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Impact of minor cannabinoids on key pharmacological targets of estrogen receptor-positive breast cancer

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ABSTRACT

Endocrine therapy for estrogen receptor-positive (ER^+) breast cancer has significantly improved over the last decades. However, it presents some limitations that make the search for novel therapeutic options mandatory. Several studies have been conducted to understand the anti-tumor potential of cannabinoids in breast cancer. Yet, most of them are focused on the major phytocannabinoids Δ^9-tetrahydrocannabinol (**THC**) and cannabidiol (**CBD**). However, *Cannabis* has other minor phytocannabinoids whose anti-cancer properties are still to be elucidated. Here, we investigated the mechanisms of action of four minor cannabinoids, cannabigerol (**CBG**), cannabidivarin (**CBDV**), cannabinol (**CBN**), and cannabichromene (**CBC**), in 2D and 3D ER^+ breast cancer models. These cannabinoids dysregulate MCF-7aro cell cycle progression, induce apoptosis by different mechanisms, and inhibit the growth of MCF-7aro spheroids. **CBG** exerts its effects through a down-regulation of both ER and AR protein levels, while **CBDV** reduces aromatase protein levels. **CBN** and **CBC** simultaneously affect the three targets, ER, aromatase, and AR. In fact, **CBN** and **CBC** present an AR-dependent cell death, down-regulate aromatase levels, and act as ER negative regulators impairing cancer cell growth. **CBN** caused the most pronounced effects. Overall, this study highlights the anti-cancer properties and the therapeutic potential of these minor cannabinoids in ER^+ breast cancer.

1. Introduction

As breast cancer incidence and mortality are rising, with female breast cancer occupying the first place as the deadliest cancer in woman worldwide [1], novel therapies are needed to fight this disease. Among the various breast cancer subtypes, estrogen receptor-positive (ER^+) breast cancer is the most frequently diagnosed, with its dependence on estrogen being the main target for the therapies applied in clinic [2–4]. In fact, endocrine therapy, comprising the aromatase inhibitors (AIs) anastrozole (Ana), letrozole (Let), and exemestane (Exe), as well as the anti-estrogens tamoxifen and fulvestrant, have been used for several years as first-line therapy for both pre- and post-menopausal women with early and advanced ER^+ breast cancers [2,5,6]. Due to some adverse effects, mainly the development of endocrine resistance [7,8], treatment modifications were introduced on the first-line therapy setting. The most recent guidelines for ER^+ advanced breast cancer recommend, as standard first-line option, the combination of endocrine therapy with CDK4/6 inhibitors, such as abemaciclib, palbociclib, and ribociclib [9–12]. However, despite the improvements achieved with CDK4/6 inhibitors, around 10 % of the patients do not respond to treatment (*de novo* resistance) and others may develop acquired resistance, causing disease relapse [5,13–16], highlighting the need for novel therapeutic approaches for ER^+ breast cancer.

Among the diverse constituents present in *Cannabis*, the therapeutic interest behind phytocannabinoids has been growing and their application has been explored in different diseases [17–19]. Regarding cancer, cannabinoids are used for the relief of chemotherapy-associated side effects, but a growing number of studies have already attributed anti-

Abbreviations: AIs, aromatase inhibitors; Ana, Anastrozole; CBC, cannabichromene; CBCA, cannabichromenic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDB, canabidiol-C4; CBDM, canabidiol monomethyl ether; CBDV, cannabidivarin; CBG, cannabigerol; CBGVA, cannabigerovarinic acid; CBN, cannabinol; CDX, Casodex/Bicalutamide; ER^+, estrogen receptor-positive; Exe, Exemestane; $HER2^+$, human epidermal growth factor receptor 2 positive; Let, Letrozole; STS, staurosporine; T, testosterone; THC, Δ^9-tetrahydrocannabinol; TNBC, triple-negative breast cancer.

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proliferative, pro-cell death, anti-invasive, and anti-angiogenic actions to these compounds [20–27]. Δ^9-tetrahydrocannabinol (**THC**) and cannabidiol (**CBD**) are the best-known and the most studied phytocannabinoids, including in breast cancer where they have been essentially investigated on human epidermal growth factor receptor 2 positive ($HER2^+$) and in triple-negative breast tumors (TNBC) [20,28,29]. Regarding ER^+ breast cancer and the mechanisms of action of cannabinoids, several groups have made important contributions, mainly focused on **CBD** and **THC**, when used alone or in combination with standard therapies [30–40]. Our group revealed that **CBD** and **THC** exert promising anti-cancer effects on ER^+ breast cancer cells, by modulating aromatase, ERα, and ERβ [41]. Moreover, we showed that **CBD**, when applied as an adjuvant therapy with AIs, may improve the anti-cancer effects of Exe [42]. Nevertheless, around 140 cannabinoids were already identified in the *Cannabis* plant and the therapeutic potential of most of them is yet to be elucidated [17,19,43,44].

Regarding the minor phytocannabinoids, cannabigerol (**CBG**), which similarly to **CBD** lacks the ability to induce psychotropic effects, has been extensively studied for its therapeutic potential in different diseases, including cancer [19,45]. In fact, several works showed that **CBG** might be beneficial for the treatment of glioblastoma [46,47], pancreatic ductal adenocarcinoma, when combined with gemcitabine and paclitaxel [48], colon adenocarcinoma, alone [49] or in combination with curcumin and piperin [50], cholangiocarcinoma [51], as well as breast cancer, both ER^+ and TNBC subtypes [30,52,53]. Cannabinol (**CBN**), originated from **THC**, is another minor phytocannabinoid that has been shown to reduce viability and impair migration of TNBC cells [53]. However, only few studies have been conducted to clarify its potential anti-tumor effects. Another minor cannabinoid with promising potential against breast cancer is cannabidiolic acid (**CBDA**), which already demonstrated ability to inhibit TNBC cells migration [54–56]. Cannabichromenic acid (**CBCA**), in turn, showed anti-proliferative effects in colorectal cancer [57]. Other minor cannabinoids, such as cannabichromene (**CBC**) and cannabidivarin (**CBDV**), have interesting anticonvulsant and anti-inflammatory properties, however, studies addressing their beneficial roles in cancer are still scarce [19]. Nevertheless, first evidence of their effects in some malignancies, including prostate cancer, colorectal cancer, and urothelial cell carcinoma, have already been revealed [58–60].

Recently, through a comprehensive *in silico* and *in vitro* study [61], our group showed that some minor phytocannabinoids namely, **CBG**, **CBDV**, **CBN**, **CBDA**, cannabidiol-C4 (**CBDB**), cannabidiol monomethyl ether (**CBDM**), **CBCA**, cannabigerovarinic acid (**CBGVA**), and **CBC**, modulate aromatase, ER and/or androgen receptor (AR), all important targets for the management of ER^+ breast cancer. Taking this into account, in this study, we aim to evaluate the cytotoxicity of these minor phytocannabinoids, their biological effects on 2D and 3D ER^+ breast cancer cell models, and their mechanisms of action and, thus contribute to expand knowledge about the anti-tumor potential of this class of compounds in this type of cancer.

2. Materials and methods

2.1. Cell culture

The possible cytotoxic effects of the nine minor phytocannabinoids, cannabigerol (**CBG**), cannabidivarin (**CBDV**), cannabinol (**CBN**), cannabidiol-C4 (**CBDB**), cannabidiolic acid (**CBDA**), cannabidiol monomethyl ether (**CBDM**), cannabichromenic acid (**CBCA**), cannabigerovarinic acid (**CBGVA**) and cannabichromene (**CBC**), were evaluated in two non-tumor cell lines: human foreskin fibroblast (HFF-1) and MCF-10A cells. The HFF-1 cell line (ATCC, Manassas, VA, USA) was originally obtained from a male's foreskin, while MCF-10A cells, (ATCC, Manassas, VA, USA) are normal human breast epithelial cells [62]. The effects of **CBG**, **CBDV**, **CBN** and **CBC** were also evaluated in two breast cancer cell lines: MCF-7aro and SK-BR-3 cells. MCF-7aro cell line, kindly provided by Dr. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, USA), is an ER^+ breast cancer cell line that overexpresses aromatase and a well-accepted *in vitro* cell model to study ER^+ breast cancer [63]. On the other hand, SK-BR-3 cells (ATCC, Manassas, VA, USA) are human ER^- breast cancer cells and, consequently, a good *in vitro* cell model to clarify the effects of those minor phytocannabinoids on ER.

All the four cell lines were maintained at 37 °C and 5 % CO_2 atmosphere. HFF-1 cells were cultured in a glucose-enriched DMEM medium without phenol-red (Gibco Invitrogen Co., Paisley, Scotland, UK) supplemented with 1 mM sodium pyruvate (Gibco Invitrogen Co., Paisley, Scotland, UK), 1 % penicillin-streptomycin-amphotericin B solution (PAN-Biotech, Aidenbach, Germany), 2 mM L-glutamine (PAN-Biotech, Aidenbach, Germany), and 10 % of heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Co., Paisley, Scotland, UK). MCF-10A cells were maintained in a DMEM F/12 culture medium without phenol red (Gibco Invitrogen Co., Paisley, Scotland, UK) and supplemented with HuMEC supplement (Gibco Invitrogen Co., Paisley, Scotland, UK), 5 % heat-inactivated horse serum (Gibco Invitrogen Co., Paisley, Scotland, UK), 1 % penicillin-streptomycin-amphotericin B solution, and 2 mmol/L L-glutamine. MCF-7aro and SK-BR-3 cells were kept in an Eagles' minimum essential medium (MEM) with phenol-red (Gibco Invitrogen Co., Paisley, Scotland, UK) supplemented with 10 % FBS, 1 mmol/L sodium pyruvate and 1 % penicillin-streptomycin-amphotericin B solution. Additionally, 100 μg/mL of geneticin (G418; Gibco Invitrogen Co., Paisley, Scotland, UK) were also added to MCF-7aro cells medium. Moreover, three days before the beginning of experiments, MCF-7aro cells were cultured in an estrogen-free MEM without phenol-red (Gibco Invitrogen Co., Paisley, Scotland, UK) supplemented with 5 % pre-treated charcoal heat-inactivated fetal bovine serum (CFBS), 1 mmol/L sodium pyruvate, 1 % penicillin-streptomycin-amphotericin B solution and 2 mmol/L L-glutamine (PAN-Biotech, Aidenbach, Germany) in order to surpass FBS hormones interference, as well as the estrogenic properties of phenol-red [64]. All the experiments on MCF-7aro cells were also carried out in the presence of 1 nM of testosterone (T; Sigma-Aldrich Co., Saint Louis, USA), used as aromatase substrate and proliferation induction agent, or 1 nM of 17β-estradiol (E_2; Sigma-Aldrich Co., Saint Louis, USA) the product of aromatization reaction [65,66].

The stock solutions of the nine minor cannabinoids, **CBG** and **CBDV** (Phytolab GmbH & Co KG, Vestenbergsgreuth, Germany), **CBN**, **CBDB**, **CBDA**, **CBC**, and **CBDM** (Cayman Chemical, Michigan, USA), **CBCA** and **CBGVA** (Biosynth Ltd., United Kingdom), as well as Exe, ICI 182780 (Fulvestrant; ICI), and Casodex (Bicalutamide; CDX; Sigma-Aldrich Co., Saint Louis, MI, USA) were prepared in 100 % DMSO (Sigma-Aldrich Co., Saint Louis, MI, USA) and stored at −20 °C. The stock solution of T and E_2 were prepared in absolute ethanol and stored at −20 °C. Before each experiment, the compounds were diluted in fresh culture medium with a final concentration of DMSO and ethanol no higher than 0.05 %. Additionally, all the controls used for each experiment contained the vehicles in these conditions.

2.2. Cell viability

The effects of the minor cannabinoids on HFF-1, MCF-10A, MCF-7aro, and SK-BR-3 cell viability were addressed using 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium (MTT) and the lactate dehydrogenase (LDH) release methods. For that, cells were cultured in 96-well plates for 3 days at 2×10^4 cell/mL, and for 6 days at 1×10^4 cell/mL for MCF-10A, MCF-7aro, and SK-BR-3 cells or at 7.5×10^3 cells/mL for HFF-1 cells. Cells were treated with the different cannabinoids at different concentrations (1, 5, and 10 μM) and MCF-7aro cells were additionally stimulated with 1 nM of T or E_2, as previously reported [42,65]. MCF-7aro cells only treated with 1 nM of T or E_2 were considered as control cells. For HFF-1, MCF-10A, and SK-BR-3 cell lines, cells treated only with culture medium were considered as control.

Control cells represent the maximum of cell viability (100 %).

After treatment with the cannabinoids, MTT (0.5 mg/mL; Sigma-Aldrich Co., Saint Louis, MO, USA) was added and the plates were then incubated at 37 °C and 5 % CO_2 for 2 h 30 min. Cell viability was quantified spectrophotometrically in a Biotek Sinergy HTX Multi-Mode Microplate Reader (Biotek Instruments, Winowski, VT, USA), while the LDH release assay was performed with 10 % of the cell culture medium and the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. All the experiments were performed in triplicate in at least three independent experiments. The results are expressed as the relative percentage of the control cells (100 %), for MTT assay, and for the LDH assay the control was set as 1.

2.3. Cell cycle analysis

In order to evaluate the anti-proliferative effects of **CBG**, **CBDV**, **CBN**, and **CBC** in MCF-7aro cells, DNA content was assessed through flow cytometry, as previously described [67]. Briefly, MCF-7aro cells were cultured in 6 well-plates at 7×10^5 cells/mL for 3 days in a medium containing 1 nM of T. Cells only treated with T were considered as control. After the 3 days of treatment, cells were fixed in 70 % cold ethanol and stained with a DNA staining solution containing 5 μg/mL Propidium Iodide (PI), 0.1 % Triton X-100, and 200 μg/mL DNase-free RNase A (Sigma-Aldrich Co., Saint Louis, MI, USA). The DNA content was assessed using the BD Accuri™ C6 cytometer through the acquisition of 40 000 events and the results were analyzed using a BD Accuri™ C6 software® (San Jose, CA, USA). The anti-proliferative effects of the four phytocannabinoids are presented as percentage of cells in the G_0/G_1, S and G_2/M phases, in relation to the T-treated control cells. All the assays were performed in triplicate, in at least three independent experiments.

2.4. Apoptotic cell death analysis

Apoptotic cell death was evaluated through the analysis of caspase-7 activity, as previously reported [68,69]. Briefly, MCF-7aro cells cultured on a 96-well white plate at 2×10^4 cells/mL were incubated with the cannabinoids (10 μM) in the presence of 1 nM of T with or without CDX (1 μM) for 24 h (**CBG**, **CBDV**) or 8 h (**CBN**, **CBC**). After that, the experiment was performed using a luminescent assay kit with Caspase-Glo® 3/7, according to manufacturer's instructions (Promega Corporation, Madison, WI, USA). Cells treated with staurosporine at 10 μM (STS, Sigma-Aldrich Co., Saint Louis, MO, USA) for 3 h before the incubation end time were used as a positive control.

Luminescence was measured using a Biotek Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, Winowski, VT, USA) and the results are expressed relative to untreated control cells with data presented as relative luminescence units (RLU). All the assays were performed in triplicate in at least three independent experiments.

2.5. Western blot analysis

Western blot analysis was performed in MCF-7aro cells. Cells were cultured in 6-well plates (1×10^6 cells/mL or 7.5×10^5 cells/mL) and exposed to **CBG**, **CBDV**, **CBN**, and **CBC** (10 μM) in the presence of 1 nM of T, for either 8, 24 or 72 h. Cells treated only with T (1 nM) were considered as negative control, while cells treated with Exe (10 μM), ICI (100 nM), CDX (1 μM), or STS (10 μM) were considered as positive controls. After treatment, cells were collected as previously described by our group [65]. 50 μg/protein per sample were subjected to electrophoresis in 10 % SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was accomplished by using rabbit anti-PARP antibody (1:200; Cell Signaling Technology Inc., Boston, MA, USA), or mouse antibodies against aromatase (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERα (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and AR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). As secondary antibodies, goat anti-mouse (1:2000) and goat anti-rabbit (1:2000) antibodies (Thermo Fisher, Waltham, MA, USA) were used. A mouse monoclonal anti-β-actin antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to control loading variations. The membranes were further exposed to a chemiluminescent substrate WesternBright™ ECL (Advansta Inc., Menlo Park, CA, USA) and the immunoreactive bands were visualized with a ChemiDoc™ Touch Imaging System (BioRad Laboratories, Melville, NY, USA). At least three independent experiments were performed for each protein. The protein expression obtained for treated cells was standardized in relation to protein expression of control cells.

2.6. RNA extraction and qPCR analysis

To perform RNA extraction and consequent qPCR analysis, MCF-7aro cells were seeded in 6-well plates at 1×10^6 cells/mL or 7.5×10^5 cells/mL and treated with **CBG**, **CBDV**, **CBN**, and **CBC** (10 μM) in the presence of 1 nM of T, for 8 h or 3 days. Cells treated only with T (1 nM) were considered as negative control, while cells treated with Exe (10 μM) or ICI (100 nM) were considered as positive controls. After treatment, cells were lysed followed by RNA extraction, as previously reported [42]. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). GRiSP Xpert cDNA Synthesis Mastermix (GRiSP Research Solutions, Porto, Portugal) was employed to obtain cDNA, which was further amplified using GRiSP Xpert Fast SYBR (GRiSP Research Solutions, Porto, Portugal), in the MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA), as previously described [70]. The sequences of the primers, as well as the respective annealing temperatures are listed in Table 1. β-Actin was the housekeeping gene used and the fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [71]. At least three independent experiments were performed for each gene. The mRNA transcript levels of treated cells were normalized in relation to the mRNA transcript levels of control.

2.7. Spheroids culture and imaging

ER^+ breast cancer spheroids were constructed using MCF-7aro cells. Cells were plated at a cellular density of 2×10^4 cells/mL in 96 U-bottom non-adherent well plates and 24 h later were exposed to the compounds under study, **CBG**, **CBDV**, **CBN**, and **CBC** at 10 μM, for 14 days, in the presence of 1 nM of T. Every three days, half of the total well volume (100 μL) was replaced with fresh medium and compounds. Spheroids only exposed to 1 nM of T were considered as control. Generation of spheroids was monitored until day 14. Moreover, the progress of the

Table 1
Primer sequences and annealing temperatures for housekeeping and target genes.

Symbol	Primers	Annealing Temperature
AR	Forward: 5'-TGTCCATCTTGTCGTCTTCG-3' Reverse: 5'-ATGGCTTCCAGGACATTCAG-3′	55 °C
AREG	Forward: 5′-TGTCGCTCTTGATACTCGGC-3′ Reverse: 5′-ATGGTTCACGCTTCCCAGAG-3′	56 °C
CYP19A1	Forward: 5'-GATGATGTAATCGATGGCTAC-3' Reverse: 5'-TTCATCATCACCATGGCGAT-3′	58 °C
EGR3	Forward: 5'-GACTCCCCTTCCAACTGGTG-3' Reverse: 5′- GGATACATGGCCTCCACGTC-3'	56 °C
ESR1	Forward: 5'-CCTGATCATGGAGGGTCAAA-3' Reverse: 5'-TGGGCTTACTGACCAACCTG-3'	55 °C
TFF1	Forward: 5'-GTGGTTTTCCTGGTGTCACG-3' Reverse: 5'-AGGATAGAAGCACCAGGGGA-3'	55 °C
β-Actin	Forward: 5′-TGCCATCCTAAAAGCCACCC-3′ Reverse: 5′-AGACCAAAAGCCTTCATACATCTC-3′	55 °C

spheroids over the 14 days was registered by capturing phase contrast pictures at days 0, 3, 7, 11, and 14. These time frames were selected to show the different phases of spheroids development.

Spheroids were measured using ImageJ software (National Institutes of Health, Maryland, USA). The program automatically determines the area and the perimeter. The values were then converted to diameter values and the absolute values of each experiment were used to describe the growth of the spheroids over the 14 days of treatment with **CBG**, **CBDV**, **CBN**, and **CBC**. The experiments were performed in triplicate and at least three independent experiments were conducted.

2.8. Spheroids viability

The resazurin reduction assay was used to evaluate spheroids viability. After the 14 days of treatment, spheroids were mechanically dissociated so that resazurin from Presto Blue™ cell viability reagent (Gibco Invitrogen Co., Paisley, Scotland, UK) is metabolized by the cells that form the spheroids. An amount of Presto Blue™ corresponding to 10 % of the total well volume was added to each well and the plate was then incubated at 37 °C and 5 % CO_2 for 2 h 30 min. Fluorescence was then measured with an excitation wavelength of 530 nm and emission 590 nm using a Biotek Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments, Winowski, VT, USA) and the results are expressed as relative fluorescence units (RFU). The percentage of spheroids viability after treatment with the cannabinoids was further quantified by normalizing RFU values in relation to T-treated control spheroids (100 % spheroids viability). All the assays were performed in triplicate in at least three independent experiments.

2.9. Statistical analysis

GraphPad Prism 8® software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. Analysis of variance (ANOVA), followed by Bonferroni tests for multiple comparisons (two-way ANOVA and one-way ANOVA, respectively), were applied for the analysis of the different experiments. Values of $p < 0.05$ were considered statistically significant. All the data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Effects of minor cannabinoids on the viability of non-tumor and tumor cells

The effects of the nine phytocannabinoids, **CBG**, **CBDV**, **CBN**, **CBDB**, **CBDA**, **CBDM**, **CBCA**, **CBGVA**, and **CBC** (1, 5 and 10 μM), in two human non-tumor cell lines, HFF-1 and MCF-10A (Fig. 1), were assessed by the MTT assay after 6 days of treatment. The results showed that **CBCA**, at 5 and 10 μM, reduced both HFF-1 ($p < 0.01$; $p < 0.001$) and MCF-10A ($p < 0.05$) cell viability (Fig. 1G), when compared to control cells. For the same concentrations, **CBDB** ($p < 0.001$; Fig. 1D), **CBDM** ($p < 0.001$; Fig. 1F) and **CBGVA** ($p < 0.01$; $p < 0.001$; Fig. 1H) only reduced HFF-1 cell viability, while **CBDA** ($p < 0.05$; $p < 0.01$; Fig. 1E) only decreased MCF-10A cell viability. On the other hand, **CBG** (Fig. 1A), **CBDV** (Fig. 1B), **CBN** (Fig. 1C), and **CBC** (Fig. 1I) did not reduce the viability of any of these cell lines. Considering these results, **CBDB**, **CBDA**, **CBDM**, **CBCA**, and **CBGVA** were considered cytotoxic and were not included in the subsequent studies on ER^+ breast cancer cells.

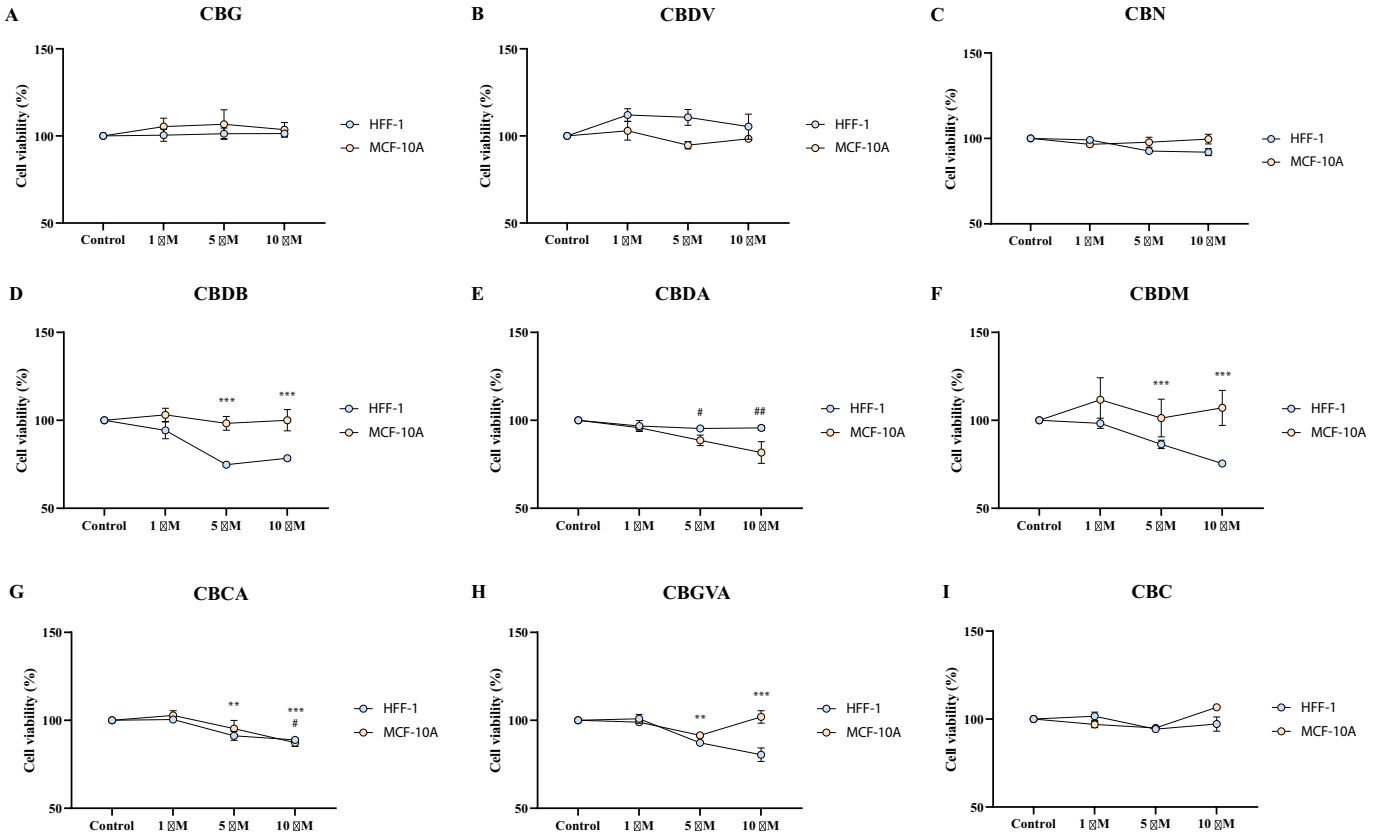

Fig. 1. Effects of the nine minor phytocannabinoids in HFF-1 and MCF-10A cells. Both cell lines were exposed to 1, 5, and 10 μM of **CBG** (**A**), **CBDV** (**B**), **CBN** (**C**), **CBDB** (**D**), **CBDA** (**E**), **CBDM** (**F**), **CBCA** (**G**), **CBGVA** (**H**), and **CBC** (**I**) for 6 days. Cells treated only with medium were considered as control, representing 100 % of cell viability. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. For HFF-1 cells, statistically significant differences between cannabinoid-treated cells and control cells are expressed as ** ($p < 0.01$) and *** ($p < 0.001$), while for MCF-10A cells those differences are represented as # ($p < 0.05$) and ## ($p < 0.01$).

The effects of **CBG**, **CBDV**, **CBN**, and **CBC** (1, 5, and 10 μM) on MCF-7aro cell viability were then evaluated, by the MTT assay, after 3 and 6 days of treatment, and through the measurement of LDH release, after 3 days of treatment. Our results showed that **CBG** (Fig. 2A) and **CBDV** (Fig. 2C) were only able to significantly ($p < 0.01$; $p < 0.001$) reduce MCF-7aro cell viability after 6 days of exposure at 10 μM, whereas **CBC** at 10 μM (Fig. 2G) decreased cell viability at both incubation times. **CBN** (Fig. 2E) significantly ($p < 0.001$) decreased MCF-7aro cell viability in a dose- and time-dependent manner. Additionally, none of the four minor phytocannabinoids induced the release of LDH (Fig. 2), indicating that they do not cause loss of cell membrane integrity.

3.2. *Anti-proliferative and pro-apoptotic effects of minor cannabinoids in ER$^+$ breast cancer cells*

To understand whether the reduction on cell viability induced by **CBG**, **CBDV**, **CBN**, and **CBC** was associated with a dysregulation of cell cycle progression, the DNA content was assessed in MCF-7aro cells exposed to 10 μM of each cannabinoid for 3 days. As shown in Table 2, when compared to control, the four minor phytocannabinoids caused a

Table 2
Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on MCF-7aro cell cycle progression. MCF-7aro cells were stimulate with T (1 nM) and treated with **CBG**, **CBDV**, **CBN**, and **CBC** (10 μM), for 3 days. Cells treated only with T (1 nM) were considered as control. Values are represented as a percentage of single cell events in each stage of the cell cycle and are the mean ± SEM of at least three independent experiments performed in triplicate. Statistically significant differences between cannabinoid-treated cells and control cells are expressed as *** ($p < 0.001$).

	G0/G1	S	G2/M
Testosterone	73.47 ± 0.46	12.45 ± 0.26	13.74 ± 0.41
CBG 10 μM	72.13 ± 1.43	4.93 ± 0.42 ***	21.51 ± 1.49 ***
CBDV 10 μM	74.09 ± 0.75	5.98 ± 0.26 ***	18.80 ± 0.75 ***
CBN 10 μM	71.73 ± 1.24	5.23 ± 0.28 ***	25.76 ± 0.76 ***
CBC 10 μM	71.51 ± 0.74	5.24 ± 0.16 ***	23.03 ± 0.71 ***

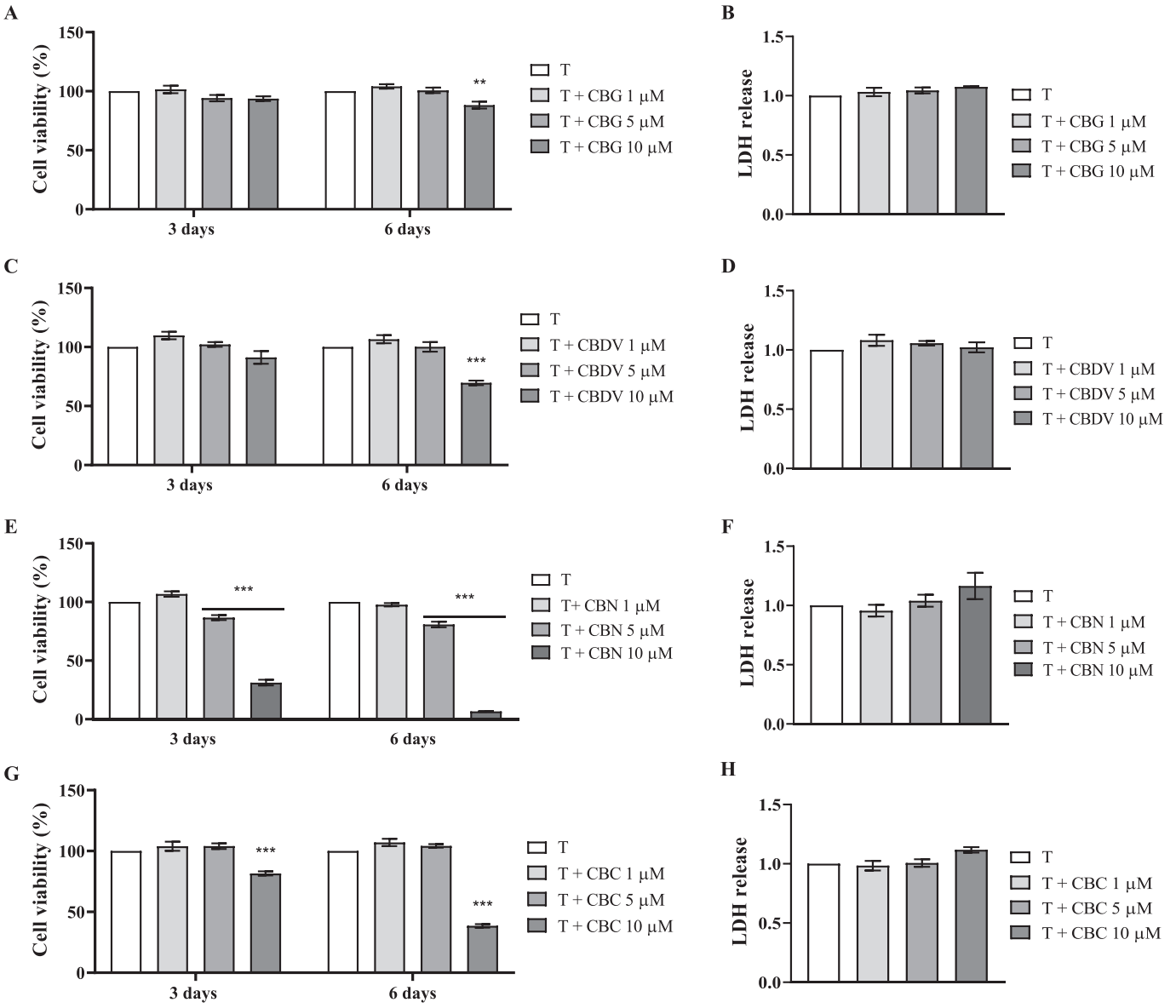

Fig. 2. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on MCF-7aro cell viability. Cells were stimulated with T (1 nM) and exposed to 1, 5, and 10 μM of **CBG** (**A, B**), **CBDV** (**C, D**), **CBN** (**E, F**), and **CBC** (**G, H**) for 3 and 6 days. Cells treated only with T were considered as control, representing 100 % of cell viability. LDH assay was also performed in the same conditions after 3 days of treatment (**B, D, F, H**). The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between cannabinoid-treated cells and control cells are expressed as ** ($p < 0.01$) and *** ($p < 0.001$).

significant ($p < 0.001$) cell cycle arrest in G_2/M phase alongside with a significant reduction ($p < 0.001$) of S population. Moreover, the involvement of apoptotic cell death was also addressed by measuring the activity of caspase-7, an effector caspase, and the expression levels of cleaved PARP (c-PARP), another hallmark of apoptosis. For that, MCF-7aro cells stimulated with T (1 nM) were treated with **CBG** and **CBDV**, for 24 h, or **CBN** and **CBC**, for 8 h, at 10 μM. It is important to note that the activity of caspase-3 was not evaluated since MCF-7aro cells do not express caspase-3 [72]. As presented in Fig. 3, all the cannabinoids significantly ($p < 0.05$, $p < 0.001$) increased the activity of caspase-7 (20–30 % in relation to control), as well as the protein levels of c-PARP, when compared to control. As expected, the positive control STS increased caspase-7 activity ($p < 0.001$) and the expression levels of c-PARP.

3.3. Involvement of aromatase in the effects induced by the minor cannabinoids in ER^+ breast cancer cells

Despite the promising results of our *in silico* studies, the further *in vitro* assessment of anti-aromatase activity, using human placental microsomes, revealed that the minor cannabinoids, **CBG**, **CBDV**, **CBN**, and **CBC** are considered weak aromatase inhibitors [61]. Nevertheless, having in mind that MCF-7aro is an aromatase-overexpressing cell line [63] and that the referred compounds significantly decreased MCF-7aro cell viability (Fig. 2), the potential involvement of aromatase in the aforementioned effects was explored by assessing the protein levels of aromatase and the mRNA transcript levels of the aromatase gene (*CYP19A1*) after 8 h of exposure to the four cannabinoids, as well as by evaluating, through MTT assay, the effects on MCF-7aro cell viability in cells stimulated with T or E_2. Except for **CBG**, a significant decrease in aromatase protein levels was observed after treatment with **CBDV** ($p < 0.001$), **CBN** ($p < 0.05$), or **CBC** ($p < 0.01$; Fig. 4A), when compared to control. Additionally, and similarly to Exe (10 μM) that, as expected, also significantly decreased aromatase protein levels ($p < 0.001$; Fig. 4A) without affecting gene transcription, none of the four cannabinoids altered the transcript levels of *CYP19A1*gene (Fig. 4B). To better understand the involvement of aromatase in the cytotoxic effects observed in MCF-7aro cells, the effects of the four phytocannabinoids in cell viability in the presence of E_2 were also explored (Fig. 4C-F). Results show that the cytotoxic effects induced by CBG (Fig. 4C), CBDV (Fig. 4D), and CBN (Fig. 4E) are aromatase-independent, while CBC causes a higher reduction in MCF-7aro cell viability in the presence of E_2 (Fig. 4F).

3.4. Involvement of ERα in the effects induced by the minor cannabinoids in ER^+ breast cancer cells

Our previous work suggested that **CBDV** displays agonistic activities on ER, while **CBN** and **CBC** are ER antagonists with inverse agonistic properties and **CBG** has no effect in this receptor [61]. Considering that ER^+ breast cancer is the focus of this study, as well as the biological and pharmacological relevance of this receptor in this breast cancer subtype, we performed additional assays in an ER^- breast cancer cell line (SK-BR-3) to better clarify the mechanism of action of these compounds and the involvement of ER on those mechanisms. The results were compared to the ones in MCF-7aro cells stimulated with T (1 nM). Apart from the absence of effects on SK-BR-3 cell viability verified for all the cannabinoids, statistically significant differences between MCF-7aro and SK-BR-3 treated cells were observed for **CBN** ($p < 0.05$; $p < 0.001$; Fig. 5C) and **CBC** ($p < 0.01$; $p < 0.001$; Fig. 5D) at 5 and 10 μM. Curiously, and contrary to **CBN**, **CBC**, at 5 μM, induced a higher reduction on SK-BR-3 cell viability than in MCF-7aro (Fig. 5D). Regarding **CBG** (Fig. 5A) and **CBDV** (Fig. 5B), no statistically significant differences between MCF-7aro and SK-BR-3 cells were identified. These results suggest that ERα might be involved in the reduction of MCF-7aro cell viability.

Additionally, the effects of cannabinoids on ERα protein and *ESR1* mRNA transcript levels were also investigated in MCF-7aro cells. Notably, **CBG**, **CBN**, and **CBC** significantly ($p < 0.001$; Fig. 5E) decreased ERα protein levels, while **CBDV** did not induce any alteration (Fig. 5E). However, only **CBN** caused a significant decrease ($p < 0.001$; Fig. 5F) on *ESR1* transcript levels, whereas **CBDV** induced a significant increase ($p < 0.001$; Fig. 5F). Furthermore, their effects on the transcript levels of three ERα-regulated genes, *AREG* (Fig. 5G), *EGR3* (Fig. 5H), and *TFF1* (Fig. 5I), were also explored. **CBN** and **CBC** significantly ($p < 0.001$) reduced the transcription of all three ERα-regulated genes, which suggests that they impair ERα signaling. On the other hand, **CBG** and **CBDV** significantly ($p < 0.001$) increased *EGR3* transcription, without affecting *AREG* and *TFF1* transcript levels. As expected, ICI (100 nM),

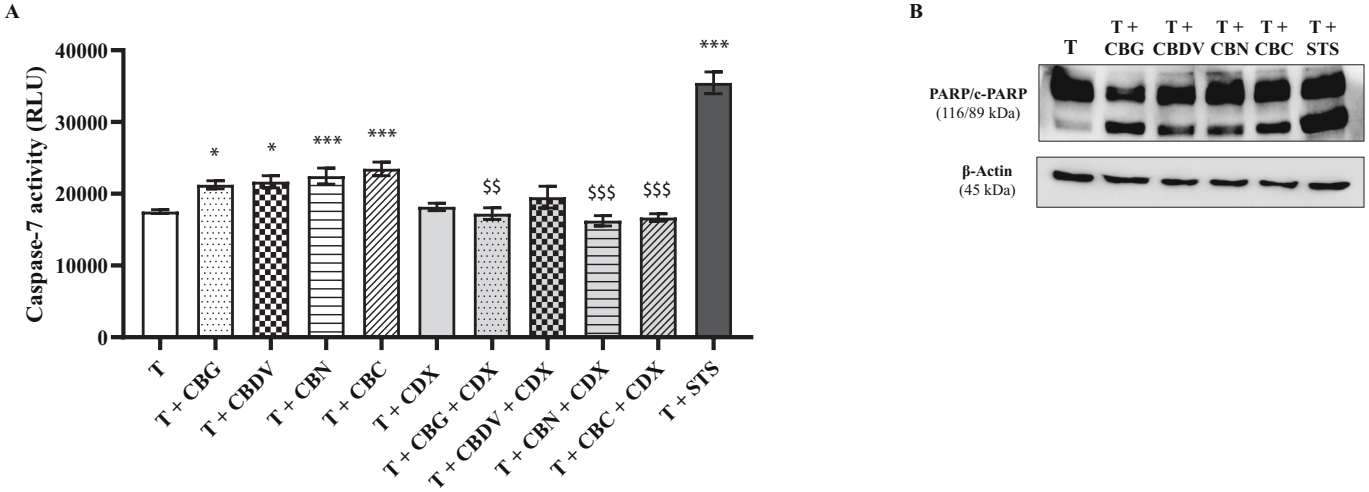

Fig. 3. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on apoptotic cell death. The impact of the four cannabinoids on apoptosis promotion was address through the analysis of caspase-7 activity (**A**) and c-PARP expression (**B**). In both cases, MCF-7aro cells were stimulated with T (1 nM) and treated with 10 μM of **CBG** or **CBDV** for 24 h, or 10 μM of **CBN** or **CBC** for 8 h. **CBG**, **CBDV**, **CBN**, and **CBC** in combination with CDX (1 μM) on caspase-7 activity were also investigated to evaluate the involvement of AR in the promotion of apoptosis. (**B**) A representative Western blot of PARP/c-PARP and β-actin, used as loading control, is presented. Cells treated only with T were used as control, while STS-treated cells (10 μM) were considered as positive control. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between cannabinoid-treated cells and control cells are expressed as * ($p < 0.05$) and *** ($p < 0.001$), while the differences between cannabinoid-treated cells and cells treated with the cannabinoids in combination with CDX are expressed as $$ ($p < 0.01$) and $$$ ($p < 0.001$).

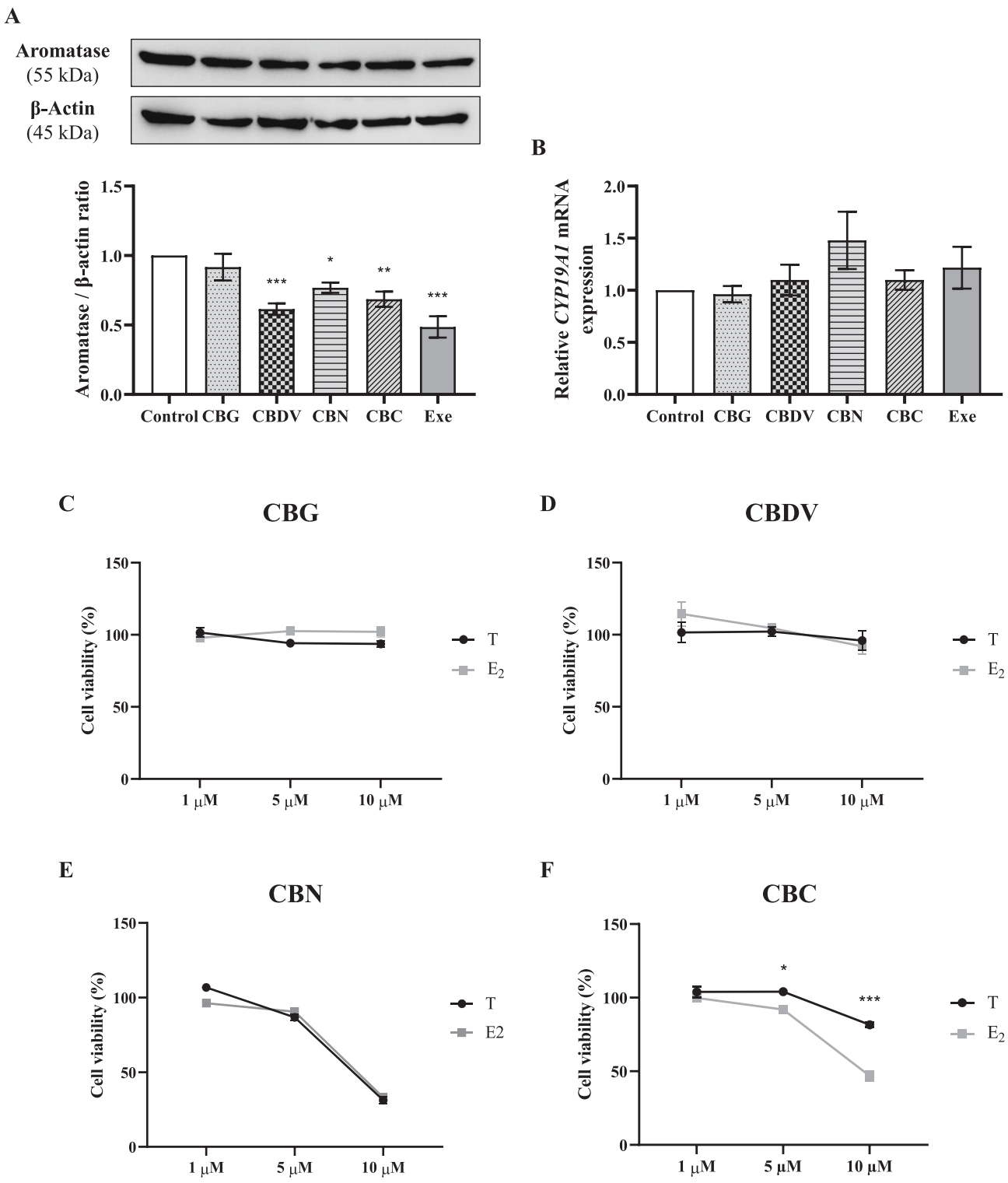

Fig. 4. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on aromatase protein and transcript levels, and on MCF-7aro cell viability in the presence of testosterone (T) or 17β-estradiol (E_2). MCF-7aro cells were exposed to **CBG**, **CBDV**, **CBN**, and **CBC** (10 μM) for 8 h, to explore their effects on aromatase protein levels and transcript levels, and for 3 days in the presence E_2 or T to evaluate the effects in the presence of E_2. (**A**) A representative Western blot of aromatase and β-actin, as well as the densitometric analysis of aromatase expression levels after normalization with β-actin levels, used as loading control, are presented. (**B**) mRNA transcript levels for *CYP19A1* gene in relation to the housekeeping gene *β-actin*. Cells treated only with medium were considered as control, while cells treated with Exe (10 μM) were used as positive control. (**C**—**F**) Cells were stimulated with T (1 nM) or E_2 (1 nM) and exposed to 1, 5, and 10 μM of **CBG** (**C**), **CBDV** (**D**), **CBN** (**E**), and **CBC** (**F**) for 3 days. Cells treated only with T or E_2 were considered as control, representing 100 % of cell viability. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between cannabinoid-treated cells and control cells, as well as between cells stimulated with T and E_2, are expressed as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

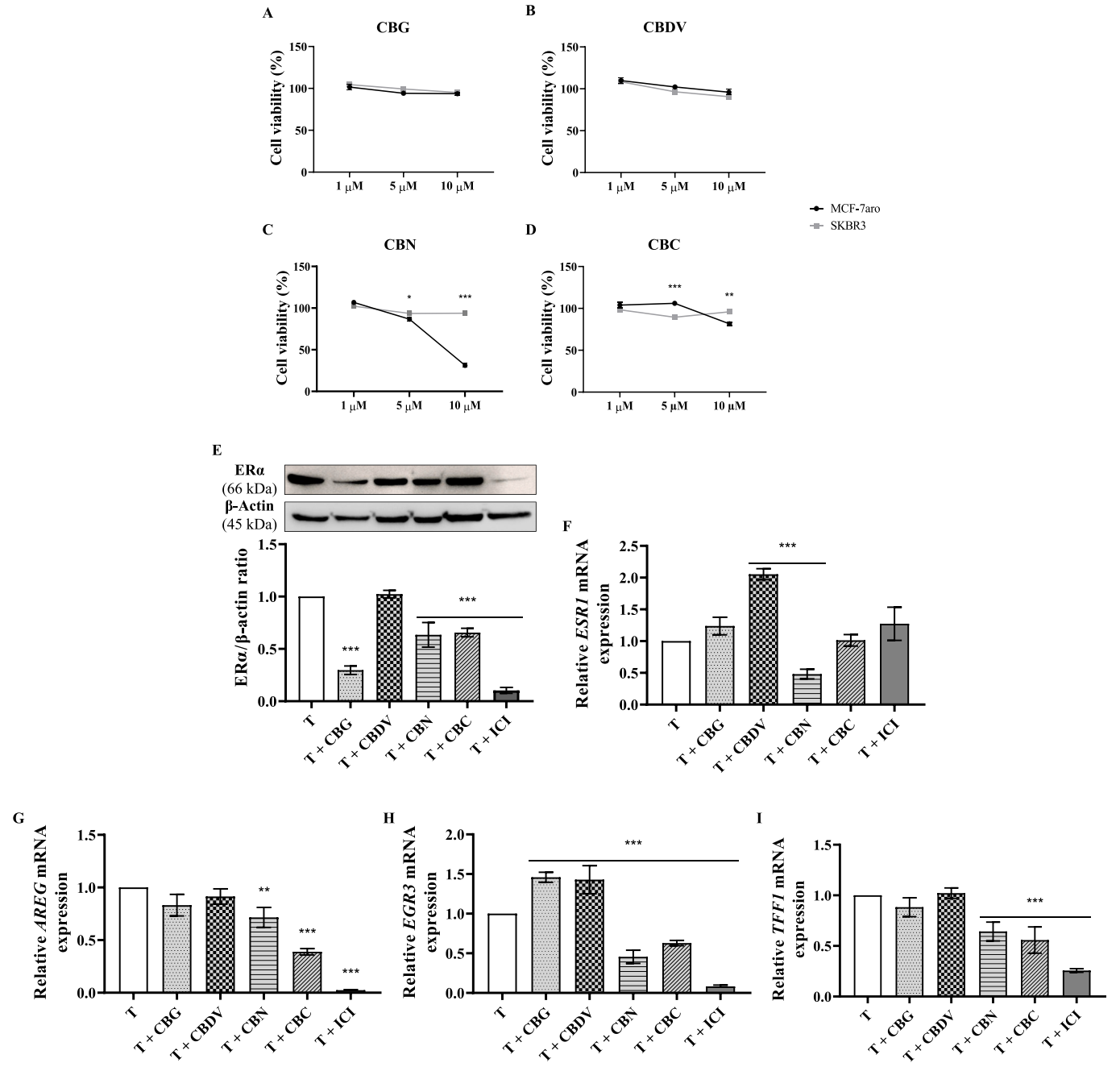

Fig. 5. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on ERα. SK-BR-3 cells were treated with **CBG** (**A**), **CBDV** (**B**), **CBN** (**C**), and **CBC** (**D**) (1, 5, and 10 μM) for 3 days and the results compared with the ones obtained in MCF-7aro cells. MC-7aro cells were stimulated with T (1 nM) and exposed to 10 μM of **CBG**, **CBDV**, **CBN**, or **CBC** during 3 days for the determination of protein (**E**) and transcript levels (**F**—**I**). (**E**) A representative Western blot of ERα and β-actin, as well as the densitometric analysis of ERα expression after normalization with β-actin, used as loading control, are presented. (**F**—**I**) mRNA transcript levels for *ESR1* (**F**), *AREG* (**G**), *EGR3* (**H**), and *TFF1* (**I**) genes in relation to the housekeeping gene *β-actin*. Cells treated only with medium or with T were used as control. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between SK-BR-3 and MCF-7aro cells and the differences between cannabinoid-treated cells and T-treated control cells are expressed as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

used as positive control, significantly reduced ERα protein levels ($p < 0.001$; Fig. 5E) and the transcription of ERα-targeted genes ($p < 0.001$; Fig. 5**G-I**).

3.5. Involvement of AR in the effects induced by the minor cannabinoids in ER$^+$ breast cancer cells

Recently, we also demonstrated, *in silico* and *in vitro*, that **CBG**, **CBDV**, **CBN**, and **CBC** act as AR antagonists with inverse agonistic properties [61]. To better understand the potential involvement of this receptor on the mechanism of action of cannabinoids in ER$^+$ breast cancer cells, the effects of cannabinoids on AR protein and transcript levels were evaluated in MCF-7aro cells. Results presented in Fig. 6 show that **CBG** induced a significant decrease in AR protein levels ($p < 0.01$; Fig. 6A) and an increase in *AR* mRNA transcript levels ($p < 0.05$; Fig. 6B). The other cannabinoids did not cause any alteration on AR protein and transcript levels (Fig. 6). Although these results suggest no direct effect of cannabinoids on AR, with the exception of **CBG**, taking into account that they may act as AR antagonists with inverse agonistic properties [61] and considering that AR may have different biological

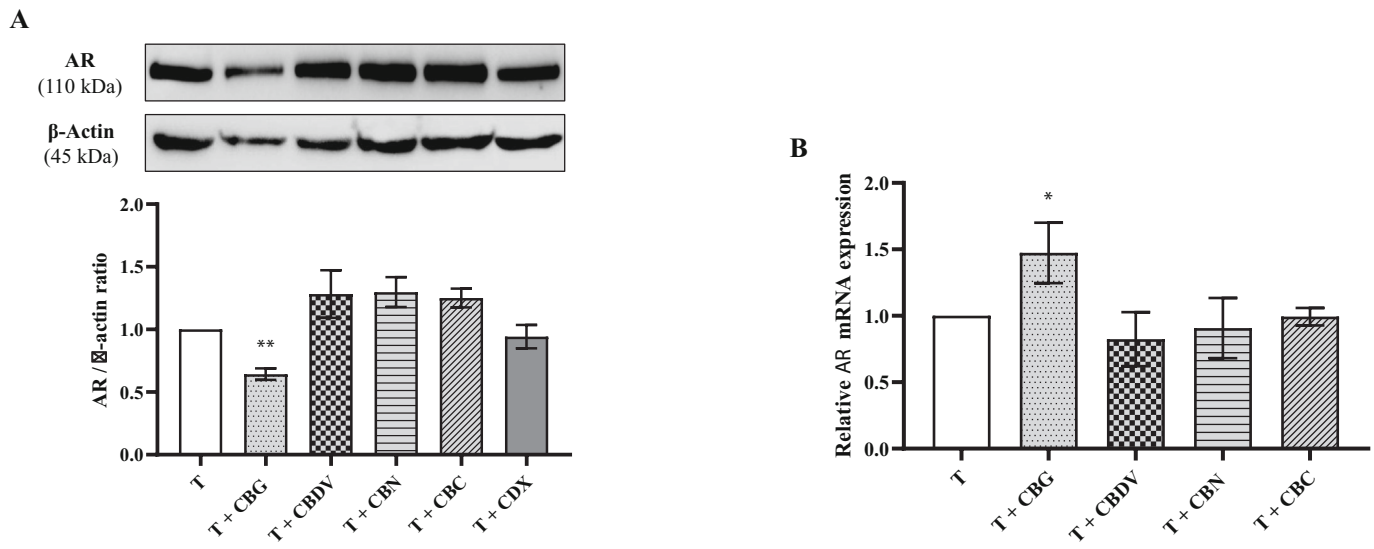

Fig. 6. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on AR. MCF-7aro cells were stimulated with T (1 nM) and exposed to 10 μM of **CBG**, **CBDV**, **CBN**, or **CBC** during 3 days for the determination of protein (**A**) and transcript levels (**B**). (**A**) A representative Western blot of AR and β-actin, as well as the densitometric analysis of AR expression after normalization with β-actin, used as loading control. (**B**) mRNA transcript levels for *AR* gene in relation to the housekeeping gene *β-actin*. Cells treated only with T were used as control, while cells treated with CDX (1 μM) were considered as positive control. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between cannabinoid-treated cells and T-treated control cells are expressed as * ($p < 0.05$) and ** ($p < 0.01$).

actions depending on hormonal status and AIs-treatment [66,70,71,73], the involvement of AR on apoptosis promotion was investigated. For that, it was evaluated the activity of caspase-7 in cannabinoids-treated cells in the presence of CDX, an AR antagonist. Under these conditions, all the cannabinoids, except **CBDV**, lost the ability to increase caspase-7 activity (Fig. 3A), suggesting that the induction of apoptosis by **CBG**, **CBN**, and **CBC** is AR-dependent.

3.6. *Effects of the cannabinoids in a 3D spheroid breast cancer cell model*

To better predict the therapeutic potential of these minor cannabinoids in ER^+ breast tumors, ER^+ breast cancer spheroids using MCF-7aro cells were generated. The spheroids were stimulated with T (1 nM) and treated with **CBG**, **CBDV**, **CBN**, or **CBC** (10 μM) for 14 days and the alterations in morphology and size were documented over time (0, 3, 7, 11 and 14 days). In Fig. 7A, it is possible to see that spheroids morphology did not change significantly over the 14 days of treatment, and that the spheroids resulting from cannabinoids treatment, mainly with **CBG**, **CBDV**, and **CBN**, were smaller than the T-treated control spheroids. This was corroborated by measuring the spheroids diameter. All the cannabinoids induced a significant ($p < 0.01$, $p < 0.001$) decrease in spheroids diameter, when compared to control, after 3, 7, and 11 days (Fig. 7B). **CBN** was the only cannabinoid capable of inducing a significant decrease ($p < 0.01$) in diameter after 14 days when compared to control (Fig. 7B). Furthermore, by using Presto Blue™ to access spheroids viability, it was demonstrated that all the cannabinoids significantly ($p < 0.01$; $p < 0.001$) reduced spheroids viability, in relation to control, after 14 days of treatment (Fig. 7C), with a reduction percentage of 28 %, 31 %, 24 %, and 26 % determined for **CBG**, **CBDV**, **CBN**, and **CBC**, respectively.

4. Discussion

The side effects induced by endocrine therapy, mainly endocrine resistance, are leading to a continuous change in the therapies used for targeting ER^+ breast tumors. Nowadays, the most recent guidelines suggest the combination of AIs or Fulvestrant with CDK4/6 inhibitors as the standard first-line therapeutic approach for advanced ER^+ breast cancer [8,9,74,75]. Nevertheless, this option is also associated with some side effects, such as resistance [13–16,76,77], making mandatory the search for novel therapeutic solutions. As cannabinoids have been showing promising anti-cancer effects in different cancers [21–25], including breast cancer [20,28,29,41,42], in this study, the *in vitro* effects of some minor cannabinoids in 2D and 3D ER^+ breast cancer models were evaluated.

Previous *in silico* and *in vitro* work from our group pointed to nine minor phytocannabinoids, namely **CBG**, **CBDV**, **CBN**, **CBDB**, **CBDA**, **CBDM**, **CBCA**, **CBGVA**, and **CBC**, as modulators of three of the most important therapeutic targets in ER^+ breast cancer: aromatase, ER, and/or AR [61]. In order to clarify their therapeutic potential in luminal A breast cancer cases, their cytotoxic behavior, anti-cancer properties, and mechanisms of action were analyzed. Our results demonstrated that 5 out of the 9 minor phytocannabinoids studied, namely **CBDB**, **CBDA**, **CBDM**, **CBCA**, and **CBGVA**, are cytotoxic in the non-tumor cell lines HFF-1 and MCF-10A, reason why they were not further studied in breast cancer cells. Corroborating a previous work [30], **CBG**, **CBDV**, **CBN**, and **CBC** are not cytotoxic for those non-tumor cells, reason why they were further assessed in MCF-7aro cells. In this ER^+ breast cancer cell line, that mimics luminal A breast cancer, **CBN** and **CBC** were the most potent in inducing a significant reduction in cell viability, at both 3 and 6 days of treatment. In all cases, the reduction in cell viability can be explained by the promotion of apoptotic cell death, demonstrated by an increase in the activity of the effector caspase-7 and on c-PARP protein levels, but also by the cell cycle arrest at G_2/M phase. It should be noted that, an increased number of cells at G_2/M phase has been associated with apoptosis promotion [78]. Furthermore, all the cannabinoids impaired the proliferation of breast cancer cells, since a significant reduction on the S phase was also observed. Therefore, altogether, these results suggest that these cannabinoids cause strong anti-proliferative effects in ER^+ breast cancer cells that lead to the occurrence of apoptosis, by disrupting cell cycle progression at G_2/M phase. Notably, previous studies have demonstrated that **CBG** induces caspase-dependent apoptotic cell death in glioblastoma, cholangiocarcinoma, and colon cancer cell models [46,47,49,51], as well as cell cycle arrest of HuCC-T1 cells, a cholangiocarcinoma cell model [51]. On the other hand, **CBN** has shown potential to induce apoptosis and to promote cell cycle arrest in HCC1806 breast cancer cells [79]. Indeed, it is widely known that apoptosis is one of the main processes by which cannabinoids, including

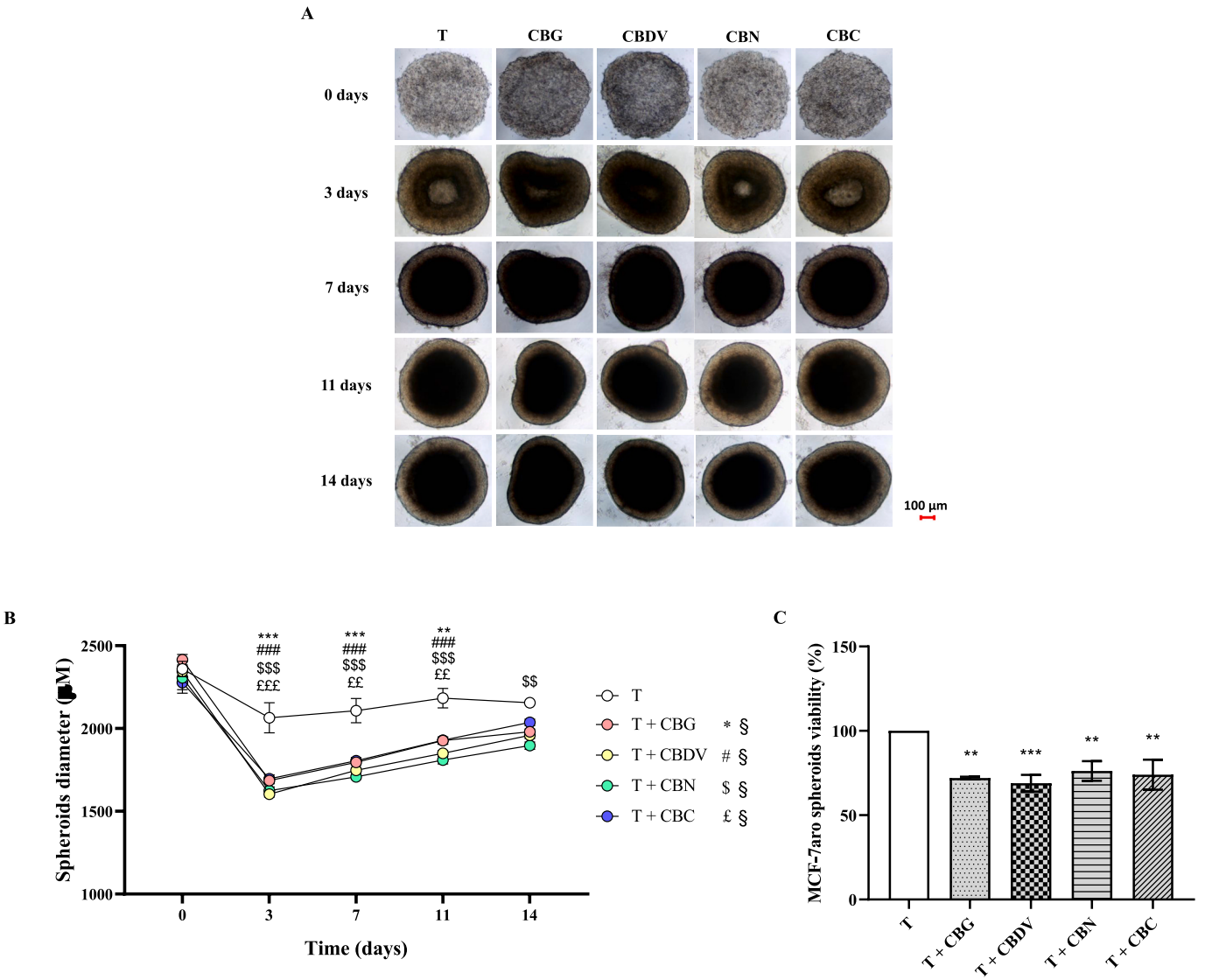

Fig. 7. Effects of **CBG**, **CBDV**, **CBN**, and **CBC** on MCF-7aro spheroids. MCF-7aro cells were used to build ER$^+$ breast cancer spheroids that were then stimulated with T (1 nM) and treated with **CBG**, **CBDV**, **CBN**, and **CBC** (10 µM) for 14 days. (**A**) Spheroids images over the 14 days of treatment. (**B**) Measurement of spheroids diameter throughout treatment. (**C**) Spheroids viability at the end of the 14 days of treatment. Spheroids treated only with T were used as control. The results are presented as mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences between the diameter of the T-treated control spheroids and cannabinoid-treated spheroids over the 14 days are expressed as ** ($p < 0.01$) and *** ($p < 0.001$), for **CBG**, as ### ($p < 0.001$), for **CBDV**, as $$ ($p < 0.01$) and $$$ ($p < 0.001$), for **CBN**, and as ££ ($p < 0.01$) and £££ ($p < 0.001$) for **CBC**. Statistically significant differences between the viability of cannabinoid-treated spheroids and T-treated control spheroids are represented as ** ($p < 0.01$) and *** ($p < 0.001$).

THC and **CBD**, exert their anti-tumor actions [23,80–83]. In fact, our group has already demonstrated that both **CBD** and **THC**, as well as the endocannabinoid anandamide (**AEA**), induce apoptosis and disrupt cell cycle progression of MCF-7aro cells [41,42].

As mentioned before, due to its fundamental role in the last step of estrogens biosynthesis, aromatase is one of the main therapeutic targets in ER$^+$ breast cancer. Recently, our group demonstrated that both **CBD** and **THC**, as well as **AEA**, target aromatase by decreasing its expression and inhibiting its activity [41,84,85]. Despite the weak anti-aromatase activity previously determined for **CBG**, **CBDV**, **CBN**, and **CBC** in human placental microsomes [61], our results in MCF-7aro cells demonstrate that, contrary to **CBG**, that did not affect aromatase levels, the other minor cannabinoids reduced aromatase protein levels without affecting the transcription of the aromatase gene (*CYP19A1*). These results are similar to the ones obtained for Exe and indicate that, besides the lack of ability to inhibit aromatase activity, **CBDV**, **CBN**, and **CBC** may induce the degradation of this enzyme, a mechanism already suggested for Exe and other compounds [41,66,67,86–88], or prevent their transcription. By affecting aromatase levels, these minor cannabinoids may compromise estrogen synthesis and consequently the levels of estrogen in the tumors will decrease, impairing cancer growth and development [5,6]. However, considering the MTT assays, apparently the effects on aromatase do not affect the overall cytotoxicity induced by the phytocannabinoids.

Regarding ER, other key target in ER$^+$ breast cancer, previous studies from our group, in MCF-7aro cells, showed that the cannabinoids **CBD**, **THC**, and **AEA** reduce ERα protein levels [41] and that **CBD** acts as an ERα antagonist with inverse agonistic properties [42]. Recently, we collected evidence that **CBN** and **CBC** also act as ER antagonists with inverse agonistic properties, while **CBDV** exert agonistic activity and **CBG** does not affect the activity of this receptor [61]. In this study, we demonstrate that **CBN** decreases ERα expression and transcription, as well as the transcription of classic E_2-induced genes *AREG*, *EGR3*, and *TFF1*. These results indicate that this cannabinoid down-regulates ERα,

thus impairing ERα signaling, which corroborates its previously determined ER antagonistic activity [61]. Interestingly, **CBC** only reduces ERα protein levels and the transcription of ERα-targeted genes, a behavior similar to the SERD Fulvestrant and to the oral SERD AZD9496 [89], as well as to **CBD** [41,42]. This corroborates its antagonistic activity [61] that might be a result of a degradation or down-regulation of ERα, a behavior similar to Fulvestrant. Regarding **CBDV**, this cannabinoid induces an upregulation of *ESR1* gene, and also of *EGR3*, the *bona fide* target gene of ERα [90], which reinforces its role as an ERα agonist predicted in our previous study [61]. Surprisingly, **CBG** only decreased ERα protein levels without affecting *ESR1*, *AREG*, and *TFF1* transcript levels. However, a significant increase in *EGR3* transcription was spotted. This behavior resembles the one exhibited by Exe, which is linked to its weak estrogenic-like effect [66].

AR is another steroid receptor that has been receiving a lot of attention due to its promising role as a therapeutic target, as it has been shown that this receptor is expressed in 70–90 % of all ER^+ breast carcinomas [70,91,92]. We previously showed that **CBD** acts as an AR antagonist with inverse agonistic properties [42] and, more recently, we demonstrated that **CBG**, **CBDV**, **CBN**, and **CBC** present the same behavior [61]. Our results indicated that only **CBG** was able to significantly reduce AR protein levels along with an increase in the transcription of *AR* gene. This increase in *AR* transcription might be an attempt to restore the levels of this receptor. On the other hand, considering that **CBG** is neither an ER agonist nor antagonist but an AR antagonist [61], we suggest that this cannabinoid, by reducing the levels of AR, may indirectly down-regulate ER levels, leading to an increased *EGR3* transcription, as a compensatory mechanism. In fact, it has been described that in systems where AR is expressed along with ER, these receptors may control each other's function [42,66,70]. In contrast, neither **CBDV**, **CBN** nor **CBC** affect AR expression, a behavior already reported for **CBD** [41,42], as well as for **THC** and **AEA** [41].

As these cannabinoids may act on ER^+ breast cancer cells trough an ER and/or AR-dependent mechanism, it was investigated the involvement of these receptors on the cell death induced by cannabinoids. As MCF-7aro cells present a high dependence on ER signaling for cell proliferation and survival, it is expected that ER inhibition leads to cell death. Therefore, as **CBN** and **CBC** impair ER signaling and **CBG**, **CBN**, and **CBC** down-regulate ERα expression levels, their cytotoxicity in breast cancer cells is ER-dependent. However, the role of AR in the promotion of cell death depends on the therapies applied [66,70,73,92]. Thus, the involvement of AR in the promotion of apoptotic cell death was explored and caspase-7 results after AR blockade demonstrated that, in such conditions, **CBDV** was the only minor cannabinoid retaining its ability to promote apoptosis. This suggests that apoptosis promoted by **CBDV** is not AR-dependent and may be the result of modulation of other signaling pathways involved in cell cycle progression. In contrast, for **CBG**, **CBN**, and **CBC** our results suggest that apoptosis is AR-dependent, as this cell death pathway is impaired after AR blockade. Curiously, the effects observed for **CBG**, **CBN**, and **CBC** are similar to the ones induced by the non-steroidal AIs Ana and Let in these cells [66].

Altogether, considering the effects in the three different targets, the minor phytocannabinoids may have distinct mechanisms of action in ER^+ breast cancer cells. Despite the reduced aromatase expression promoted by **CBDV**, **CBN**, and **CBC**, only **CBDV** appears to act as an ERα agonist, which from a clinical point of view is not beneficial. On the contrary, **CBN** and **CBC** act as ERα antagonists. In fact, **CBN** induces a down-regulation of ERα gene and protein and, consequently, a down-regulation of ERα-targeted genes, whereas **CBC,** contrary to **CBN**, does not affect *ESR1* levels. Thus, we postulate that **CBC** may act as Fulvestrant, since its effects on ERα protein levels and ERα-targeted genes resemble the ones exhibited by this SERD and the oral SERD AZD9496 [89]. Finally, despite the down-regulation of ERα protein levels, **CBG** increases *EGR3* transcription, presenting a weak estrogenic-like effect similar to the AI Exe [66], that might be a result of a compensation mechanism, due to its effect on AR protein levels, a behavior that might not be beneficial. In addition, **CBG**, **CBN**, and **CBC** may act as AR antagonists to promote cell death. Despite the previous AR antagonistic activity determined for **CBDV** [61], this behavior may be tissue-specific and, apparently, our results suggest that its effects on breast cancer cells are AR-independent, a behavior similar to other cannabinoids [41].

Despite the wide use of 2D cell cultures to evaluate the anti-cancer potential of compounds, these systems fail to recapitulate the architecture and the structure of a solid tumor and most of the physiological aspects of *in vivo* tumor microenvironment, such as cell-cell interactions and access to oxygen and nutrients. As an attempt to surpass these issues, recently, 3D cell culture models have been developed and applied in several studies that aim to explore the anti-tumor effects of compounds. By having cells growing into clusters rather than monolayers, these models can better simulate the *in vivo* microenvironment [93–97], reason why they are being applied in drug discovery, replacing sometimes *in vivo* studies. Considering this, to better understand the possible therapeutic application of these four minor cannabinoids in ER^+ breast cancer treatment, as well as to clarify their anti-tumor effects, ER^+ breast cancer spheroids were developed, using MCF-7aro cells, and exposed to cannabinoids. Regardless the absence of significant morphological differences between control and cannabinoids-treated spheroids, all the cannabinoids decreased spheroids diameter and cell viability. The reduction on spheroids diameter was more pronounced for **CBN**, but regarding cell viability, the reduction was similar for all of them. In fact, even **CBG** and **CBDV**, that slightly reduced MCF-7aro cell viability after 6 days of treatment (11.8 % and 30.4 %, respectively), were able to significantly decrease both spheroids diameter and cell viability (27.9 % and 31.1 %, respectively), indicating that they may be also important anti-cancer agents. On the other hand, the results of **CBN** and **CBC** treated spheroids corroborate the MCF-7aro cell viability and cell proliferation results, although the reduction on spheroids cell viability in these cases (23.8 % and 26.0 %, respectively) was not as pronounced as the one verified on MCF-7aro 2D cell culture after 6 days of treatment (93.3 % and 61.5 %, respectively). Albeit that, these 3D cell culture results reinforce the potential clinical benefit of these minor cannabinoids in ER^+ breast cancer, as well as the feasibility of 3D breast cancer models in predicting the anti-tumor activities of compounds.

Overall, taking into account the results both in the 2D and 3D breast cancer models, as well as the effects on the specific ER^+ breast cancer molecular targets, that corroborate previous data from our group [61], among the minor phytocannabinoids studied, **CBN** is the most promising. On the other hand, despite the induction of apoptosis and impaired cell proliferation, **CBDV** presents ERα agonistic activity and **CBG** a weak estrogenic-like effect that may limit their potential as anti-cancer drugs for this subtype of breast cancer. Nonetheless, it should be pointed that other targets and signaling pathways not explored in this work may also contribute to cannabinoids effects.

5. Conclusions

Here, we demonstrated that four minor phytocannabinoids, namely **CBG**, **CBDV**, **CBN**, and **CBC** exert relevant anti-cancer effects in 2D and 3D ER^+ breast cancer models. The mechanism of action by which **CBG** may exert its anti-proliferative and cell death effects involves the downregulation of both ER and AR protein levels, while **CBDV** only reduces aromatase protein levels. Regarding **CBN** and **CBC**, their anti-cancer effects are the result of a simultaneous effect on the three targets, ER, aromatase, and AR. In fact, to impair breast cancer growth, these cannabinoids present an AR-dependent cell death, down-regulate aromatase protein levels, and act as ER negative regulators. Notably, although all the cannabinoids affected the growth of MCF-7aro spheroids, **CBN** caused the most pronounced effect.

Overall, by exploring the mechanisms of action of some less known phytocannabinoids in specific targets, this study expands the knowledge regarding the anti-cancer potential of cannabinoids and boosts the idea that these compounds might be applied not only in ER^+ breast cancer

treatment, but also in other diseases relying on the same signaling pathways. It should be highlighted that to best of our knowledge, this is the first *in vitro* study that unveils the effects, mechanism of action, and targets of these minor cannabinoids in luminal A breast cancer cases.

CRediT authorship contribution statement

Cristina Ferreira Almeida: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Georgina Correia-da-Silva:** Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Ana Paula Ribeiro:** Methodology, Investigation. **Natércia Teixeira:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Cristina Amaral:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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