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The role of soybean extracts and isoflavones in hormone-dependent breast cancer: aromatase activity and biological effects

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Estrogen receptor-positive (ER^+) breast cancer is the most common cause of cancer death in women worldwide. Nowadays, the relationship between soya diet and breast cancer is controversial due to the unknown role of its isoflavones, genistein (**G**) and daidzein (**D**). In this work, we investigated not only the anti-tumor properties of a soybean extract (**NBSE**) but also whether the biotransformation of extract (**BSE**) by the *fungus Aspergillus awamori* increased its effectiveness. The **BSE** showed a stronger anti-aromatase activity and anti-proliferative efficacy in ER^+ aromatase-overexpressing breast cancer cells. **D** and **G** were weak aromatase inhibitors, but inhibited cancer cell growth, being **G** the isoflavone that contributed to the **BSE**-induced effects. This work demonstrated that the biotransformation increased the anti-aromatase activity and the anti-tumoral efficacy of soybean extract in breast cancer cells. Moreover, it elucidated the potential use of soya in the prevention and/or treatment of ER^+ breast cancer.

1. Introduction

The most common subtype of breast cancer is estrogen receptor-positive (ER^+), being approximately 70%–80% of all breast tumors. Targeted therapies include selective estrogen receptor modulators and down-regulators (SERMS and SERDS, respectively) and aromatase inhibitors (AIs). The latter prevent the conversion of androgens to estrogens, and have been considered as a successful therapeutic approach for ER^+ breast cancer treatment in post-menopausal women.[1–3] However, there are some drawbacks concerning their use, such as the development of acquired resistance and the occurrence of bone loss,[1] which emphasizes the importance to find new therapeutic alternatives.

In recent years, the relationship between soya foods and breast cancer has become controversial due to the estrogen-like properties of the isoflavones. However, according to epidemiological studies, in countries with high soya intake there is a low risk and incidence of breast cancer cases. On the other hand, it is known that Asian, in comparison with Western breast cancer patients, exhibit better survival rates.[4] Corroborating these observations, *in vitro* and *in vivo* studies have also suggested a strong relationship between isoflavones and a lower risk of carcinogenesis.[5] These remarks led to the hypothesis that this class of flavonoids may play a role in the prevention of breast cancer development.[6,7]

Although, isoflavones can be present in legumes, fruits or vegetables, soybeans are their main source. These molecules can be glycosylated, acetylglycosylated, malonylglycosylated or aglycones,[6,8] being the two main aglycones the genistein and daidzein. In soya, the isoflavones are present in their β-D-glycoside form, genistin and daidzin, which are further biotransformed by intestinal bacteria glucosidases into the biologically active aglycone forms of isoflavones.[6,9] The composition and total amount of these compounds depend on soybean treatment,[8,10,11] which influences their bioavailability and biological activities. Furthermore, the final concentration of isoflavones may differ from individual to individual due to their specific metabolism.[8,10,12,13]

Isoflavones are a group of compounds that have structural similarity to the endogenous steroid hormone, estradiol. The presence of the phenolic ring allows them to bind to ER and induce a similar response, being also called phytoestrogens. It has been described that isoflavones, depending on the concentration and cell type, can act as ER-agonists or ER-antagonists[6]

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and for this reason, they are classified as selective estrogen receptor modulators (SERMs).[14] Indeed, genistein and daidzein are described as natural SERMs[6] that bind to ERα or ERβ, but with an approximately 20-fold higher affinity to the latter.[12] This may explain their protective role against breast cancer, since the binding to this receptor may lead to breast cancer cell growth inhibition. In fact, in breast cancer cell lines, it was shown that genistein induced up-regulation of ERβ,[12,15] though it also exhibits a weak estrogenic action when compared with estradiol.[8,15,16] On the other hand, the isoflavones also inhibit several important enzymes involved in estrogen biosynthesis, namely the cytochrome P450 enzyme aromatase,[17–20] 17β-HSD[20,21] and 3β-HSD.[22] It has already been demonstrated that genistein and daidzein inhibit aromatase, the enzyme responsible for the last step of estrogen biosynthesis, in human placental microsomes,[20] in human preadipocyte cells[18] or in primary cultures of human granulosa-luteal cells and also induce a decrease in aromatase expression.[23] Nevertheless, the efficacy of these isoflavones in inhibiting aromatase is controversial, since other studies reported that daidzein or genistein did not affect aromatase activity.[20,24–27]

Although a variety of health benefits have been attributed to soya consumption, there is also a growing concern over their estrogenic properties that may increase the risk of recurrence or growth stimulation of pre-existing tumours. Some studies showed that, *in vitro*, genistein and daidzein stimulate the growth of breast cancer cells[28–31] and of breast tumors in xenograft studies.[14,31,32] Moreover, it has been suggested that soy-based supplements might affect the efficacy of breast cancer treatment with aromatase inhibitors.[29] Nonetheless, recent studies indicate that soya intake has no detrimental effects on the risk of breast cancer recurrence and in some cases significantly reduced the risk.[33] In addition, several studies showed that genistein and daidzein could promote cell cycle arrest and inhibit growth of different cancer cell lines,[34] including breast cancer cells[12,16,35–44] and inhibit angiogenesis.[45] So, as the role of isoflavones in breast cancer prevention remains unclear, the impact of soya intake on breast cancer patients and on women at high risk of this disease needs to be clarified. In this work, we explored the anti-aromatase activity of soybean extracts of *Glycine max* with or without biotransformation by the fungus *Aspergillus awamori*, a β-glucosidase producer,[46] using human placental microsomes and an ER^+ aromatase overexpressing human breast cancer cell line (MCF-7aro). In addition, the biological effects of the soybean extracts and of the isoflavones, genistein and daidzein isolated or in combination were explored. It has already been described that the fungus *Aspergillus awamori* acts as a β-glucosidase producer in order to enhance the conversion of glucosidase isoflavones to their bioactive aglycone forms (genistein and daidzein).[46] Moreover, recent studies have demonstrated that the soybean extract biotransformed by the fungus *Aspergillus awamori* has antioxidant,[46] anti-inflammatory[47] and anti-tumoral properties,[48,49] being, therefore, an attractive extract to be explored.

2. Materials and methods

2.1. Soybean extracts and isoflavones

The soybean extract *Glycine max* biotransformed by the fungus *Aspergillus awamori* (**BSE**) and the soybean extract *Glycine max* without biotransformation (**NBSE**) were produced and kindly donated by Dr M. J. V. Fonseca (Faculty of Pharmaceutical Sciences of Ribeirão Preto, Laboratory of Photochemoprotection and Quality Control, University of São Paulo, Brazil).[46] The characterization of the content of the soybean extracts and the quantification of the isoflavones, daidzein and genistein, in the extracts were previously described.[46,48,49] Both extracts contain isoflavone glycosides and aglycones, and other soybean polyphenols, though the major contents are isoflavone aglycones, daidzein and genistein.[48] The concentrations used for **BSE** and **NBSE** were determined taking into account the total amount of isoflavones, daidzein (**D**) and genistein (**G**), present in each extract and the molecular weight for each isoflavone. The isoflavones **D** and **G** were used as the standard control and were purchased from Sigma-Aldrich Co. (Saint Louis, USA). The stock solutions of **BSE** and **NBSE** and the standards **D** and **G** were prepared in DMSO and stored at −20 °C. The stock solution of testosterone (T) was prepared in absolute ethanol and also stored at −20 °C. Just prior to the assays, appropriate dilutions were prepared with medium and the final concentrations of DMSO and ethanol in culture medium were less than 0.05% and 0.01%, respectively.

2.2. Biochemistry – aromatase activity

2.2.1. Preparation of human placental microsomes. The human placental microsomes were obtained as described previously.[50–52] Human term placentas obtained from a local hospital were placed in cold 67 mM potassium phosphate buffer (pH 7.4) containing 1% KCl. The cotyledon tissue was separated and homogenized in a Polytron homogenizer with 67 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and 0.5 mM dithiothreitol (DTT, 1 : 1, w/v). The homogenate was centrifuged at 5000*g* for 30 min and the supernatant was centrifuged at 20 000*g* for 30 min and at 54 000*g* for 45 min to yield the microsomal pellet. The microsomes were washed and resuspended in 67 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol, and 0.5 mM DTT and stored at −80 °C. All the procedures were carried out at 4 °C. The protein content was estimated by the Bio-Rad protein assay (Bio-Rad Labs, Munich, Germany) using bovine serum albumin (BSA) as a standard.

2.2.2. Aromatase assay procedure. Aromatase activity was evaluated as previously described by our group,[50–52] by measuring the tritiated H_2O released from [1β-^{3}H] androstenedione (PerkinElmer Life Sciences, Boston, MA, USA), during the aromatization process. The soybean extracts **BSE** and **NBSE** and the isoflavones **G** and **D** were diluted in 67 mM potassium phosphate buffer (pH 7.4). To determine the percentage of aromatase inhibition of each compound we performed a screening assay. For the aromatization reaction

we used 20 μg of microsomal protein, 40 nM of [1β-^{3}H] androstenedione (1 μCi) and **BSE** and **NBSE** (1.5–10 μM of the total amount of isoflavones **D** and **G**), **G** (2 μM), **D** (2 μM) or the combination of **G** + **D** (4 μM, 1 : 1) in a final reaction volume of 1 mL. All the experiments were performed in triplicate in three independent experiments. As a reference AI we used formestane (**F**) (0.5 μM) (Sigma-Aldrich Co., Saint Louis, USA).

2.3. Cell biology – cell culture

In this study, we used an ER$^+$ aromatase-overexpressing human breast cancer cell line, MCF-7aro, stably transfected with the human placental aromatase gene and Geneticin selection from the parental MCF-7 cells.[53,54] As these cells express higher levels of aromatase, they are considered a good model to study potential AIs.[55] MCF-7aro cells were maintained with Eagle's minimum essential medium (MEM) supplemented with Earle's salts and 1 mmol L^{-1} sodium pyruvate, 1% penicillin–streptomycin–amphotericin B, 100 μg ml^{-1} G418 and 10% of heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Co., Paisley, Scotland, UK), regularly grown at 37 °C under a 5% CO_2 atmosphere and the medium was changed every three days.

For the biological studies and in order to avoid the interference of steroids present in FBS and of the estrogenic effects of phenol-red,[56] three days before the experiments, cells were cultured in an estrogen-free MEM medium without phenol-red containing 5% pre-treated charcoal heat-inactivated fetal bovine serum (CFBS), 1 mmol L^{-1} sodium pyruvate, 2 mmol L^{-1} glutamine and 1% penicillin–streptomycin–amphotericin B. The assays were performed under these conditions, with 1 nM of testosterone (T) (Sigma-Aldrich Co., Saint Louis, USA), which was used as an aromatase substrate and a proliferation inducing agent. As control, MCF-7aro cells were incubated with 1 nM of testosterone (T) plus 0.05% of DMSO. MCF-7aro cells were kindly provided by Prof. Shiuan Chen from the Beckman Research Institute, City of Hope, Duarte, CA, USA

2.3.1. In cell aromatase assay. The anti-aromatase activity of each steroidal compound in MCF-7aro cells was determined as previously described by our group.[52,57] Briefly, confluent MCF-7aro cells plated in a 24-well plate were cultured in a serum-free medium containing the soybean extracts **BSE** and **NBSE** (1.5–10 μM of the total amount of isoflavones **D** and **G**) with 50 nM of [1β-^{3}H] androstenedione, as a substrate, and 500 nM of progesterone (that was used to suppress the 5α-reductase activity, which also uses the androgen as a substrate) and incubated at 37 °C for 1 h. The aromatase reaction was finished by addition of 100 μl of 20% TCA. All the experiments were carried out in triplicate in three independent experiments. Exemestane and formestane at 1 μM were used as reference AIs.

2.3.2. Morphological studies. MCF-7aro cells were cultured in 24-well plates, in a cellular density of 2 × 10^5 cells per ml, and incubated with 1 nM of T and different concentrations of **BSE** (0.74–2.3 μM of the total amount of isoflavones **D** and **G**) for 2 days. The morphological alterations were analysed by phase contrast microscopy, Giemsa (Merck, Whitehouse Station, USA) and Hoechst staining (Sigma-Aldrich Co., Saint Louis, USA). The presence of acid vesicular organelles (AVOs) was evaluated by acridine orange (AO) staining (Sigma-Aldrich Co., Saint Louis, USA). Giemsa stained cells were observed under the microscope Eclipse E400, Nikon equipped with image analysis software LeicaQwin. For Hoechst staining, fixed cells were exposed to 0.5 μg ml^{-1} Hoechst 33258 and mounted with a fluoroshield mounting medium (Sigma-Aldrich Co., Saint Louis, USA). The nuclear morphology was examined under a fluorescence microscope (Nikon Eclipse Ci), equipped with an excitation filter with maximum transmission at 360/400 nm, and processed by using Nikon NIS Elements v 4.0 image software.

For AO staining, cells were stained with AO at 0.1 μg ml^{-1} for 15 min under a 5% CO_2 atmosphere at 37 °C and then mounted in an aqueous mounting medium. The presence of AVOs was indicated by the yellow/orange/red fluorescence, analysed using the fluorescence microscope (Nikon Eclipse Ci) equipped with a 490 nm band-pass blue excitation filter and a 515 nm long pass-barrier filter and processed by using Nikon NIS Elements v 4.0 image software.

2.3.3. Cell viability and cell proliferation. MCF-7aro cells were cultured in 96-well plates at a cellular density of 2.5 × 10^4 cells per ml in an estrogen-free MEM medium. After 24 h of cell adhesion, the cells were incubated with 1 nM of T and different concentrations of **BSE** or **NBSE** (0.11–2.3 μM of the total amount of isoflavones **D** and **G**), **G** (1–25 μM), **D** (1–25 μM) and with the combination of **G** + **D** (0.11–50 μM, 1 : 1) for 1, 2 and 3 days of incubation.

The effects on cell viability were assessed by using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay. After the incubation period, MTT (0.5 mg ml^{-1}) (Sigma-Aldrich Co., Saint Louis, USA) was added and cells were incubated for 2 h and 30 min at 37 °C in 5% CO_2. The formazan was quantified spectrophotometrically (540 nm) by addition of a DMSO : isopropanol mixture (3 : 1).

The effects on DNA synthesis were evaluated by thymidine incorporation assay. At each exposure time [^{3}H]–thymidine (0.5 μCi) (Amersham International, Amersham, UK) was added 8 h before the end of the treatment. After a cycle of freezing/defrosting, cells were harvested using a semi-automated cell harvester (Skatron Instruments, Norway), a scintillation cocktail was added and [^{3}H]–thymidine incorporation was determined using a scintillation counter (LS 6500, Beckman Instruments, CA, USA).

The results are expressed as the relative percentage of the untreated control cells (cells treated only with T) (100%).

2.3.4. Cell cycle analysis. To explore the anti-proliferative effects, we analysed the cell cycle by flow cytometry. MCF-7aro cells (7 × 10^5 cells per ml) were incubated with 1 nM of T and **BSE** (1.5–2.3 μM of the total amount of isoflavones **D** and **G**), **G** (10 μM) and **D** (10 μM) for 2 days. Adherent cells were harvested, mixed with non-adherent cells, washed with PBS, fixed with 70% cold ethanol and resuspended in 0.5 ml of DNA staining solution (5 μg ml^{-1} propidium iodide (PI), 0.1% Triton X-100 and 200 μg ml^{-1} DNase-free RNase A (Sigma-Aldrich Co., Saint Louis, USA) in PBS) for 30 min at room temperature. The DNA content was analysed by flow cytometry

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based on the acquisition of 20 000 events on a Becton Dickinson FACS Calibur (San Jose, CA, U.S.A) equipped with CELLQuest Pro software. Detectors for the three fluorescence channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris, cell doublets and aggregates were gated out using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. Data were analysed using FlowJo Software (Tree Star, Inc.). The anti-proliferative effects were indicated by the percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle.

2.4. Statistical analysis

All the assays were performed in triplicate in three independent experiments. The data presented are expressed as the mean ± SEM. Statistical analysis of data was performed using GraphPad Prims 6, through analysis of variance (ANOVA) followed by Tukey and Bonferroni *post-hoc* tests for multiple comparisons. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Anti-aromatase activity in human placental microsomes and in breast cancer cells

The anti-aromatase activity of **BSE** and **NBSE** in human placental microsomes and in MCF-7aro cells was evaluated by performing a radiometric assay in which tritiated water released from [1β-^{3}H] androstenedione into the incubation medium, was used as an index of estrogen formation.[58] Since these extracts are mainly composed of isoflavones, genistein (**G**) and daidzein (**D**), we also assessed their anti-aromatase activity in human placental microsomes.

Firstly, we determined the percentage of aromatase inhibition for different concentrations of the **BSE** and **NBSE** (1.5–10 μM) and also for **G** (2 μM), **D** (2 μM) and for the combination of **G** with **D** (**G** + **D**) (2 μM each) (Fig. 1, Table 1). As presented in Table 1 and Fig. 1A, in human placental microsomes and for the same concentrations, the **NBSE** presented an aromatase inhibition higher than **BSE**. In relation to the isoflavones **G** and **D**, and the combination of **G** + **D**, we obtained an aromatase inhibition lower than 30% (Fig. 1B, Table 1). These results indicate that, in placental microsomes, **NBSE** presents higher anti-aromatase activity than **BSE** and that the isoflavones **G**, **D** or **G** + **D** did not efficiently inhibit aromatase. Moreover, using the same concentrations (4 μM), it was observed that both extracts presented an anti-aromatase activity higher than the combination of isoflavones **G** + **D** (Fig. 1B). In addition, the IC_{50} value was also determined, being 2 μM for **NBSE** and 4.5 μM for **BSE** (Fig. 1A), which confirms that **NBSE** inhibits more efficiently the enzyme aromatase.

We also evaluated the anti-aromatase activity of the extracts in MCF-7aro cells. In this case and for the same

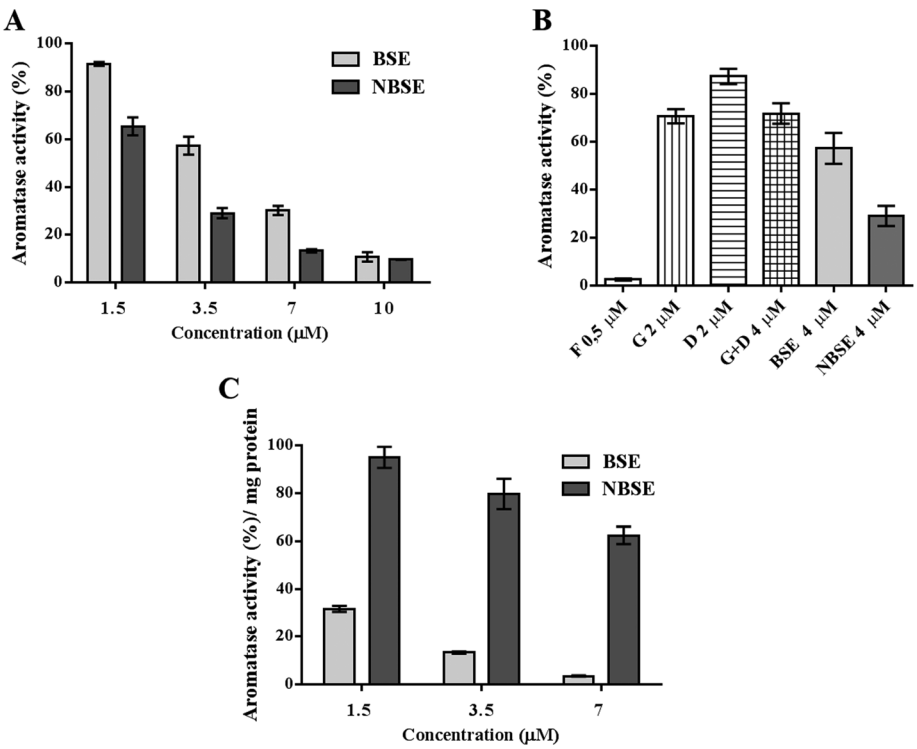

Fig. 1 Aromatase activity of the extracts **BSE** and **NBSE**, as well as isoflavones **G** and **D**, alone or in combination in human placental microsomes and in MCF-7aro cells. (A) Graphical representation of the percentage of aromatase activity with **BSE** and **NBSE** in human placental microsomes. (B) Comparison of the aromatase activity with **BSE**, **NBSE**, **G**, **D** and **G** + **D** in placental microsomes. (C) Graphical representation of the percentage of aromatase activity with **BSE** and **NBSE** in MCF-7aro cells. All the data are presented as a percentage of the tritiated water in relation to the control and correspond to three independent experiments carried out in triplicate. Formestane (F) was used as a reference AI.

Table 1 Aromatase inhibition (%) of the extracts **BSE** and **NBSE** in human placental microsomes and in MCF-7aro cells

	Aromatase inhibition	
	Human placental microsomes[a] (%) ± SEM	MCF-7aro cells[b] (%) ± SEM
BSE (1.5 μM)	8.57 ± 0.80	68.31 ± 1.24
BSE (3.5 μM)	42.73 ± 3.73	86.63 ± 0.46
BSE (7 μM)	69.87 ± 1.93	96.33 ± 0.25
BSE (10 μM)	89.17 ± 1.96	—
NBSE (1.5 μM)	41.27 ± 2.94	4.91 ± 4.43
NBSE (3.5 μM)	71.03 ± 2.11	23.88 ± 5.10
NBSE (7 μM)	86.62 ± 0.65	37.52 ± 3.64
NBSE (10 μM)	90.25 ± 0.12	—
F (0.5 μM)	98.82 ± 1.06	95.90 ± 0.61

[a] Concentrations of 40 nM [1β-^{3}H] androstenedione, 20 μg of protein from human placental microsomes and different concentrations of the compounds were used for 15 min of incubation. [b] Concentrations of 50 nM [1β-^{3}H] androstenedione, confluent MCF-7aro cells and different concentrations of the compounds were used for 1 h of incubation. The results represent the mean ± SEM of three different experiments performed in triplicate.

concentrations, the **BSE** induced an aromatase inhibition higher than **NBSE** (Fig. 1C, Table 1). Thus, contrary to what was observed in placental microsomes, the biotransformed extract presented higher inhibitory properties in cells. As the **BSE** was the extract more effective in inhibiting aromatase in breast cancer cells, it was further selected for the biological studies.

2.2. Morphological studies

To explore the morphological alterations induced by **BSE** and by isolated or combined isoflavones, MCF-7aro cells were treated for 2 days with compounds and observed by phase contrast microscopy, Giemsa and Hoechst staining. As presented in Fig. 2, we observed a reduction in cell density for **BSE**-treated cells, which was more evident for the highest concentration, and the presence of some morphological alterations typical of an apoptotic process, such as membrane blebbing and chromatin condensation. Moreover, we also observed some vacuolization of the cytoplasm. The appearance of vacuoles was further evaluated by acridine orange (AO) staining. As presented in Fig. 2, cells treated with the extract induced a shift from the green fluorescence to the yellow/orange fluorescence, which suggests the presence of acid vesicular organelles (AVOs).

2.3. Effects on MCF-7aro cell viability

Although only **BSE** strongly inhibited aromatase activity in MCF-7aro cells, we also explored the effects of the **NBSE**

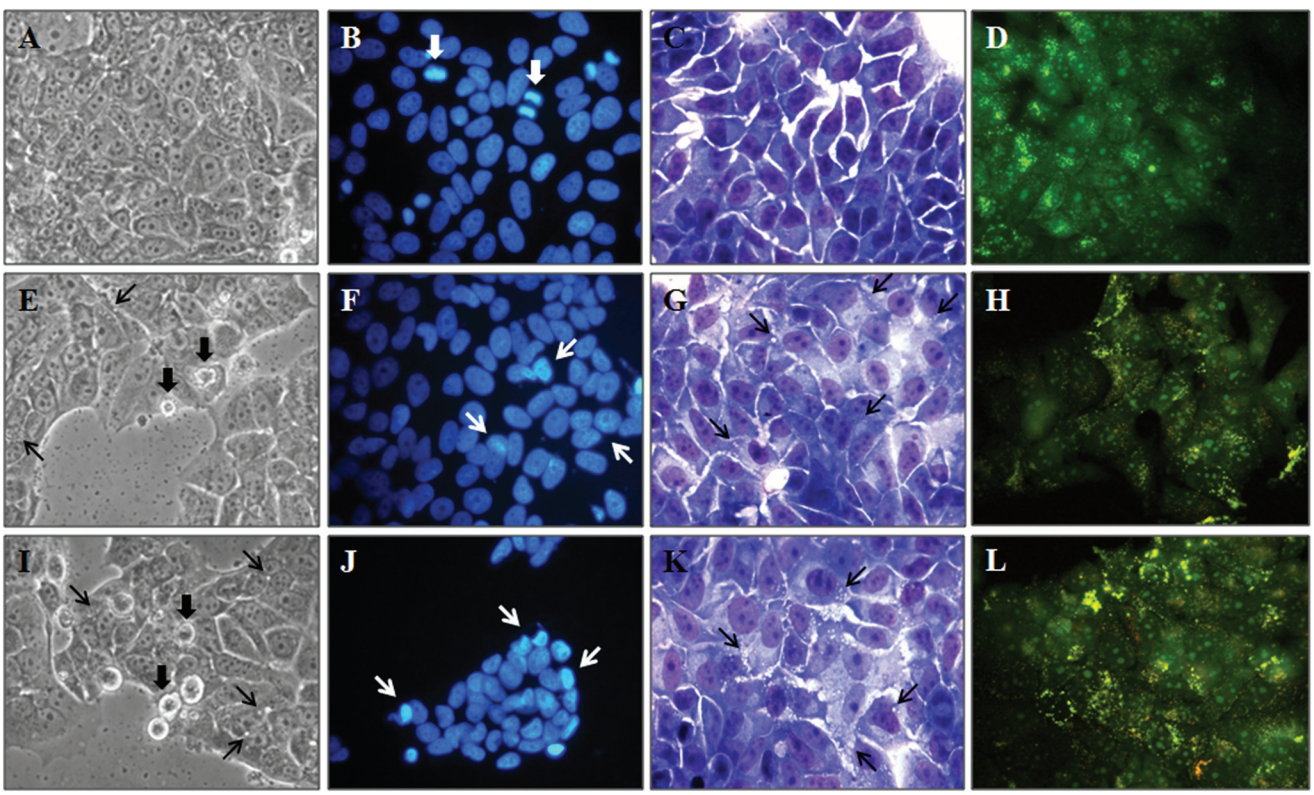

Fig. 2 Effects of **BSE** on the MCF-7aro cell morphology, examined by phase contrast microscopy (A, E and I), Hoechst staining (B, F and J), Giemsa staining (C, G and K) and AO staining (D, H, L). Cells were cultured in the absence (A–D) or in the presence of 0.7 μM (E–H) and 1.5 μM (I–L) of **BSE** after 3 days of treatment. Treated cells presented cytoplasm vacuolization (black arrows), membrane blebbings (black filled arrows) and chromatin condensation (white arrows). The presence of AVOs was indicated by the yellow/orange/red fluorescence in AO staining.

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extract and the isoflavones on MCF-7aro cell viability. Thus, the effects of **BSE** (0.11–2.3 μM), **NBSE** (0.11–2.3 μM), **G** (1–25 μM), **D** (1–25 μM) and the combination of **G** + **D** (2–50 μM) were investigated after 1, 2 and 3 days of treatment, using the MTT assay. Cells treated only with T (1 nM) were considered as the control.

The **BSE** (Fig. 3A), caused a significant reduction ($p < 0.01$; $p < 0.0001$) in MCF-7aro cell viability, in a dose- and time-dependent manner, except for the lowest concentration (0.11 μM) after 1 and 2 days of treatment. The isoflavones **G** and **D** and their combination (**G** + **D**) also induced a decrease in MCF-7aro cell viability in a dose- and time-dependent manner (Fig. 3). This decrease was only significant ($p < 0.05$; $p < 0.001$; $p < 0.0001$) for the higher doses (5–25 μM) and longer periods of treatment. On the other hand, by comparing **BSE** with **G**, **D** or **G** + **D**, it was noted that the **BSE**-induced effects on cell viability were more pronounced than the ones caused by the isoflavones (Fig. 3). We also compared the effects of **BSE** and **NBSE** on MCF-7aro cell viability at the same concentration and incubation time (2 days). Although **NBSE** caused a significant ($p < 0.05$; $p < 0.0001$) reduction in cell viability, the **BSE** effect was more accentuated (Fig. 3E). In fact, significant differences ($p < 0.0001$) were observed when comparing **BSE** with **NBSE** effects.

2.4. Effects on MCF-7aro cell proliferation

To understand if **BSE** also caused alterations in MCF-7aro cell proliferation, we also evaluated, by thymidine incorporation assay, its effects on the rate of DNA synthesis after 2 days of treatment (0.11–2.3 μM). These effects were compared with cells treated with **G** (0.5–25 μM), **D** (1–25 μM) or the mixture **G** + **D** (0.11–50 μM) for the same incubation period. Cells with T (1 nM) represent the maximum of cell proliferation and were considered as the control. Our results demonstrate that **BSE** induced a significant ($p < 0.0001$) decrease in the rate of DNA synthesis in a dose- and time-dependent manner (Fig. 4). The isoflavones **G** and **D** and their mixture (**G** + **D**) also caused a significant ($p < 0.05$; $p < 0.01$; $p < 0.001$; $p < 0.0001$) reduction in MCF-7aro cell proliferation, though **D** was less effective (Fig. 4). **BSE** was more potent in decreasing MCF-7aro cell proliferation even at the same concentrations as those of the mixture **G** + **D** (Fig. 4). These results confirm that **BSE** presented potent anti-proliferative properties in breast cancer cells.

As **BSE** caused a reduction in the rate of DNA synthesis, we also analysed, after 2 days of treatment (1.5 and 2.3 μM), its effects on MCF-7aro cell cycle progression, using flow cytometry. In order to compare with **BSE**, we also evaluated the effects of isolated (**G** or **D**) or combined isoflavones (**G** + **D**) on the

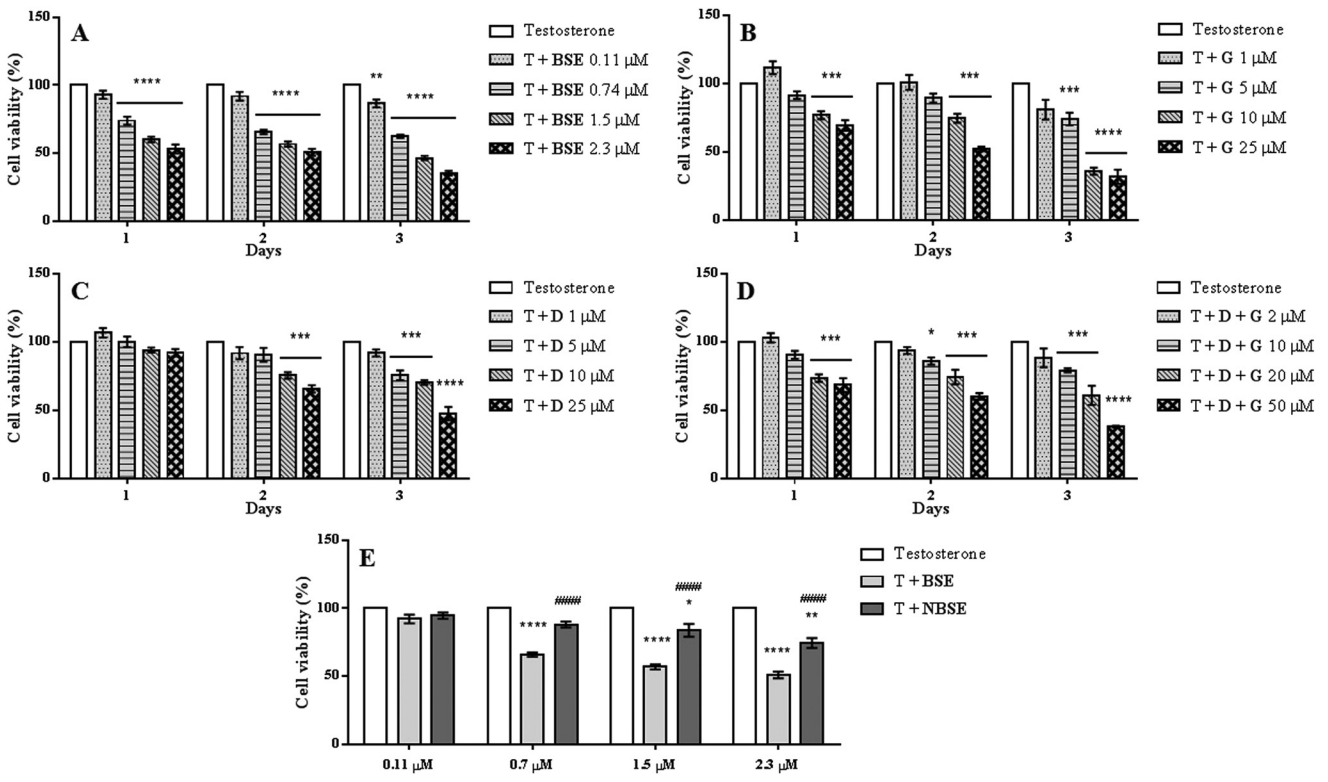

Fig. 3 Effects of **BSE** (A), **G** (B), **D** (C) and **D** + **G** (D) at different concentrations (0.11–50 μM) on MCF-7aro cell viability, after 1, 2 and 3 days of treatment. (E) Comparison of the effects on MCF-7aro cell viability between **BSE** and **NBSE**, after 2 days of treatment. Cells cultured with testosterone (T) represent the maximum of cell viability (100%) and were considered as the control. The results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells treated with compounds are denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$; between **BSE**-treated cells with **NBSE**-treated cells indicated by $^{\#\#\#\#}p < 0.0001$.

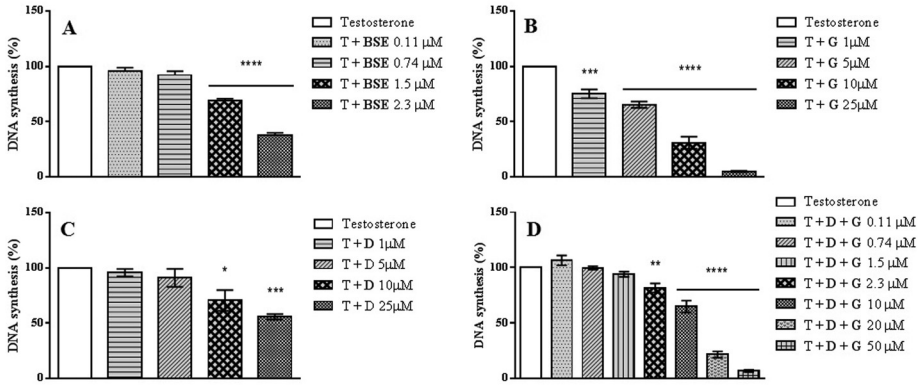

Fig. 4 Effects of **BSE** (A), **G** (B), **D** (C) and **D** + **G** (D) on MCF-7aro cell proliferation, by measuring the rate of DNA synthesis. Cells were treated with different concentrations of compounds (0.11–50 μM) for 2 days. Cells cultured with T represent the maximum of cell proliferation (100%) and were considered as the control. The results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells treated with compounds are denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

progression of the cell cycle. The results demonstrate that **BSE**, only at 2.3 μM, induced a significant ($p < 0.001$) cell cycle arrest in the G_2/M phase (Table 2 and Fig. 5) with a significant reduction of cells in the G_0/G_1 ($p < 0.001$) and S ($p < 0.05$) phases. Curiously, and contrarily to **BSE** the isoflavone **G** (10 μM) was able to cause a significant ($p < 0.01$) cell cycle arrest in the G_0/G_1 phase (Table 2 and Fig. 5). The isoflavone **D** and the mixture of **G** + **D** did not cause any alteration in the progression of the cell cycle (data not shown). Compared to **BSE**, the isolated or combined isoflavones have different effects on cell cycle progression and, consequently, in MCF-7aro cell proliferation.

Table 2 Effects of **BSE** and **G** on the cell cycle distribution of MCF-7aro cells, after 2 days of treatment

	Cell cycle phases		
	G_0/G_1	S	G_2/M
Testosterone	74.43 ± 0.25	7.04 ± 0.37	15.62 ± 0.46
T + **BSE** 1.5 μM	73.83 ± 0.89	6.2 ± 0.29	16.36 ± 0.61
T + **BSE** 2.3 μM	66.75 ± 0.67***	5.26 ± 0.10*	23.83 ± 0.18***
T + **G** 10 μM	79.07 ± 0.56***	3.72 ± 0.41***	14.31 ± 0.12

Cells were treated with different concentrations of **BSE** (1.5 and 2.3 μM) as well as **G** (10 μM) for 2 days. Treated cells were harvested, fixed and their DNA content was evaluated by PI labelling, followed by flow cytometry analysis. Data are presented as single cell events in the G_0/G_1, S and G_2/M phases of the cell cycle. The data represent means ± SEM of triplicates and are representative of three independent experiments. Significant differences between the control *versus* treated cells are indicated by *$p < 0.05$ and ***$p < 0.001$.

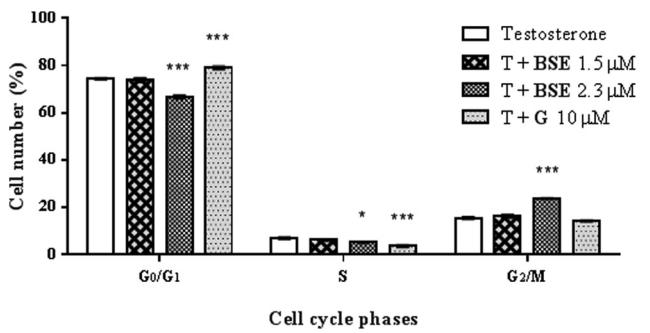

Fig. 5 Effects of **BSE** (1.5 and 2.3 μM) and **G** (10 μM) on MCF-7aro cell cycle progression, after 2 days of treatment. Cells cultured with T were considered as the control. The results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells treated with compounds are denoted by *$p < 0.05$ and ***$p < 0.001$.

3. Discussion

Epidemiological studies, as well as *in vitro* and *in vivo* studies, have evidenced that high soya intake is associated with a reduced breast cancer risk and lower rates of breast cancer cases.[4,5] Over the last few years, the relationship between soya diet and breast cancer has become controversial, since the main soya isoflavones, genistein and daidzein present estrogen-like properties, and can induce proliferation[14,28–32] or cause cell death[12,35–40] of breast cancer cells. Therefore, the role and impact of soya intake in breast cancer remains unclear. Previous studies have demonstrated that a soybean extract *Glycine max* biotransformed by the fungus *Aspergillus awamori* (**BSE**) presents antioxidant,[46] anti-inflammatory[47] and anti-tumoral properties.[48,49] Taking these into account, in this study, we investigated the anti-aromatase activity and the biological effects of the soybean extract **BSE** in MCF-7aro cells, a breast cancer cell line that overexpress aromatase and is considered a good model to study AIs.[55] Its effects were compared to a soybean extract *Glycine max* without biotransformation (**NBSE**) and to the isolated or combined isoflavones, genistein (**G**) and daidzein (**D**), in order to understand if the biotransformation reaction by the fungus increased the anti-tumor properties of soya extract as well as the role of isoflavones in soya effects.

Firstly, we evaluated the ability of soybean extracts and isoflavones, isolated or combined, to inhibit aromatase, a key

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enzyme responsible for estrogen synthesis and, consequently, a therapeutic target for ER^+ breast cancer treatment.[1] **BSE** and **NBSE** inhibited aromatase activity in human placental microsomes, being **NBSE** more potent than **BSE**. This suggests that, in placental microsomes, the biotransformation of soya did not abolish, only attenuated, the anti-aromatase properties of the soybean extract. However, the isoflavones, combined or isolated, did not efficiently inhibit aromatase, being, therefore, considered weak AIs. This observation is in accordance with other studies that demonstrated that **G** and **D** did not inhibit aromatase in placental microsomes[20,24,26] and in other biological samples.[25,27] Kao *et al.* (1998) showed that isoflavones, such as **G** and **D**, were weak inhibitors of aromatase because the 4′-hydroxyphenyl group at C-3 greatly reduced the ability of these compounds to bind aromatase.[59] Later, Le Bail *et al.* (2000) also confirmed that isoflavonoids with the phenolic B-ring in the C-3 position on the pyran ring, as **G** and **D**, are weak AIs, while flavonoids with the B-ring at the C-2 position are more potent AIs.[20] Thus, by comparing the ability of soybean extracts and isoflavones to inhibit aromatase it can be suggested that the anti-aromatase activity of the former may be due to other polyphenolic molecules, as already described.[46,48,49]

To clarify if the extracts are also potent AIs in breast cancer cells, we investigated their anti-aromatase activity in MCF-7aro cells. Contrary to the results obtained in placental microsomes, the **BSE** was more efficient in inhibiting aromatase. One possible explanation is that the cell membrane may interfere with the uptake rate of the **NBSE** components, affecting consequently their ability to inhibit aromatase, which suggests an advantage for the biotransformation of soya by the fungus. Nevertheless, it cannot be excluded the hypothesis of the existence of other molecules in the biotransformed extract that may have the ability to modulate the activity of aromatase. In fact, as the fungus acts as a β-glucosidase producer,[46] the biotransformation process by the fungus in **BSE** not only increased the content of isoflavones **G** and **D**, but also the content in other polyphenolic molecules when compared to **NBSE**.[46,48,49] It was also described that *Aspergillus awamori* can produce not only β-glucosidase with different substrate specificities but also other enzymes able to hydrolyse glycosidic linkages.[46]

Since **BSE** presented high anti-aromatase activity in breast cancer cells, to clarify its anti-cancer effectiveness, we investigated the effects on cell viability and proliferation, and the results were compared with the ones obtained for the isoflavones. **BSE** and the isolated or combined isoflavones (**G**, **D** and **G** + **D**) caused a significant reduction in MCF-7aro cell viability and a significant reduction in the rate of DNA synthesis. Moreover, the reduction in MCF-7aro cell viability was more pronounced for **BSE** than for isoflavones or **NBSE**. This reinforces the idea that biotransformation of soya by the fungus has anti-tumoral advantages. On the other hand, comparing the effects of isoflavones, we also observed that **G** and **G** + **D** induced similar anti-proliferative effects, while **D** has weaker effects. The latter results are in accordance with Theil *et a1*. (2011), who have already described that the isoflavone **D** did not affect the viability and proliferation of breast cancer cells.[16] Furthermore, other authors have also demonstrated that only higher concentrations of **D** (25–50 μM) reduced the proliferation of MCF-7 cells.[21,41,44] Curiously, the results obtained for **G**, in similar concentrations to those existing in **BSE**, indicated that this isoflavone may be the constituent of the extract that is responsible for its cellular effects. In addition, by comparing our results with a recent study performed with the same soybean extracts, but in MCF-7 cells, it was observed that lower concentrations of **BSE** increased MCF-7 cell viability and only higher concentrations reduced cell viability.[48] Taking into account that MCF-7aro cells are a cell line model that expresses high aromatase levels[55] and **BSE** presents potent anti-aromatase activity, it can be suggested that the **BSE** anti-proliferative effects may be aromatase-dependent. However, as mentioned previously,[46,48,49] this soya extract besides aglycones **G** and **D**, also contains a high content of polyphenols[46] that may contribute to the anti-proliferative efficacy in breast cancer cells.

In order to explore the anti-proliferative effectiveness of **BSE** in breast cancer cells, we also investigated its effects on cell cycle progression. Our data demonstrated that, contrary to **BSE** that induced cell cycle arrest in the G_2/M phase, **G** caused cell cycle arrest in the G_0/G_1 phase, while **D** did not affect cell cycle progression. These results are not in accordance with what has been reported for the isoflavones. In fact, **D** caused cell cycle arrest at the G_1 and G_2/M phases, due to a decrease in the expression of cyclins B and D,[41] whereas **G** induced an arrest in the G_2/M phase in MCF-7 cells[40,42] and in other breast cancer cells,[43] also due to a decrease in the expression of cyclin B.[40] Even so, this study confirms that the isoflavones can disrupt cell cycle progression and inhibit growth of breast cancer cells, as already demonstrated,[12,16,35–44] but contrary to other studies that indicate that isoflavones stimulate the growth of breast cancer cells[28–31] due to their estrogenic effects.[28]

The anti-proliferative effects induced by **BSE** in MCF-7aro cells were accompanied by marked morphological alterations, such as membrane blebbing and chromatin condensation, typical features of the apoptotic process, as well as by the presence of AVOs, which also suggests the involvement of an autophagic process that may be responsible for cell survival or cell death. In a recent study, **BSE** induced apoptosis in MCF-7 cells, by increasing cell permeability, DNA degradation, phosphatidylserine externalization, and by decreasing the expression of anti-apoptotic factors and increasing pro-apoptotic proteins.[48] Altogether, it can be suggested that **BSE** can also induce apoptosis in MCF-7aro cells, since it caused a reduction in MCF-7aro cell viability, the presence of typical apoptotic morphological features and an arrest in the G_2/M phase of the cell cycle that has been referred to be associated with enhanced apoptosis.[60,61] Nonetheless, further studies should be performed with **BSE** to confirm the involvement of apoptosis and the elucidation of the underlying mechanisms.

In summary, our study demonstrates that the soybean extract *Glycine max* biotransformed by the fungus *Aspergillus*

awamori (**BSE**) inhibits aromatase, in human placental microsomes and in MCF-7aro cells, and the growth of ER^+ breast cancer cells, by decreasing MCF-7aro cell viability and proliferation, causing cell cycle arrest in the G_2/M phase. Its anti-proliferative efficacy in MCF-7aro cells seems to be aromatase-dependent and be affected by the isoflavone **G**, one of the major constituents of the extract. Importantly, this study demonstrates that the biotransformation of the extract by the fungus *Aspergillus awamori*, which is a β-glucosidase producer,[46] increases the anti-aromatase activity and the anti-proliferative efficacy of the soybean extract in breast cancer cells and therefore its anti-tumoral properties. It must be also pointed out that besides the anti-tumoral properties, the biotransformation has other advantages, since it has been described that fermented soybean contains higher amounts of isoflavone aglycones that are absorbed more rapidly and in bigger quantities than the glycoside forms, as well as high contents of other polyphenolic molecules.[46,62] This work contributed to the clarification of the potential use of soya in the prevention and/or treatment of ER^+ breast cancer.

Abbreviations

AIs	Aromatase inhibitors
AO	Acridine orange
AVOs	Acid vesicular organelles
BSE	Soybean extract *Glycine max* biotransformed by the fungus *Aspergillus awamori*
CFBS	Pre-treated charcoal heat-inactivated fetal bovine serum
D	Daidzein
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ER	Estrogen receptor
ER^+	Estrogen receptor-positive
Exe	Exemestane
F	Formestane
FBS	Fetal bovine serum
FSC	Forward light scatter
G	Genistein
G + D	Combination of genistein with daidzein
KCl	Potassium chloride
MCF-7aro cells	ER^+ aromatase-overexpressing breast cancer cell line
MEM	Eagle's minimum essential medium
MTT	Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NBSE	Soybean extract *Glycine max* without biotransformation
PI	Propidium iodide
SERMS	Selective estrogen receptor modulators
SERDS	Selective estrogen receptor down-regulators
SSC	Side light scatter
T	Testosterone
TCA	Trichloroacetic acid

Author contributions

Conceived and designed the study: CA, GCdS, and NT. Performed the experiments: CA and LDV. Provided the soybean extracts: MRTT and MJVF. Analyzed and interpreted the data: CA, MRTT, LDV, GCdS, and NT. Wrote the paper: CA, GCdS, and NT. Revised the manuscript: CA, MRTT, GCdS, and NT. Read and approved the manuscript for publication: CA, MRTT, LDV, MJVF, GCdS, and NT.

Conflict of interest

The authors have no conflict of interest to declare.

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