anti-diabetic, anti-bacterial, anti-tumour, and

antimicrobial actions as well as cardiovascular, liver, thyroid, and lung protective effects (Sharma and Lee, 2021). The suppression of carcinogenesis was facilitated through various approaches, including the use of dietary supplements, intravenous and intraperitoneal administration of cyanidin 3-O-glucoside (C3G), blue honeysuckle extracts, as well as the prevention of DNA damage and oxidative stress, the stimulation of antioxidant defense enzymes, and the inhibition of factors promoting cancer cell proliferation and metastasis (Rupasinghe et al., 2018). C3G, isolated from blue honeysuckle showed cytotoxic effect on human hepatocarcinoma cells SMMC-7721, can induce apoptosis in live cancer cells via flow cytometric analysis (Luo et al., 2017).

Ovarian cancer is both extremely frequent and fatal. Its etiology and molecular biology can vary greatly (Torre et al., 2018). Research on the active constituents of various food products may yield crucial information regarding alternative cancer treatment methods (Lang et al., 2015).

In the current study, ovarian cells HGL5 and OVCAR-3 did not decrease viability after treatment with blue honeysuckle extract at any of the doses used (Fig. 1). Similarly, treatment with blue honeysuckle extract up to 300 μ g/mL had no impact on the viability of rat liver cells BRL-3A (Wang et al., 2017).

Conversely, blue honeysuckle extract was observed to inhibit the growth of cancer cells HeLa cultured with a protein film. The rate of proliferation also declined whereas the rate of apoptosis increased (Li et al., 2017). Anthocyanin (extracted and purified from blue honeysuckle) inhibited the proliferation of human hepatoma cells SMMC-7721 and inhibited the G2/M phase of the cell cycle. It further induced DNA damage and eventually led to apoptosis. Blue honeysuckle extract also inhibited the viability of prostate cancer cells *in vitro*, as reported by Ali et al. (2017). After 48 hours of treatment, cyanidin glycosides inhibited the proliferation of human cervical cancer cells HeLa and increased the production of reactive oxygen species (ROS), suggesting the involvement of blue honeysuckle in the antiproliferative activity (Rugină et al., 2012).

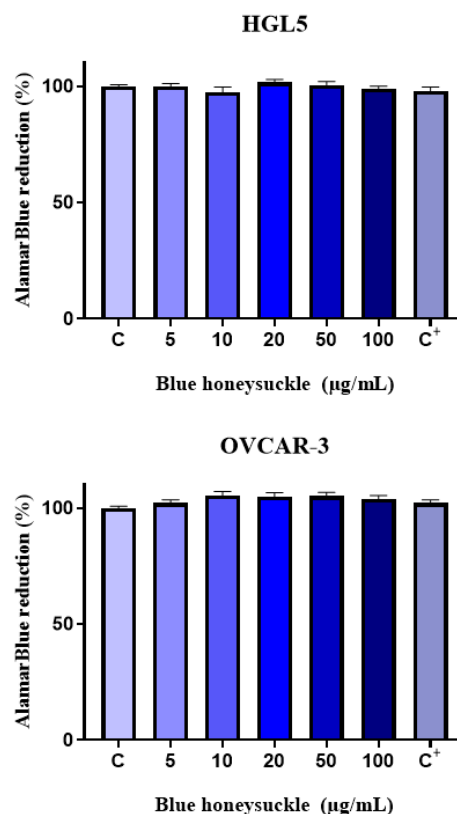


Figure 1. Viability of human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 after treatment with blue honeysuckle extract. In the control group (C), cells were incubated without treatment. In the positive control (C⁺), cells were incubated with the maximum dose of the extract in ethanol. Experimental groups of cells were incubated with blue honeysuckle extract at doses of 5, 10, 20, 50 and 100 μ g/mL.

Like cell viability, blue honeysuckle extract treatment did not impact the secretion of steroid hormones 17 β -estradiol and progesterone by HGL5 cells at all the doses used in the study, as revealed by ELISA (Fig. 2). Granulosa cells are the most numerous type of somatic cells in the ovarian follicle and are responsible for steroidogenesis and folliculogenesis (Ai et al., 2019). Their primary constituents are 17 β -estradiol and progesterone, which are steroid hormones. These hormones are involved in a vast array of physiological processes because they affect a variety of tissues. Moreover, they serve an essential role in modulating ovarian functions and female fertility (Kolesarova et al., 2015). In a study on the impact of C3G

(which is found in blue honeysuckle), an increase in progesterone synthesis was noted in rat Leydig cells R2C. For 24 hours, cells were pre-treated with C3G (10–80 mol/L) (Li, et al., 2019).

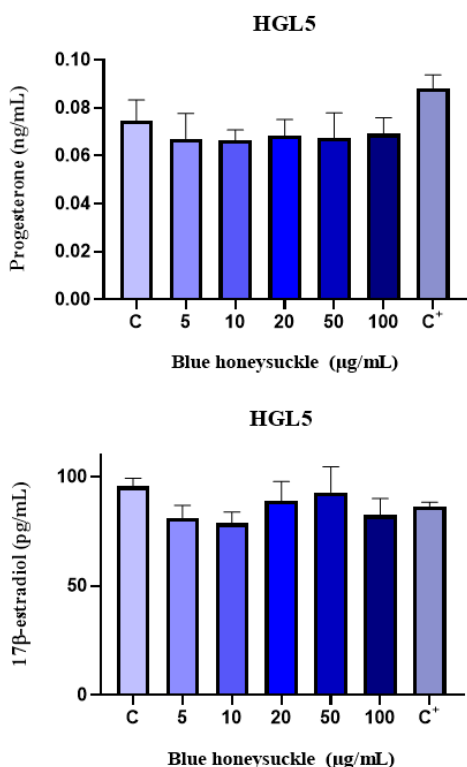


Figure 2. Progesterone and 17β-estradiol secretion by HGL5 cells after treatment with blue honeysuckle extract. In the control group (C), cells were incubated without treatment. In the positive control (C⁺), cells were incubated with maximum dose of the extract in ethanol. Experimental groups of cells were incubated with blue honeysuckle extract at doses of 5, 10, 20, 50 and 100 μg/mL.

Treatment with blue honeysuckle extract had no effect on the levels of TGF-β2 (Fig. 3) and TGFBR2 (Fig. 4) at all the doses used in the study. The disruption of TGF-β signalling is strongly associated with cancer progression. TGF signalling pathway exerts tumour suppressive effects in normal cells and early carcinomas; however, these protective and cytostatic activities are frequently lost as tumours progress. TGF-β signalling switches promote the progression, invasion, and spread of cancer (Lebrun, 2012). In another *in vivo* study, microRNA2911 from blue honeysuckle was able to target TGF-β1 by targeting tumour growth inhibition (Liu et al., 2021). Wu et al. (2022) observed that after 24 hours, the dose-dependent inhibition of metastasis-related factors (TGF, CD44, epidermal growth factor receptor EGFR, and vascular endothelial growth factor VEGF) in C3G-treated lung carcinoma cells H661 was significant.

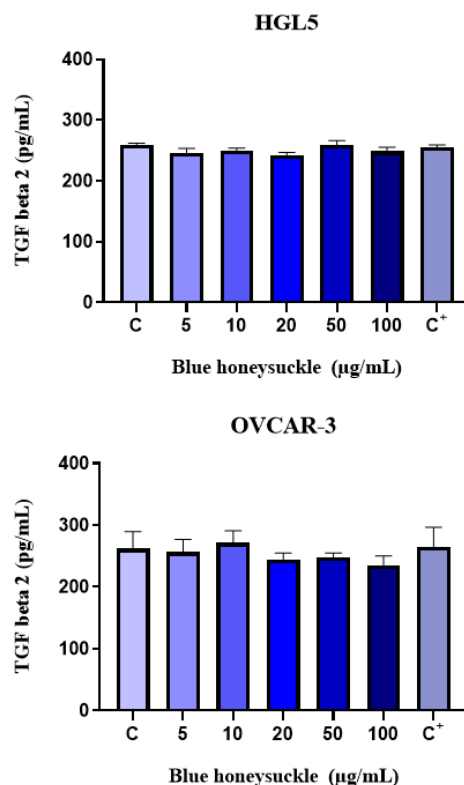


Figure 3 Release of human transforming growth factor-β2 (TGFβ-2) by human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 after treatment with blue honeysuckle extract (5, 10, 20, 50 and 100 μg/mL). In the control group (C), cells were incubated without treatment. In the positive control (C⁺), cells were incubated with the maximum dose of the extract in ethanol.

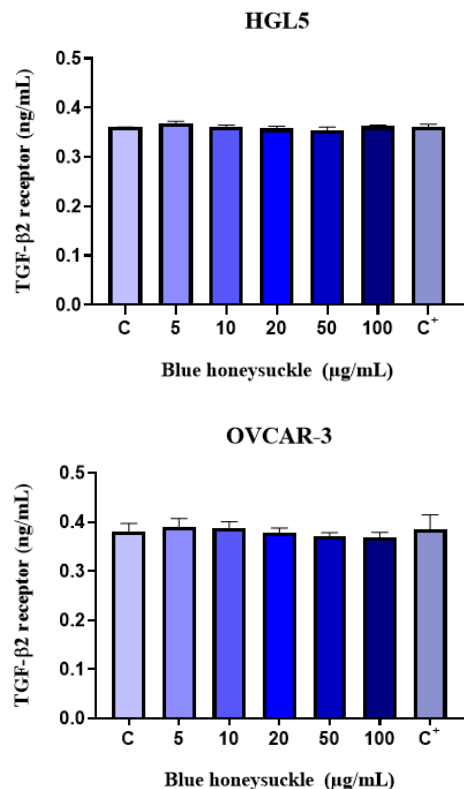


Figure 4 Release of human transforming growth factor-β2 receptor (TGFBR2) by human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 after treatment with blue honeysuckle extract (5, 10, 20, 50 and 100 μg/mL). In the control group (C), cells were incubated without treatment. In the positive control (C⁺), cells were incubated with the maximum dose of the extract in ethanol.

Both caspase-dependent and caspase-independent pathways may be activated during apoptosis. AIF was discovered as the first protein to mediate caspase-independent cell death (Norberg et al., 2010). Miyake et al. (2012) proposed that IFN-2a (interferon-alpha-2a) induced apoptosis in OVCAR-3 cells is facilitated through the mitochondria-associated pathway, involving the cleavage of BH3-interacting domain death agonist (Bid), AIF release, Bcl-2 homologous antagonist killer (Bak) activation, and translocation of AIF from mitochondria to nucleus. C3G-rich blue honeysuckle extract, C3G-rich extracts from other berries, or pure C3G can arrest cell cycle and/or induce apoptosis in tumour cells through different mechanisms both *in vitro* and *in vivo* (Rupasinghe et al., 2018). Luo et al. (2017) reported that C3G isolated from honeysuckle inhibits the proliferation of liver cancer cells by promoting apoptosis. However, in our study, the blue honeysuckle extract did not significantly influence the quantity of AIF in cell lysates HGL5 and OVCAR-3 (Fig. 5).

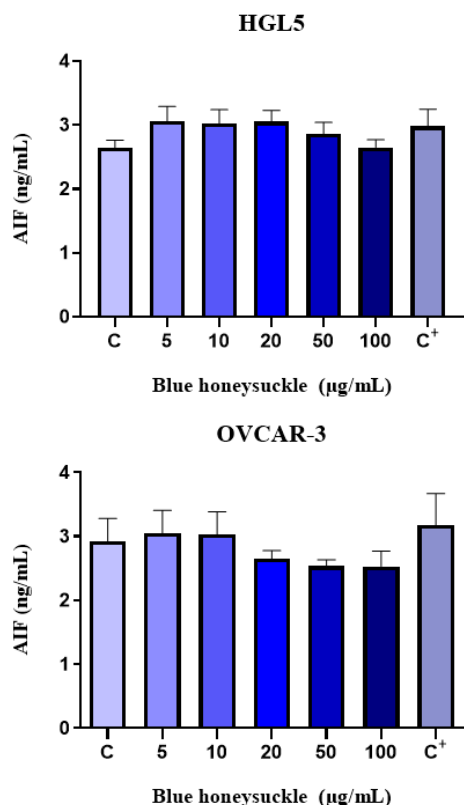


Figure 5 Release of apoptosis-inducing factor (AIF) by human ovarian granulosa cells HGL5 and human ovarian carcinoma cells OVCAR-3 after treatment with blue honeysuckle fruit extract (5, 10, 20, 50 and 100 µg/mL). In the control group (C), cells were incubated without treatment. In the positive control (C⁺), cells were incubated with the maximum dose of the extract in ethanol.

CONCLUSION

In this pilot *in vitro* study, we investigated the impact of blue honeysuckle extract on both non-cancerous HGL5 and cancerous OVCAR-3 human ovarian cells. We explored various cellular parameters, including cell viability, secretion of steroid hormones (17β-estradiol and progesterone), levels of human transforming growth factor-β2 (TGFβ-2) and its receptor (TGFBR2), and the presence of apoptosis-inducing factor (AIF).

Our findings indicate that blue honeysuckle extract, at doses ranging from 5 – 100 µg/mL, did not affect human ovarian cells' viability. It also had no significant impact on the secretion of steroid hormones. Furthermore, the extract did not interfere with the TGFβ signalling pathway, crucial in cancer progression, as evidenced by unchanged levels of TGFβ-2 and TGFBR2. Additionally, blue honeysuckle extract did not induce apoptosis in the tested cells.

In summary, this study suggests that blue honeysuckle extract, within the tested dose range, does not affect the viability and secretory function of human ovarian cells, nor does it induce apoptosis. These results provide initial insights into the safety and potential utility of blue honeysuckle extract, warranting further investigation into its therapeutic applications. In further research, it is possible to examine a longer exposure time to see if it affects the cells.

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