https://doi.org/10.31925/farmacia.2024.5.16 **ORIGINAL ARTICLE**

IN VITRO ASSESSMENT OF 17ß-ETHINYLESTRADIOL AND LEVONORGESTREL ON BREAST CANCER MCF-7 AND MDA-MB-231 CELL LINES

FLORINA BOROZAN [1], IOAN SAS [1], ALEXANDRU BLIDISEL [1]*, DIANA HAJ ALI [2], AIDA PASC [2], CRISTINA DUMITRESCU [2, 3], DALIANA MINDA [2, 3], CRISTINA TRANDAFIRESCU [2, 3], STEFANIA DUMITREL [2], SIMONA ARDELEAN [4]

[1]*Faculty of Medicine, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041 Timișoara, Romania*
[2]*Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041 Timișoara, Romania*
[3]*Research Center for Pharmaco-Toxicological Evaluations, Faculty of Pharmacy, "Victor Babeș" University of Medicine and Pharmacy Timișoara, 2 Eftimie Murgu Square, 300041 Timișoara, Romania*
[4]*Faculty of Pharmacy, "Vasile Goldiș" Western University of Arad, Revolutiei Bvd. 94, 310130 Arad, Romania*

**corresponding author: blidy@umft.ro*

Manuscript received: July 2024

Abstract

Oral contraceptives are widely used drugs, especially in the prevention of unwanted pregnancies. Although oral contraceptives are used in the management of several pathologies, their toxicity on the female body raises a question mark. The specialised literature highlights a connection between their use and the development of breast cancer, but there are uncertainties. The current study evaluates *in vitro* two recognised contraceptives - levonorgestrel (LG) and 17β-ethinylestradiol (EE) regarding the cytotoxicity and the changes occurring in two breast cancer cell lines MCF-7, a cell line positive for the oestrogen receptor (ER+) and MDA-MB-231, a triple-negative cell line (TNBC), without oestrogen and progesterone receptors. The obtained results reported that after 24 hours of exposure at LG, EE and LG-EE, the cell viability decreases with the increase of the dose, while with the increase of the exposure period, stimulation of proliferation is evident. Moreover, differences are observed between the two cell lines. At the level of MCF-7 cells, EE has a more pronounced stimulatory effect, while on the MDA-MB-231 line, LG induces cell proliferation and a low cytotoxic effect. Also, 24 hours after stimulation, signs of apoptosis are recorded at the dose of 7.5, while at the lowest concentration, a migratory effect is observed. Following the results, an affinity of EE for the MCF-7 line is observed due to the receptors present. However, additional investigations are needed to determine the mechanism of action of oral contraceptives on breast cancer.

Rezumat

Contraceptivele orale sunt medicamente utilizate pe scară largă, mai ales în prevenirea sarcinilor nedorite. Deși contraceptivele orale sunt folosite în gestionarea mai multor patologii, toxicitatea lor asupra organismului feminin ridică un semn de întrebare. Literatura de specialitate evidențiază o legătură între utilizarea lor și dezvoltarea cancerului de sân, dar există incertitudini. Studiul actual evaluează *in vitro* două contraceptive recunoscute - levonorgestrel (LG) și 17β-etinilestradiol (EE) privind citotoxicitatea și modificările care apar la nivelul a două linii celulare de cancer de sân MCF-7, o linie celulară pozitivă pentru receptorul de estrogen (ER+) și MDA-MB-231, o linie celulară triplu negativă (TNBC), fără receptori de estrogen și progesteron. Rezultatele obținute au relatat că după 24 de ore de expunere la LG, EE și LG-EE, viabilitatea celulară scade odată cu creșterea dozei, în timp ce odată cu creșterea perioadei de expunere este evidentă stimularea proliferării. Mai mult, se observă diferențe între cele două linii celulare. La nivelul celulelor MCF-7, EE are un efect stimulator mai pronunțat, în timp ce pe linia MDA-MB-231, LG induce proliferarea celulară și un efect citotoxic scăzut. De asemenea, la 24 de ore de la stimulare se inregistreaza semne de apoptoza la doza de 7,5 µM, în timp ce la cea mai mică concentrație se observă un efect migrator. În urma rezultatelor, se observă o afinitate a EE pentru linia MCF-7 datorită receptorilor prezenți. Cu toate acestea, sunt necesare investigații suplimentare pentru a determina mecanismul de acțiune al contraceptivelor orale asupra cancerului de sân.

Keywords: levonorgestrel, ethinylestradiol, breast cancer, proliferation

Introduction

In the United States, the contraceptive pill is one of the most widely prescribed forms of contraception, with about 25% of women aged 15 to 44 years reporting this method as their contraceptive choice. In general, the underlying mechanism of these methods is the inhibition of follicular development and hence the subsequent inhibition of ovulation [6]. Many research studies have highlighted the applications of oral contraceptives, among which it has been suggested that they can help to increase bone density and decrease

the risk of fractures, relieve acne and reduce the risk of major gynaecologic malignancies, namely ovarian and endometrial cancer [16], and additionally, they have also been shown to improve menstrual regularity with less dysmenorrhea [46]. One health concern among women who have used oral contraceptives is the risk of breast cancer. It has been found that women who have used oral contraceptives have a 7% higher risk of breast cancer than women who have never used them [36]. Various epidemiological studies have shown that long-term use of oral contraceptives is correlated with an increased risk of breast cancer [5].

Worldwide, breast cancer represents a serious health problem, with an increasing incidence and mortality [48]. Breast cancer is the most common type of cancer diagnosed in women and the main cause of cancer-related mortality among women [49]. The causes of this pathology's appearance and development are complex and not fully known. Breast cancer is a disease that includes molecular and intracellular changes and even epigenetic changes [24, 57]. However, hormonal disorders and the level of hormones in the body are the main factors in the development of cancer [51].

Data from the literature support that oestrogens are associated with a high risk of breast tumours in post-menopausal women, while antioestrogens (tamoxifen) reduce the incidence of cancer [8, 18]. Furthermore, animal studies have reported that oestrogens stimulate breast cancer, while a small exposure to them has the opposite effect [27].

Levonorgestrel represents a 17-beta-hydroxy steroid and a synthetic progestin often used individually or in association with oestrogen for contraception [35]. It is also recognised as a morning-after pill, being approved by the World Health Organization for the prevention of pregnancy, being used within 72 hours of unprotected sexual intercourse, or when the contraception method has failed. The mechanism of action of levonorgestrel is briefly that it binds to progesterone and androgen receptors and delays the release of gonadotropin-releasing hormone from the hypothalamus, which subsequently attenuates the increase in luteinizing hormone that occurs during the pre-ovulation phase and inhibits ovulation [54]. 17beta-Ethinylestradiol ($C_{20}H_{24}O_2$) [1]. was synthesized around 1930 by substituting oestradiol at C17 with an ethynyl group. It is the main oestrogen found in oral contraceptives used today [47]. There is a lack of consistency in the findings regarding the relationship between oral contraceptives and breast cancer. However, the risk is higher in women who start using oral contraceptives at a young age on a long-term basis [14].

As controversies exist regarding the risks and benefits of oral contraceptives on the female organism about breast cancer and because these medications are also often prescribed for gynaecological conditions [16], the present study evaluates *in vitro* two recognised contraceptives – Levonorgestrel and 17β-Ethinyl-oestradiol – concerning their cytotoxicity on two distinct breast cancer cell lines MCF-7, an oestrogen receptor-positive cell line (ER+) and MDA-MB-231, a cell line triple-negative (TNBC).

The study aimed to investigate whether the compounds affect the proliferation of breast cancer cells and to investigate the possible mechanisms underlying hormone-mediated breast cancer development *in vitro*.

Materials and Methods

Reagents, instruments and two-dimensional cell culture conditions

Several reagents were used to perform the *in vitro* analysis. Culture medium – Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS) and foetal bovine serum (FBS) were acquired from PAN-Biotech GmbH (Aidenbach, Germany). Sigma Aldrich, Steinheim, Germany provided levonorgestrel, 17β-ethinylestradiol, trypsin-EDTA and penicillin/streptomycin (Pen/Strep) solution. Dimethyl sulfoxide (DMSO), MTT (3-(4,5-di-methylthiazol2-yl)-2,5-diphenyltetrazolium bromide), lactate dehydrogenase (LDH) kit and the Hoechst 33342 dye were procured from ThermoFisher Scientific (Waltham, MA, USA). All the chemicals were analytically pure for use in cell culture.

The cells were furnished from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were grown in the EMEM supplemented with 10% FBS and 1% antibiotic mixture [40], while MDA-MB-231 cells were cultured in DMEM with 10% FBS and 1% antibiotic solution. During all the experiments, the cells were maintained in a humidified incubator, in standard conditions (5% CO_2 and 37°C). The devices used were Cytation 5 (plate reader) and Lionheart FX (automated microscope) by BioTek Instruments Inc. (Winooski, VT, USA).

Computational evaluation of levonorgestrel and ethinylestradiol

For the computational description of the tested compounds, regarding their potency and toxicity, OSIRIS Property Explorer was used, as previously reported by the study conducted by Dinu *et al*. [10]. The canonical simplified molecular-input line-entry system (SMILES) for ethynylestradiol was achieved from Pubchem [1].

Treatment regimen

The compounds, levonorgestrel (LG), ethinylestradiol (EE) and levonorgestrel-ethinylestradiol (LG-EE) were dissolved in the DMSO to prepare a stock solution. Four other concentrations to be tested are obtained from the prepared solution (0.1, 2.5, 5 and 7.5 μM).

MTT test - cell viability assay

The MTT test was performed to determine the effect on cell viability of the two compounds, as well as their combination on two breast cancer cell lines. The method was carried out by going through several

steps: (i) cells were cultivated in plates with 96 wells (10^4 cells/well); (ii) at 85% confluence the cells were tested with the compounds; (iii) after the stimulation period reagent 1 (10 µL) was added and cells were incubated for 3 h; (iv) finally reagent 2 (solubilizing buffer – 100 µL) was added and left in contact for 30 min at room temperature and in the dark; (v) the absorbance value was measured at 570 nm using a Cytation 5 device from BioTek Instruments Inc. (Winooski, VT, USA), as in the study conducted by Dinu *et al*. [11].

Assessment of Lactate Dehydrogenase (LDH) leakage

The LDH technique was used to determine the cytotoxic effects induced by the samples of interest on breast cancer cell lines. The principle of the analysis refers to the measurement of the cytosolic enzyme LDH leaked into the extracellular medium, which can only be quantified if the cell wall is damaged. The LDH method was developed following the steps provided by the manufacturer, as detailed in previously published articles [19, 43].

Nuclear morphology assessment

To evaluate the activity induced by the compounds (LG, EE, LG-EE) at the nuclear level in MDA-MB-231 and MCF-7 cells, Hoechst 33342 staining (1:2000 solution in PBS) was performed, as described in previous research [38]. Nuclei labelling was performed 24 hours after stimulation with the three samples (7.5 µM). A Lionheart FX automated microscope from BioTek Instruments Inc (Winooski, VT, USA) was used to identify changes that occurred after the application of the samples.

Based on the formula below [10], the apoptotic index (AI) was calculated:

$$\text{AI}\ (\%) = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \text{x}\ 100 \quad (1).$$

Migration assay

The migratory effect of the two cell lines after applying LG, EE and LG-EE was evaluated using a Scratch assay. The work protocol consisted of (i) culturing the cells in Corning plates with 24 wells; (ii) at 95% confluence, a scratch was made in the middle with the AutoScratch™ Wound Making Tool from BioTek® Instruments Inc., Winooski, VT, USA; (iii) after washing with PBS, the cells were treated with the 3 samples at the highest concentration. Scratch widths were measured with Gen5™ Microplate Data Collection and Analysis Software provided by BioTek® Instruments Inc., Winooski, VT, USA. The rate of migration and proliferation of scratches was calculated according to the formula applied by Khaled *et al*. [19]:

$$\text{Scratch closure rate}\ (\%) = \frac{\text{At0}-\text{At}}{\text{At0}} \text{x}\ 100 \quad (2)$$

where, At_0 - wound closure at 0h; At – wound closure at 24 h.

Statistical analysis

The one-way ANOVA test followed by Dunnett's multiple comparisons post-test was used to identify the statistical differences between multiple treatment groups. The results are signalled as mean ± standard deviation (SD) using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The statistically significant differences between the results were noted with *, as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results and Discussion

The regulation and development of the reproductive system in women are significantly influenced by oestrogens. Female hormones act through various receptors to generate signals in target cells, affecting many oestrogen response elements. Breast cancer is mostly a hormone-dependent pathology, so more than 60% of breast cancers express sex hormone receptors (oestrogen/progesterone) which are an important marker in the prognosis of the disease [2].

Over the years, it has been shown that the risk of breast cancer is more pronounced for women who have used or are using hormonal contraceptives, compared to women who have not used this type of hormonal therapy. Moreover, it has been shown that the risk is greater with increasing use for a longer time [31].

First, LG and EE toxicity were exposed computationally with the help of the OSIRIS Property Explorer program. The OSIRIS Property Explorer program is a popular web tool to aid in the computational prediction of drug-like characteristics, physicochemical properties and toxic potential [20]. The results of the two compounds are described in Table I and show that both LG and EE have an overall positive score but with a negative drug-likeness. Moreover, EE does not have a tumorigenic, mutagenic, irritant, or reproductive toxicity potential, while LG has a reproductive toxicity potential.

Table I

Computational prediction of the properties exercised by LG and EE

Compound	MW	Solubility	cLogP	Drug-Likeness	Drug Score	Mutagenic, tumorigenic, irritant toxic potential	Reproductive toxic potential
LG	312.0	-4.59	3.54	1.42	0.37	No indication	Risk
EE	296.0	-4.45	3.8	0.64	0.57	No indication	

To understand the effect produced by oral contraceptives on breast cancer, two breast cancer cell lines were used, MCF-7 (oestrogen receptor-positive breast cancer (ER+)) and MDA-MB-231 (triple-negative breast cancer).

Breast cancer is one of the types of cancer with the greatest impact on women. Surgical interventions, radio- and chemotherapy represent the basis for treating breast cancer, but the effectiveness of the therapy is unsatisfactory. Therefore, targeted treatment with compounds directed to the receptors of specific molecules seems to have an important role in the management of the disease [12, 28].

The exact molecular mechanisms by which oestrogens influence the production and development of breast cancer are not fully understood. Tumour cell proliferation is dependent on oestrogens and growth factors. Oestrogens act stimulatory in a paracrine manner through the production of cytokines, their receptors, or stromal derived growth factors [22]. Oestrogens work by binding to ER-α and ER-β oestrogen receptors that have different roles in the development of breast tumour cells [38]. Most experimental evidence states that oestradiol stimulates cell growth by activating ER-α and can produce mutations that occur during DNA replication, [41, 42], while ER-β receptors have suppressive activities [45].

Meanwhile, progesterone acts through two receptor isoforms PR-A and PR-B. Most breast cancers are positive for hormone receptors, and ER and PR are important markers in the prognosis and management of cancer therapy [13].

MCF-7 breast cancer cells represent an efficient *in vitro* model to study hormone-responsive breast cancer because they contain receptors for oestrogen and progesterone [53]. The MCF-7 cell line is one of the tumour cell lines that is HR+/HER2-, being considered a relevant model for the investigation of invasive breast cancer [4].

The type of triple-negative breast cancer (MDA-MB-231) has a poor survival prognosis and the highest probability of recurrence [9]. Triple-negative breast cancer is a different pathology, lacking the expression of ER-α, PRs and HER2 [29]. But MDA-MB-231 cells can express receptors ER-β, ERR (oestrogen-related receptor), GPER-1 (oestrogen receptor coupled to G protein) and mPR-α (membrane receptor) [35, 52].

Thus, following the data from the literature, the current study was developed to determine the effect of the two sex hormones on the behaviour of MCF-7 and MDA-MB-231 breast tumour cells to understand their mode and role in the development of cancer. The impact of LG, EE and the combination of the two oestrogens on the two cancer lines was tested, at 24 and 72 hours, respectively.

Initially, the action exerted on cell viability was tested. On the MCF-7 cell line, for levonorgestrel, a slight decrease in viability percentages can be observed after exposure for 24 hours, reaching a rate of 70% at the highest tested concentration; while after 72 hours of treatment, the results show for the concentration of 0.1 and 2.5 μM a higher viability compared to the control (>100%) (Figure 1A). Regarding ethinylestradiol, at 24 hours a slight antiproliferative effect is observed, and at 72 hours a decrease in viability is observed with the increase of the dose, but for all 4 concentrations viability percentages >105% are exposed (Figure 1B). Also, the combination of the two compounds exerts a dose- and time-dependent effect, with viability values of approximately 80% and respectively 90% at the concentration of 7.5 μM (Figure 1C).

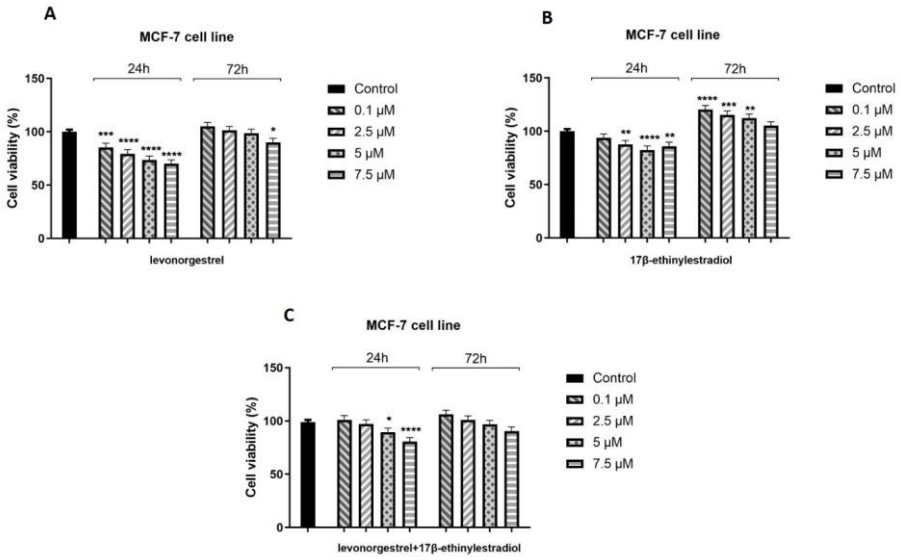

Figure 1.

Cell viability effect of LG, EE and LG-EE (0.1, 2.5, 5 and 7.5 μM) determined by the MTT test, 24 hours and 72 hours post-stimulation of MCF-7 cells

The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (*p < 0.05; **p < 0.01; ***p < 0.001; **** p < 0.0001)

On the line of triple-negative breast cancer, EE decreased cell viability more pronounced (≈ 69% at 5 μM after 24 hours), also with longer exposure the percentages of viability decreased dose-dependently starting from 103% (Figure 2A). For LG, exposure for 72 hours, recorded high percentages of viability, with cell proliferation when applying the dose of 0.1 and 2.5 μM, respectively, greater than 100% (Figure 2B). LG-EE exhibited a slight antiproliferative effect at both time intervals, but without exerting a cytotoxic effect (Figure 2C).

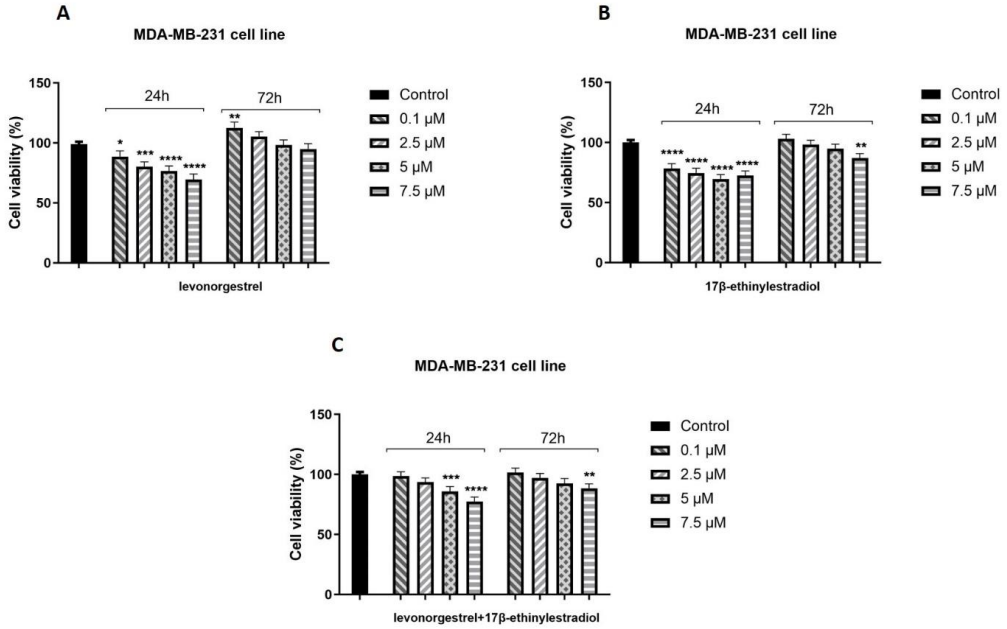

Figure 2.

Cell viability effect of LG, EE and LG-EE (0.1, 2.5, 5 and 7.5 μM) determined by the MTT test, 24 hours and 72 hours post-stimulation of MDA-MB-231 cells

The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (*p < 0.05; **p < 0.01; ***p < 0.001; **** p < 0.0001)

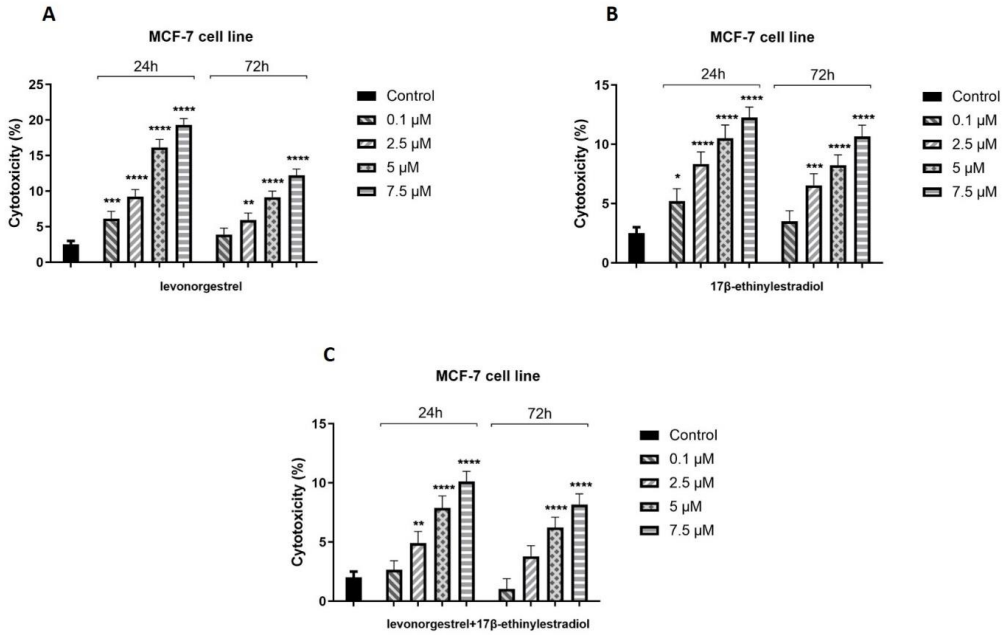

Figure 3.

Graphical illustration of LDH release obtained at 24 hours and 72 hours after treatment of MCF-7 cells with LG, EE and LG-EE

The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)

It can be highlighted that the application of EE stimulates to a greater extent the cell proliferation of MCF-7, which is a type of breast cancer with positive oestrogen receptors (ER+), a tumour that spreads with increasing oestrogen levels.

To complete the data regarding the impact of the two compounds on the two breast cancer lines, the action on the permeability and integrity of the cell membrane was investigated by quantifying the release of lactate dehydrogenase (LDH).

On the MCF-7 line, it can be seen that LG exhibited the most pronounced cytotoxic effect both at 24 and at 72 hours, results that are consistent with those regarding viability. For EE, after exposure for 24 hours, a release of LDH of up to 12.22% is observed, while for LG-EE a lower cytotoxic effect is noted, after 72 hours of stimulation reaching a percentage of only 8.11%. The application of the compounds on the MDA-MB-23 line exhibits slightly different results, but just as on the MCF-7 cells, time- and concentration-dependent effects are recorded. On triple-negative breast cancer, a greater cytotoxic action was evident when applying EE (26.12%) at 24 hours. In all cases, a more pronounced cytotoxic effect is shown at 24 hours, which decreases with increasing exposure time. For LG-EE, an LDH release of only 13.32% and 10.12% was observed after 24 and 72 hours of stimulation, respectively.

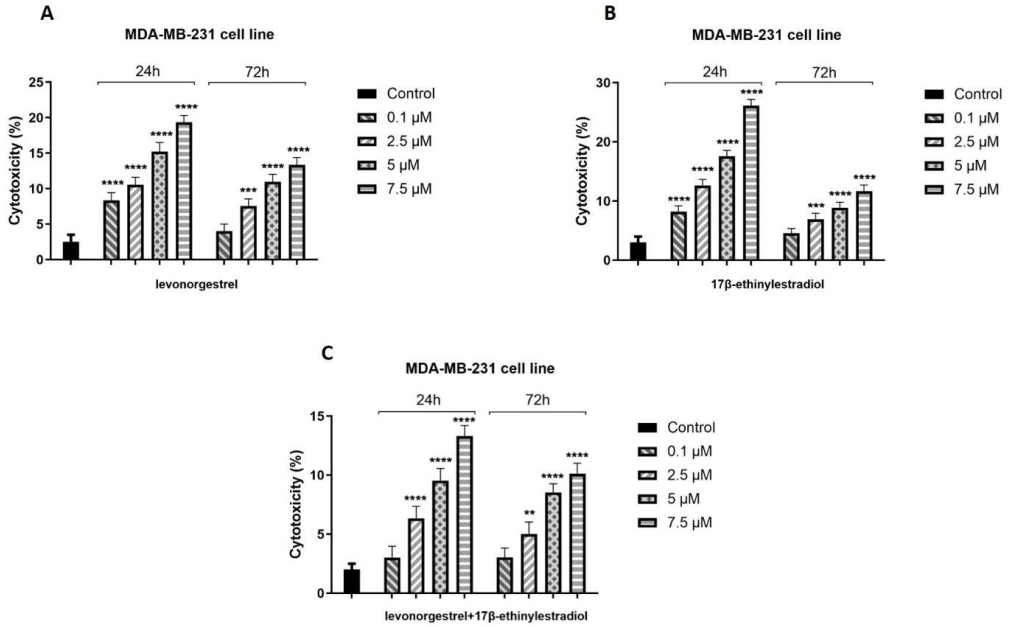

Figure 4.

Graphical illustration of LDH release obtained at 24 hours and 72 hours after treatment of MDA-MB-231 cells with LG, EE and LG-EE

The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (**p < 0.01; ***p < 0.001; **** p < 0.0001)

In a previous study on the MDA-MB-231 cell line, it was shown that 17β-ethinylestradiol exerts a significant antiproliferative action after stimulation for 24 hours, with a decrease in viability of up to 64.32%, at the concentration of 10 μM. However, with the time interval increase, a stimulatory effect was observed, especially at the dose of 0.05 μM. In the same way, for levonorgestrel, there was a decrease in viability, but less, up to 72.79%, followed by a stimulation of proliferation at 48 and 72 hours, respectively (> 100% at all doses tested). Moreover, for the combination of EE-LG, the effect was to the same extent dependent on the dose, after 72 hours of exposure the percentages were similar to control cells [47].

Coricovac *et al.*, evaluated the two compounds as well as their combination regarding the cytotoxic profile on healthy and tumourous human and murine skin lines, to evaluate hormonal therapy in association with UV radiation and malignant diseases. Through the Alamar Blue method, it was shown that on healthy skin lines (HaCaT, 1BR3, HEMa and JB6 Cl 41-5a) the compounds decrease cell viability to a small extent, without having a cytotoxic effect. While in the presence of UVB irradiation (40 mJ/cm^2), a considerable decrease in the viability of healthy cells was observed, the most pronounced being for HEMa (58.25%). On A375 human melanoma cells, there was a significant dose-dependent decrease in cell viability, especially after UVB irradiation, reaching 10 μM at a viability percentage of 56% *vs*. 49.69% for EE + LNG *vs*. LG + EE + UVB. An opposite effect was exhibited for B164A5 murine melanoma cells. Thus, after irradiation, higher viability was quantified for the cells exposed to the

compounds and irradiated with UVB, higher percentages being observed at the dose of 1 μM [7].

In addition to cell viability and cytotoxicity assays, Hoechst 33342 nuclear staining was performed to investigate potential cell death effects induced by LG, EE and LG-EE. Following the previous results, we observed a decrease in viability after 24 hours of exposure, especially at the concentration of 7.5 μM, so the nuclear analysis was performed at this time interval and after exposure to the highest dose. Regarding the nuclear changes, we can say that on both cell lines slight signs of nuclear fragmentation and apoptotic bodies are observed, being more pronounced for LG on MDA-MB-231 cells, without signs of necrosis (Figure 5 and Figure 6).

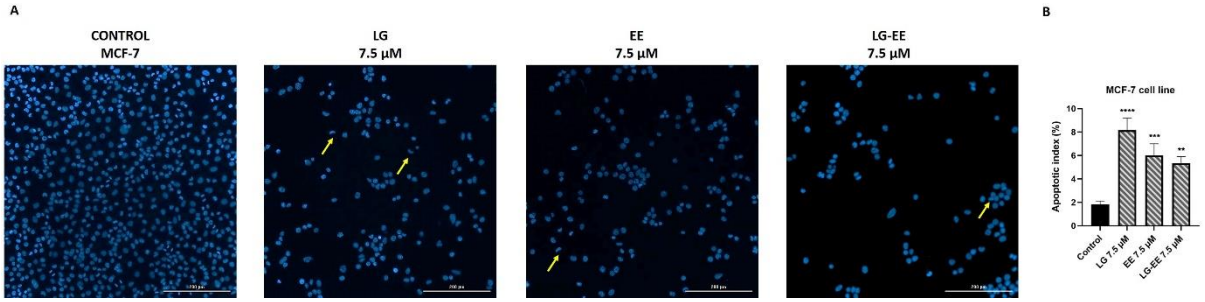

Figure 5.

(A) Cell nuclei staining of MCF-7 cells using Hoechst 33342 at 24 hours post-exposure to LG, EE and LG-EE (7.5 μM) and (B) apoptotic index (AI) percentages

The yellow arrows mark signs of apoptosis. The scale bars represent 200 μm. The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (**p < 0.01; ***p < 0.001; **** p < 0.0001)

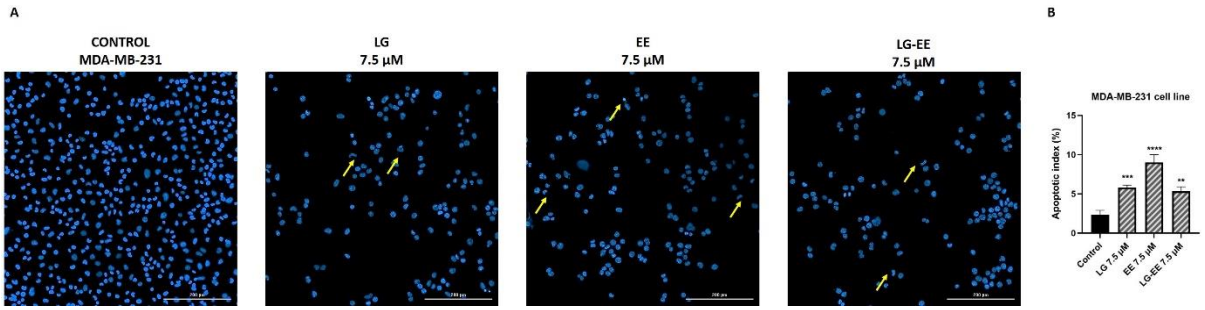

Figure 6.

(A) Cell nuclei staining of MDA-MB-231 cells using Hoechst 33342 at 24 hours post-exposure to LG, EE and LG-EE (7.5 μM) and (B) apoptotic index percentages

The yellow arrows mark signs of apoptosis. The scale bars represent 200 μm. The statistical differences between the untreated and the treated group were evaluated by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (**p < 0.01; ***p < 0.001; **** p < 0.0001)

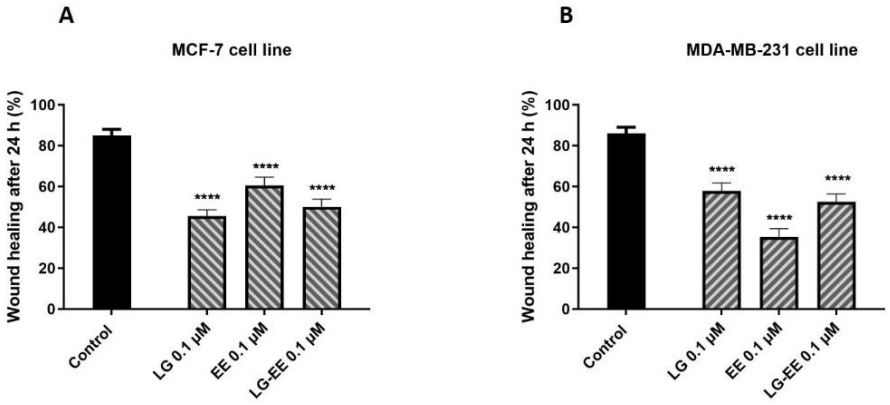

Figure 7.

Graphical representation of the migratory capacity of MCF-7 (A) and MDA-MB-231 (B) cells following the treatment with LG, EE, LG-EE (0.1 μM) for 24 h

In the study led by Simu *et al.*, oral contraceptives were investigated regarding the possible changes at the nuclear level by the Hoechst staining method on MDA-MB-231 cells. In this study, changes such as membrane blebbing, nuclear fragmentation, and the presence of apoptotic bodies were highlighted at concentrations of 0.05 and 10 μM. Also, no signs of necrosis were exposed, results that are consistent with those obtained in the current study [47].

Figure 7 shows the migratory activity of MCF-7 and MDA-MB-231 cells after stimulation with LG, EE and LG-EE. The lowest concentration at which a stimulation of cell viability was observed in previous tests was analysed.

The *in vitro* Scratch test reported that the migration capacity of MCF-7 and MDA-MB-231 cells was not inhibited by the application of the samples. For 17 β-ethinylestradiol, on the MCF-7 cell line, wound closure of up to 60.61% was recorded, while on MDA-MB-231 cells the wound healing rate was 35%. For LG, a stronger stimulation of migration was observed on MDA-MB-231 cells, with a migration rate of 57.87%, while for LG-EE, a wound closure percentage of 50.15% and 52.66% was reported on the MCF-7 and MDA-MB-231 cell lines, respectively (Figure 7).

In previous research, Scratch analysis was performed to determine the effect produced by EE and LG and the combination of the two on cell migration, using the lowest concentrations (0.05 and 1 μM). On the triple-negative breast cancer line, the highest migration rate was observed for control cells (85.9%), followed by cells exposed to the lowest concentration of LG (85%) and respectively to the dose of 1 μM of LG -EE (81.6%) [47]. In the present study, we observed the same trend of proliferation and migration of MDA-MB-231 cells, more pronounced after the application of LG, while the hormone-responsive breast cancer line exhibited a more obvious migration rate for EE.

In the study conducted by Coricovac and the research group, the anti-migratory effect of LG, EE and LG-EE at a dose of 1 μM was evaluated. Exposure of healthy skin cells to EE exhibited a slight stimulation compared to untreated cells, more evident on the 1BR3 line. For LG, no effect on migration was observed on any healthy line, and for LG-EE, an antimigratory effect was demonstrated on HaCaT cells and a stimulatory effect on 1BR3 and JB6 Cl 41-5a lines. The migration capacity of human and murine melanoma cells was not inhibited by the application of LG, EE and LG-EE, but stimulated, with a less pronounced effect for LG [7].

Moreover, in another study, it was demonstrated that ethinylestradiol produces a stimulation of the proliferation of MCF-7 cells as well as HCC1500 (metastatic breast cancer cells and primary breast cancer cells with positive human oestrogen and progesterone receptors), in a continuous treatment regimen and intermittent (10^{-9} - 10^{-10} M). Thus, on the MCF-7 line, EE significantly stimulated cell growth. Values of 49% and 54% were recorded at the lowest dose and percentages of 28% and 39% for the concentration of 1 nM, for the period of intermittent and continuous exposure [30].

Other studies have used MCF-7 tumour cells as well as other types of breast cancer cell lines (T47D) to evaluate the effect of oestrogens and progestogens on tumour development and define their potential risk, demonstrating a stimulatory effect [15, 21, 22, 32].

Zhou *et al.* demonstrated concentration-dependent inhibition of MDA-MB-231 cell growth after stimulation with progesterone (20 - 80 ng/mL) [58].

Also, in another study, the influence of the two hormones on the breast cancer line Hs 578 Bst was evaluated. A decrease in proliferation was found when applying LG and the LG-EE combination, while exposure to EE led to a stimulation of cell growth [17].

Grubczak demonstrated that blocking CTLA-4 in lymphocytes can lead to the suppression of the development of breast cancer cells, showing that the responses of tumour cells can differ depending on the surface expression of the receptors [12].

In another study, data were presented that prove that the development and proliferation of breast cancer can be influenced by oestradiol through different mechanisms that do not depend on the ER receptor [56].

Therefore, breast cancer is a complex pathology, with many mutated genes that can develop the tumour [3]. Most breast cancers in postmenopausal women are ER+/PR+ [37]. However, new membrane receptors that can facilitate the non-classical (non-genomic) signalling of steroid hormones [33] have been identified, which can be clinically successful.

Non-genomic signalling allows hormones to bind receptors on cell membranes that do not have DNA-binding domains, but by activating secondary messengers can produce fast-acting responses [55].

Progestins are widely used in hormonal contraception, despite their association with a high risk of breast cancer. Although some progress has been made in understanding the mechanism of action underlying the risk of oral contraceptives, studies that have evaluated the effects of progestins on the behaviour of cancer cells *in vitro* are rare. Moreover, research studying the role of kinase signalling pathways (ERK1/2 and JNK) with an important role in the development and progression of breast cancer is limited [26].

Cell proliferation is a complex process that involves the regulation of different genes [23]. The study carried out by Louw-du Toit R highlights that the activation of the ERK1/2 and/or JNK pathways supports progestins such as LNG in stimulating the proliferation of breast cancer cells, as well as their migration [26].

In another study, it was shown on the T47D cell line that cell proliferation occurred following the regulation of the MKI67 and CCND1 genes by progestogens, while on the MCF-7 BUS line, no regulation of these genes was observed. Moreover, it was shown that progesterone, medroxyprogesterone acetate and

drospirenone regulate c-MYC mRNA expression in MCF-7 BUS breast cancer cells [39].

As we pointed out in this study, oral contraceptives can influence the development of breast cancer, but a clear understanding of the mechanism is difficult due to the heterogeneity observed in the studies carried out over the years. Therefore, the translation of the results into practice must be carried out with caution, and the decision to use contraceptives among women during the fertile period should be based on a personalised risk-benefit balance [50].

To have a broader view of the mechanism by which contraceptives act on breast cancer cells, future studies are needed to detect and characterize the proteins that regulate gene expression. Gene-specific mRNA analysis, siRNA and various chromatin immunoprecipitated tests (ChIP) can be performed in this regard [25, 37].

Conclusions

The study wanted to determine the influence of the two compounds (17β-ethinylestradiol and levonorgestrel), as well as their association on tumour cell lines MCF-7, an oestrogen receptor-positive cell line (ER+) and MDA-MB-231, a triple-negative cell line (TNBC). The effects of the compounds were dependent on the dose and time of application. On both cell lines, after 24 hours of stimulation, a decrease in cell viability was observed with increasing dose, but with increasing exposure time, cell proliferation was observed. On MCF-7 cells, the most pronounced cytotoxic effect was given by LG, and on MDA-MB-231 cells a stronger effect was highlighted for EE. In addition, 24 hours after the application of the compounds, signs of apoptosis were observed at the highest dose, while at the lowest concentration, a migratory effect was observed. These results can be attributed to the affinity of EE for oestrogen receptor-positive cell lines. The data obtained support the idea that oral contraceptives influence the two types of breast cancer. However, detailed studies are needed to understand the mechanisms underlying these effects.

Conflict of interest

The authors declare no conflict of interest.

References

1. 17-beta-Ethinylestradiol. https://pubchem.ncbi.nlm.nih.gov/compound/17beta-Ethinylestradiol
2. Al-Shami K, Awadi S, Khamees A, Alsheikh AM, Al-Sharif S, Bereshy RA, Al-Eitan SF, Banikhaled SH, Al-Qudimat AR, Al-Zoubi RM, Al Zoubi MS, Estrogens and the risk of breast cancer: a narrative review of literature. *Heliyon*, 2023; 9(9).
3. Bernhardt SM, Dasari P, Walsh D, Townsend AR, Price TJ, Ingman WV, Hormonal Modulation of Breast Cancer Gene Expression: Implications for Intrinsic Subtyping in Premenopausal Women. *Front Oncol.*, 2016; 6: 241.
4. Booms A, Coetzee GA, Pierce SE, MCF-7 as a model for functional analysis of breast cancer risk variants. *Cancer Epidemiol Biomarkers Prev.*, 2019; 28(10): 1735-1745.
5. Conz L, Mota BS, Bahamondes L, Teixeira Dória M, Derchain SFM, Rieira R, Sarian LO, Levonorgestrel-releasing intrauterine system and breast cancer risk: A systematic review and meta-analysis. *Acta Obstet Gynecol Scand.*, 2020; 99(8): 970-982.
6. Cooper DB, Patel P, Oral Contraceptive Pills. *Stat Pearls*, 2024, www.ncbi.nlm.nih.gov/books/NBK430882/.
7. Coricovac D, Farcas C, Nica C, Pinzaru I, Simu S, Stoian D, Soica C, Proks M, Avram S, Navolan D, Dumitru C, Popovici RA, Dehelean CA, Ethinylestradiol and levonorgestrel as active agents in normal skin and pathological conditions induced by UVB exposure: *in vitro* and *in ovo* assessments. *Int J Mol Sci.*, 2018; 19(11): 3600.
8. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P, Overview of the main outcomes in breast-cancer prevention trials. *Lancet*, 2003; 361: 296-300.
9. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka C, Lickley LA, Rawlinson E, Sun P, Narod SA, Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res.*, 2007; 13: 4429-4434.
10. Dinu S, Matichescu A, Buzatu R, Marcovici I, Geamantan-Sirbu A, Semenescu AD, Bratu RC, Bratu DC, Insights into the cytotoxicity and irritant potential of chlorhexidine digluconate: an *in vitro* and *in ovo* safety screening. *Dentistry J.*, 2024; 12(7): 221.
11. Dinu S, Olteanu ND, Matichescu A, Zetu IN, Szuhanek C, Geamantan A, Semenescu AD, Anton A, Buzatu R, *In vitro* biocompatibility and *in ovo* irritant potential screening of two clear aligners with orthodontic applications. *Farmacia*, 2024; 72(3): 513-520.
12. Grubczak K, Kretowska-Grunwald A, Groth D, Poplawska I, Eljaszewicz A, Bolkun L, Starosz A, Holl JM, Mysliwiec M, Kruszewska J, Wojtukiewicz MZ, Moniuszko M, Differential response of MDA-MB-231 and MCF-7 breast cancer cells to *in vitro* inhibition with CTLA-4 and PD-1 through cancer-immune cells modified interactions. *Cells*, 2021; 10(8): 2044.
13. Hilton HN, Clarke CL, Graham JD, Estrogen and progesterone signalling in the normal breast and its implications for cancer development. *Mol Cell Endocrin.*, 2018; 466: 2-14.
14. Institute of Medicine (US) Committee on the Relationship Between Oral Contraceptives and Breast Cancer. Oral Contraceptives and Breast Cancer. *National Academies Press (US)*; 1991, www.ncbi.nlm.nih.gov/books/NBK234348/.
15. Jeng MH, Parker CJ, Jordan VC, Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. *Cancer Res.*, 1992; 52: 6539-6546.
16. Jensen JT, Speroff L, Health benefits of oral contraceptives. *Obstet Gynecol Clin North Am.*, 2000; 27(4): 705-721.

17. Karandeep S, Lauren A, Breast and ovarian cell behaviour in the presence of contraceptive ingredients. *J Appl Biotechnol Bioeng.*, 2020; 7: 67-80.
18. Key T, Appleby P, Barnes I, Reeves G, Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst.*, 2002; 94: 606-616.
19. Khaled Z, Vlaia L, Semenescu AD, Dumitrescu C, Coneac G, Kis AM, Moacă A, Vlaia V, Iurciuc S, Ștefănescu EH, *In vitro* study of topical caffeine and ascorbyl magnesium phosphate formulations: preparation, physicochemical characterization and pharmaco-toxicological assessment. *Farmacia*, 2024; 72(2): 381-396.
20. Khan T, Ahmad R, Azad I, Raza S, Joshi S, Khan AR, Computer-aided drug design and virtual screening of targeted combinatorial libraries of mixed-ligand transition metal complexes of 2-butanone thiosemi-carbazone. *Comput Biol Chem.*, 2018; 75: 178-195.
21. Kloosterboer HJ, Schoonen WG, Deckers GH, Klijn JG, Effects of progestagens and Org OD14 *in in vitro* and *in vivo* tumour models. *J Steroid Biochem Mol Biol.*, 1994; 49: 311-318.
22. Krämer EA, Seeger H, Krämer B, Wallwiener D, The effects of progesterone, medroxyprogesterone acetate and norethisterone on growth factor- and estradiol-treated human cancerous and noncancerous breast cells. *Menopause*, 2005; 12: 468-474.
23. Liao DJ, Dickson RB, c-Myc in breast cancer. *Endocr Relat Cancer.*, 2000; 7(3): 143-64.
24. Li L, Gao P, Li Y, Shen Y, Xie J, Sun D, Xue A, Zhao Z, Xu Z, Zhang M, Li B, Jiang J, JMJD2A-dependent silencing of Sp1 in advanced breast cancer promotes metastasis by downregulation of DIRAS3. *Breast Cancer Res Treat.*, 2014; 147: 487-500.
25. Louw-du Toit R, Hapgood JP, Africander D, Medroxy-progesterone acetate differentially regulates interleukin (IL)-12 and IL-10 in a human ectocervical epithelial cell line in a glucocorticoid receptor (GR)-dependent Manner*. *JBC*, 2014; 289(45): 31136-31149.
26. Louw-du Toit R, Simons M, Africander D, Progestins and breast cancer hallmarks: The role of the ERK1/2 and JNK pathways in estrogen receptor positive breast cancer cells. *J Steroid Biochem Mol Biol.*, 2024; 237: 106440.
27. Maynadier M, Chambon M, Basile I, Gleizes M, Nirde P, Gary-Bobo M, Garcia M, Estrogens promote cell-cell adhesion of normal and malignant mammary cells through increased desmosome formation. *Mol Cell Endocrinol.*, 2012; 364: 126-133.
28. McDonald ES, Clark AS, Tchou J, Zhang P, Freedman GM, Clinical diagnosis and management of breast cancer. *J Nucl Med.*, 2016; 57(Suppl 1): 9S-16S.
29. Mehanna J, Haddad FGH, Eid R, Lambertini M, Kourie HR, Triple-negative breast cancer: current perspective on the evolving therapeutic landscape. *Int J Womens Health.*, 2019; 11: 431-437.
30. Merki-Feld GS, Seeger H, Mueck AO, Comparison of the proliferative effects of ethinylestradiol on human breast cancer cells in an intermittent and a continuous dosing regime. *Horm Metab Res.*, 2008; 40(3): 206-209.
31. Mørch LS, Skovlund CW, Hannaford PC, Iversen L, Fielding S, Lidegaard Ø, Contemporary hormonal contraception and the risk of breast cancer. *N Engl J Med.*, 2017; 377(23): 2228-2239.
32. Mueck AO, Seeger H, Wallwiener D, Comparison of the proliferative effects of estradiol and conjugated equine estrogens on human breast cancer cells and impact of continuous combined progestogen addition. *Climacteric.*, 2003; 6: 221-227.
33. Mueck AO, Ruan X, Seeger H, Fehm T, Neubauer H, Genomic and non-genomic actions of progestogens in the breast. *J Steroid Biochem Mol Biol.*, 2014; 142: 62-67.
34. Norgestrel. https://pubchem.ncbi.nlm.nih.gov/compound/Norgestrel.
35. Nouriemamzaden F, Word B, Cotton E, Hawkins A, Littlejohn K, Moore R, Miranda-Carbon G, Orish CN, Lyn-Cook B, Modulation of estrogen α and progesterone receptors in triple negative breast cancer cell lines: the effects of vorinostat and indole-3-carbinol *in vitro*. *Anticancer Res.*, 2020; 40: 3669-3683.
36. Oral Contraceptives and Cancer Risk. www.cancer.gov/about-cancer/causes-prevention/risk/hormones/oral-contraceptives-fact-sheet.
37. Pedroza DA, Subramani R, Tiula K, Do A, Rashiraj N, Galvez A, Chatterjee A, Bencomo A, Rivera S, Lakshmanaswamy R, Crosstalk between progesterone receptor membrane component 1 and estrogen receptor α promotes breast cancer cell proliferation. *Lab Invest.*, 2021; 101(6): 733-744.
38. Perkins MS, Louw-du Toit R, Africander D, A comparative characterization of estrogens used in hormone therapy *via* estrogen receptor (ER)-α and -β. *J Steroid Biochem Mol Biol.*, 2017; 174: 27-39.
39. Perkins MS, Louw-du Toit R, Jackson H, Simons M, Africander D, Upregulation of an estrogen receptor-regulated gene by first generation progestins requires both the progesterone receptor and estrogen receptor alpha. *Front Endocrinol (Lausanne)*, 2022; 13: 959396.
40. Popescu R, Daliborca CV, Cimporescu A, Vlad CS, Verdes D, Botau D, Filimon MN, Pauliuc I, Citu C, Dumitrascu V, Chemical properties and *in vitro* antitumour effects of *Momordica charantia* extracts in different solvents. *Revista de chimie*, 2016; 67(1): 69-73,
41. Preston-Martin S, Pike MC, Ross RK, Henderson BE, Epidemiologic evidence for the increased cell proliferation model of carcinogenesis. *Environ Health Perspect Suppl.*, 1993; 101: 137-138.
42. Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE, Increased cell division as a cause of human cancer. *Cancer Res.*, 1990; 50: 7415-7421.
43. Semenescu AD, Moacă EA, Iftode A, Dehelean CA, Tchiakpe-Antal DS, Vlase L, Rotunjanu S, Muntean D, Chiriac SD, Chioibaș R, Recent updates regarding the antiproliferative activity of *Galium verum* extracts on A375 human malignant melanoma cell line. *Life*, 2024; 14(1): 112.
44. Semenescu AD, Moacă EA, Iftode A, Dehelean CA, Tchiakpe-Antal DS, Vlase L, Vlase AM, Muntean D, Chioibaş R, Phytochemical and nutraceutical screening of ethanol and ethyl acetate phases of Romanian *Galium verum* herba (*Rubiaceae*). *Molecules*, 2023; 28(23): 7804.

45. Settings C, Vingiani A, Pruneri G, Vernieri C, Hormone receptor loss in breast cancer: molecular and therapeutic implications. *Cells*, 2020; 9(12): 2644.
46. Sherif K, Benefits and risks of oral contraceptives. *Am J Obstet Gynecol.*, 1999; 180(6 Pt 2): S343-348.
47. Simu S, Marcovici I, Dobrescu A, Malita D, Dehelean CA, Coricovac D, Olaru F, Draghici GA, Navolan D, Insights into the behaviour of triple-negative MDA-MB-231 breast carcinoma cells following the treatment with 17β-ethinylestradiol and levonorgestrel. *Molecules*, 2021; 26(9): 2776.
48. Tian JM, Ran B, Zhang CL, Yan DM, Li XH, Estrogen and progesterone promote breast cancer cell proliferation by inducing cyclin G1 expression. *Braz J Med Biol Res.*, 2018; 51(3): 1-7.
49. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A, Global cancer statistics, 2012. *CA Cancer J Clin.*, 2015; 65: 87-108.
50. Torres-de la Roche LA, Acevedo-Mesa A, Lizarazo IL, Devassy R, Becker S, Krentel H, De Wilde RL, Hormonal Contraception and the Risk of Breast Cancer in Women of Reproductive Age: A Meta-Analysis. *Cancers (Basel)*, 2023; 15(23): 5624.
51. Travis RC, Key TJ, Oestrogen exposure and breast cancer risk. *Breast Cancer Res.*, 2003; 5: 239-247.
52. Treeck O, Schüler-Toprak S, Ortmann O, Estrogen actions in triple-negative breast cancer. *Cells*, 2020; 9(9): 2358.
53. Vickers PJ, Dickson RB, Shoemaker R, Cowan KH, A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumour growth *in vivo*. *Mol Endocrinol.*, 1988; 2(10): 886-892.
54. Vrettakos C, Bajaj T, Levonorgestrel. *StatPearls*, 2024; www.ncbi.nlm.nih.gov/books/NBK539737/.
55. Wilkenfeld SR, Lin C, Frigo DE, Communication between genomic and non-genomic signalling events coordinate steroid hormone actions. *Steroids*, 2018; 133: 2-7.
56. Yue W, Wang JP, Li Y, Fan P, Liu G, Zhang N, Conaway M, Wang H, Korach KS, Bocchinfuso W, Santen R, Effects of estrogen on breast cancer development: role of estrogen receptor independent mechanisms. *Int J Cancer.*, 2010; 127(8): 1748-1757.
57. Zhang H, Stephens LC, Kumar R, Metastasis tumour antigen family proteins during breast cancer progression and metastasis in a reliable mouse model for human breast cancer. *Clin Cancer Res.*, 2006; 12: 1479-1486.
58. Zhou L, Zhou W, Zhang H, Hu Y, Yu L, Zhang Y, Progesterone suppresses triple-negative breast cancer growth and metastasis to the brain *via* membrane progesterone receptor α. *Int J Mol Med.*, 2017; 40: 755-761.