CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of biochemistry

SOYA ISOFLAVONES, GENISTEIN AND DAIDZEIN: THEIR BIOLOGICAL EFFECTS ON BREAST CANCER CELL LINES

in cooperation with

UNIVERSIDADE DO PORTO

FACULDADE DE FARMÁCIA

Laboratório de Bioquímica

Supervisors: Prof[a]. Dr[a]. Natércia Teixeira

Prof[a]. Dr[a]. Georgina Correia da Silva

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Porto 2011

Kristýna Hůrková

KARLOVA UNIVERZITA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra biochemických věd

SOJOVÉ ISOFLAVONOIDY, GENISTEIN A DAIDZEIN: JEJICH BIOLOGICKÉ EFEKTY NA BUNĚČNÉ LINIE RAKOVINY PRSU

ve spolupráci s

UNIVERSIDADE DO PORTO

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Vedoucí diplomové práce: Prof[a]. Dr[a]. Natércia Teixeira
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„Prohlašuji, že tato práce je mým autorským dílem. Veškerá literatura a další zdroje, z nichž jsem čerpala při zpracování, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.“

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ABSTRACT

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Supervisors:	Prof[a]. Dr[a]. Natércia Teixeira, Prof[a]. Dr[a]. Georgina Correia da Silva, Doc.Pharm.Dr. Tomáš Šimůnek Ph.D.
Title of diploma thesis:	Soya isoflavones, genistein and daidzein: Their biological effects on breast cancer cell lines

Cancer is one of the leading causes of death worldwide. Epidemiological studies suggested that there is a low incidence of breast cancer in countries with high soya intake. Soya contains the isoflavones daidzein (D) and genistein (G), which are responsible for the protective properties, although their exact effects have not yet been clarified. In this study we evaluated and compared the biochemical and biological effects of a soya extract biotransformed by *Aspergillus Awamori* (SBE) and of the pure major isoflavones, G and D in placental microsomes and in an estrogen-dependent breast cancer cell line stably transfected with the aromatase gene, the MCF-7aro cell line. The results showed that D did not induce marked alterations in any of the parameters studied. In placental microsomes G was not a potent aromatase inhibitor, inducing only a moderate reduction in the aromatase activity. In MCF-7aro cells it was observed a significant decrease in cell viability, after 48 h treatment, with SBE, G and mixture of D and G in a dose-dependent manner. In addition, it was detected a decrease in cell proliferation evaluated by the thymidine assay. Morphological studies, using phase contrast microscopy, Giemsa and Hoechst staining, demonstrated the appearance of membrane blebbings and chromatin condensation, considered to be apoptotic features, as well as some vacuoles in the cytosol. By acridine orange staining these structures were identified as acidic vesicular organelles, a characteristic of autophagic cell death. In addition, while G induced cell cycle arrest in G_0/G_1 phase, SBE caused an arrest in G_2/M phase. This study demonstrated that G is a moderate aromatase inhibitor and induces cell cycle arrest and cell death. However, further studies are required to clarify if autophagy is associated to apoptosis and the mechanisms that underly G and extract effects on cell cycle progression.

ABSTRAKT

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Název diplomové práce: Sojové isoflavonoidy, genistein a daidzein: Jejich biolgické efekty na buněčné linie rakoviny prsu

Rakovina je jednou z hlavních příčin smrti na celém světě. Epidemiologické studie ukázaly, že ve státech s vysokým příjmem soji je nízká incidence rakoviny prsu. Soja obsahuje isoflavonoidy daidzein (D) a genistein (G), které mohou být zodpovědné za protektivní vlastnosti, ačkoli jejich přesný účinek ještě nebyl zcela objasněn. V této práci jsme hodnotili a srovnávali biochemické a biologické efekty sojového extraktu biotransformovaného houbou *Aspergillus awamori* (SBE) a čistých hlavních isoflavonoidů, G a D na placentární mikrosomy a estrogen-dependentní buněčnou linii rakoviny prsu stabilně transfektovanou genem aromatasy, MCF-7aro. Výsledky ukázaly, že D nezpůsobuje žádné patrné změny v žádném ze studovaných parametrů. V placentárních mikrosomech G způsobil pouze mírné snížení aktivity aromatasy. U buněk MCF-7aro bylo pozorováno na koncentraci závislé snížení viability po 48 hodinovém působení SBE, G a směsi G a D. Navíc bylo zjištěno i snížení v proliferaci buněk hodnocené testem inkorporace thymidinu. Morfologické studie, za použití mikroskopie ve světelném kontrastu, Giemsova a Hoechstova barvení, demonstrovaly vznik membránových blebů a kondenzaci chromatinu považované za znaky apoptosy a vakuoly v cytosolu. Tyto struktury byly barvením akridinovou oranží identifikovány jako kyselé vesikulární organely charakteristické pro autofagickou buněčnou smrt. Navíc, zatímco G způsobil zastavení buněčného cyklu ve fázích G_0/G_1, SBE navodil zástavu ve fázích G_2/M. Tato studie ukázala, že G je mírný inhibitor aromatasy, zastavuje buněčný cyklus a navozuje buněčnou smrt. Jsou nezbytné další studie, které by objasnily, jestli je autofagie asociovaná s apoptosou a jakým mechanismem působí G a extrakt na buněčný cyklus.

CONTENT

1.

1. INTRODUCTION

1.1. Breast cancer

Cancer is one of the leading causes of death worldwide. According to The World Health Organization (Internet 1) cancer killed 7.9 million people in 2007, which is approximately 13% of all deaths. Around 72% of these occurred in low- and middle-income countries. WHO estimates that this number will reach 12 million in 2030.

The most frequent type of cancer is lung cancer in men and breast cancer in women (Internet 2). The latter occupies the first place in Europe in 2008, both in incidence of new cases and in cancer deaths of females. The number of female deaths and of new cancer cases has increased from 2006 to 2008, respectively, 1.3% and 3.7% (Karim-Kos et al. 2008, Internet 2). The incidence and mortality rates of different types of cancers in the European countries, in 2008, are shown in Figure 1.

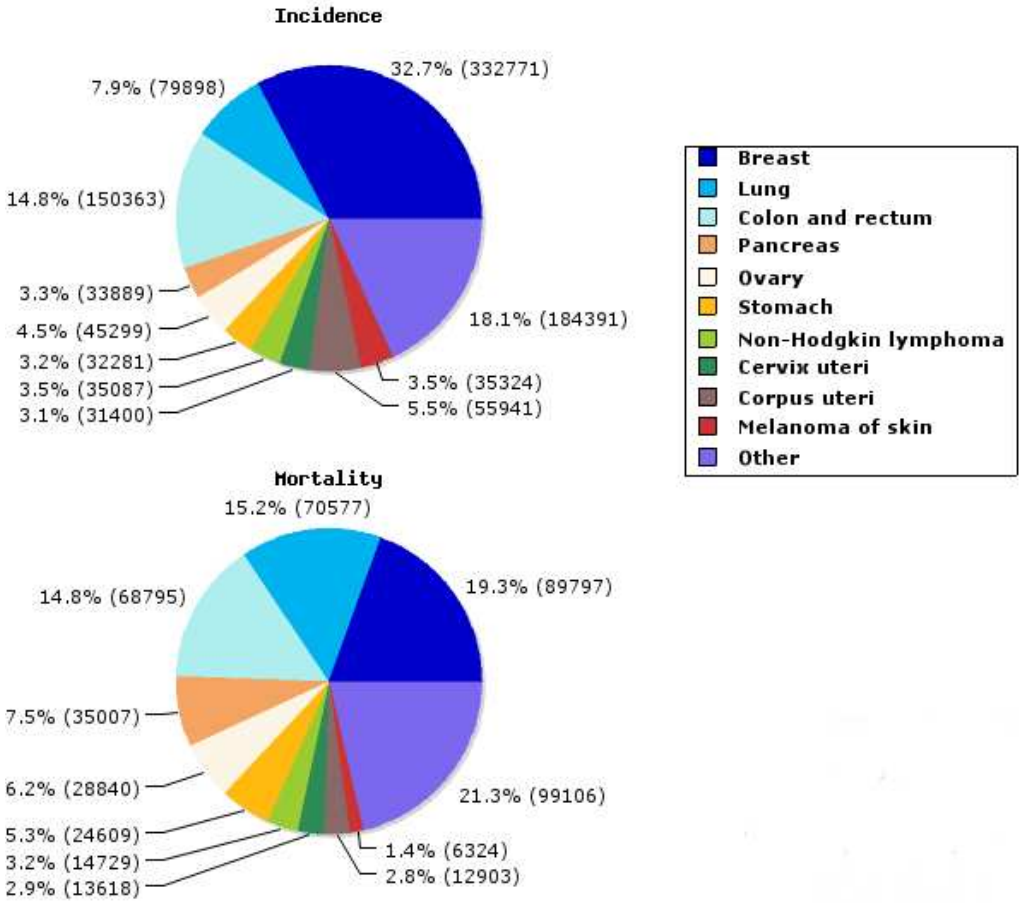


Fig. 1 Estimated incidence and mortality in women in European Union, 2008 (Internet 2)

There are more than one million new cases of breast cancer in the world every year (Coughlin and Ekwueme 2009). Nevertheless, the death rates decreased in most of European countries between 1989 and 2006. In some of them the reduction was more than 20%. This improvement is generally attributed to earlier detection and better treatment. The earlier detection is possible due to the establishment of breast cancer screening programmes, whereas is generally known that the earlier detection the better prognosis and efficacy of treatment (Internet 3). For example, in Czech Republic, the incidence of breast cancer increased from 55 to 62.5 per 100000, between 1994 and 2004. Though, the mortality has decreased during the same period from 23.5 to 19 per 100000. In addition, five years relative survival rates have improved in whole Europe, with a relative increase of 11% for Czech Republic (Karim-Kos et al. 2008).

However, in developing countries the problem number of new cases is higher, due to the lack of screening and worse treatment conditions (such as radiotherapy or chemotherapy). Moreover the number of oncologists in the countries with low resources is inadequate. So, most of new cases, in these countries, are diagnosed in clinical stages III or IV, instead of stages 0 and I. All these factors lead to much higher mortality rates when compared with developed countries (Coughlin and Ekwueme 2009). Figure 2 represents the mortality and incidence rates of breast cancer in various countries.

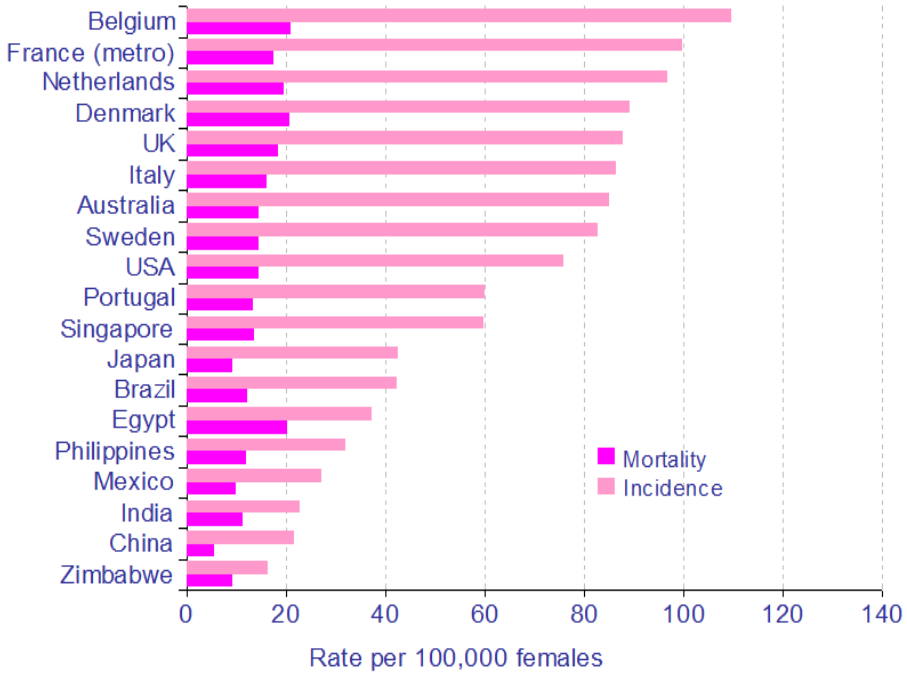


Fig. 2 Mortality and incidence of breast cancer in women in selected countries (adapted from Ferlay et al. 2010)

Breast cancer can also be found in men, but, due to the lower oestrogens levels, is 100 times less common than in women. It was thought that men´s prognosis was worse than women´s, however, studies suggest that, for the same stage of disease, the prognosis is identical to men and women (Internet 4).

Cancer development is associated to different risk factors. One of the main factors contributing to cancer development is age. This is due to longer exposure to carcinogens whether they are physical, chemical or biological. Another reason is lower activity and efficacy of cellular repair mechanisms. In addition to age, other factors have been referred as long menstrual history, early menarche (before 12 years) and late menopause (after 50 years), age of first birth (higher than 30), no children, no breastfeeding, age between 50 – 60 year (70% breast cancer cases are diagnosed in postmenopausal women) (Brooks and Thompson 2005), physical inactivity, unbalanced energy intake, obesity, regular alcoholic beverages consumption (one and more per day) and postmenopausal hormone replacement therapy. As for young pre-menopausal women the number of breast cancer cases is increasing and tumours are more aggressive, like in Chinese women (Porter 2009), other risk factors are also investigated.

As most of breast cancer cases are estrogen-dependent, in the past, androgens were used to treat them. But its use was reduced by the adverse effects caused, such as masculinization (Miller et al. 2008). Nowadays, the first option after breast cancer diagnosis, beside the surgical approach, is anti-estrogenic treatment with selective estrogen receptor modulator (SERM). One example is tamoxifen, the first targeted anti-cancer agent, also used as a chemopreventive agent. It is a partial estrogen receptor (ER) agonist. Its behaviour depends on type of tissue and also on the expression of co-regulators (Oseni et al. 2008). The standard treatment with tamoxifen is five years. Prolonged treatment does not bring any increase in efficacy due to development of drug resistance and enhanced agonist effect of tamoxifen (Orlando et al. 2010). Moreover, approximately 20% to 30% cases do not respond to this treatment. As far, any other SERM has demonstrated as good results as tamoxifen in breast cancer treatment or prevention (Orlando et al. 2010).

After tamoxifen discovery a group of selective estrogen receptor downregulators (SERD) was found. They act as pure ER antagonists. One of them is fulvestrant, which binds to ER causing changes in its conformation. In this way, ER loses its ability to control gene transcription and undergo degradation. Fulvestrant was described to reduce the growth of both human breast cancer cell lines, sensitive and resistant to tamoxifen

treatment (Orlando et al. 2010).

The other possibility to treat breast cancer is with aromatase inhibitors of third generation. They have showed to be an effective adjuvant therapy for hormone-sensitive early breast cancer in post-menopausal women. Aromatase inhibitors (AIs) are divided into two groups: non-steroidal and steroidal. The former group (letrozole and anastrozole) compete, with the substrate of aromatase, for enzyme active site where they bind reversibly. Steroidal aromatase inhibitors, like exemestane, are converted to the active intermediates by aromatase and these bind irreversibly to the enzyme´s substrate-binding site (Miller et al. 2008). Clinical trials demonstrated that AIs have increased efficacy over tamoxifen (Coates et al, 2007, Coombes et al, 2004). Moreover, with AI treatment there is less occurrence of blood clot formation compared to tamoxifen but superior bone loss, followed by increased risk of osteoporosis. This is due to systemic estrogen depletion and the effects of AIs on the human skeleton are becoming clinically important due to their increasing use as an adjuvant therapy in post-menopausal women with breast cancer. However, it was suggested that exemestane had a potential protective effect on osteoblast and osteoblastist-like cell lines via androgen receptor dependent and independent pathways (Miki et al. 2007). In addition, it has been shown that some AIs, like letrozole and anastrazol, and tamoxifen inhibit proliferation of breast cancer cells by inducing cell cycle arrest in G_0/G_1 phase and cell death by apoptosis (Sasano et al. 1999, Thiantanawat et al. 2003). Though, the mechanisms that modulate breast cancer cell death by aromatase inhibition are not totally clarified.

1.2. Estrogens

Estrogens belong to the group of steroid hormones. They occur naturally in both males and females, but their levels are higher in premenopausal women. Their production takes place mainly in ovary and adrenal cortex, but we can find enhanced synthesis in breast tissue of post-menopausal women. The first precursor of steroid synthesis is cholesterol. Estrogens are synthesized from C19-androgens. Ovaries are capable to synthesize androgen´s precursors, whereas breast tissue uses the blood androgens to synthesize estrogens. The main source of C19-androgens is the adrenal cortex and the first step is the conversion of cholesterol to pregnenolone. The following steps include conversion of pregnenolone to dehydroepiandrosterone, which is further metabolised to testosterone. The other pathway produces progesterone from pregnenolone, which is a precursor of androstenedione (Blair 2010).

There are three main estrogens in human: estrone (E_1), 17β-estradiol (E_2) and estriol (E_3). E_1 is synthesized from androstendione, whereas E_2 is from testosterone. Both of these reactions are catalysed by the enzyme aromatase. Moreover, E_1 can be converted to E_2 and E_2 back to E_1. These reactions are catalysed by 17β-hydroxysteroid-dehydrogenase 1 and 2, respectively. Conversion of E_2 to E_1 is one of the mechanisms, which are used to control the levels of 17β-estradiol in breast cancer (Blair 2010).

The most important estrogen is E_2, which controls reproductive system and sexual behaviour. It promotes secondary sexual signs such as breasts or pubic hair and plays a key role in menstrual cycle via thickening of endometrium (Chen et al. 2008). 17β-estradiol has the highest affinity to ERα and ERβ and is the estrogen that reaches the highest concentration in blood.

After menopause, ovaries gradually stop to synthesize estrogens, but E_2 can be produced by extragonadal tissues, like liver, skin and adipose tissue, acting by a paracrine pathway (Simpson et al. 2002).

Estrogens have shown an ability to contribute to the development of breast cancer. This hypothesis was formulated in 1936 by Antoine Lascassagne. He believed that breast cancer was caused by a special hereditary sensitivity (Oseni et al. 2006).

Estrogen carcinogenesis can be induced by three mechanisms. One is associated to the oxidative products formed during estrogens conversion to their metabolites. These oxidative products are able to damage DNA. Second, estrogens are known to promote not only cell proliferation by its direct or indirect effect on growth factors synthesis, in breast cancer cells included, but also to protect cells against apoptosis through up regulation of Bcl-2 protein, which is encoded by estrogen-responsive gene. There is also evidence that estrogen may induce breast cancer through a non-ERα mediated mechanism. All these mechanisms may lead to tumours growth (Blair 2010).

Estrogens may activate two pathways: the genomic and the non-genomic. Non-genomic pathway activation induces a faster cellular response that can take seconds to minutes. Estrogen´s effect is mediated through a plasma membrane-associated estrogen-binding proteins or an ER located in plasma membrane. Estrogens are able to enhance the levels of nitric oxide or calcium. They also possess ability to activate different signalling cascades including phospholipase C/protein kinase C, cAMP/protein kinase A, phosphatidyl inositol- kinase/AKT and Ras/Raf/MAPK (Marino et al. 2006, Chen et al. 2008).

Genomic pathway is provided via nuclear estrogen receptors (ER) and after hormone binding to the nuclear receptor there is a modification of their conformation.

They form homo- or heterodimers and these dimers are capable to bind to a DNA specific sequence, the estrogen response elements (ERE) (Simoncini et al. 2004). This induces the binding of co-activators, release of protein co-repressors and up regulation or down regulation of genes associated to cell survival or cell proliferation. The whole procedure takes usually hours (Marino et al. 2006, Chen et al. 2008). Recently, it was also shown that ER bind not only to ERE but there are alternative elements, like activator protein 1 binding sites (AP-1), cAMP response elements or specificity protein 1 (SP-1) response elements, to which other transcriptional factors bind (Fig 3). Binding of AP-1 to ER involves indirect binding through Jun/Fos-proteins (Hartman et al. 2009). Interestingly, ERβ is more potent than ERα on AP-1 binding sites, whereas the contrary is observed for ERE.

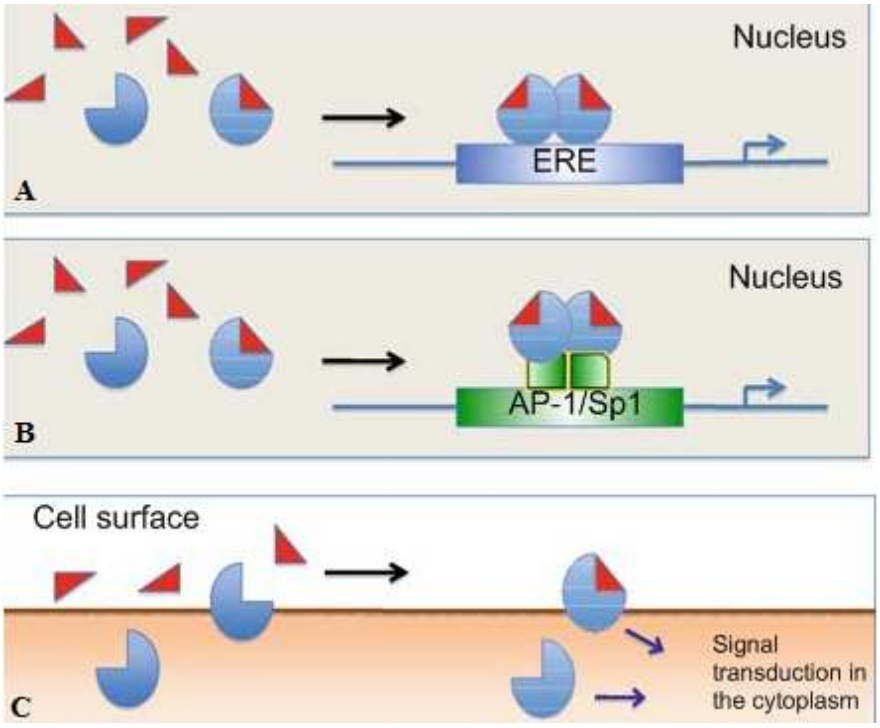


Fig. 3 A ‘‘Classical’’ ER activity through direct binding to (A) estrogen response elements (EREs); (B) activated ERs signal through protein–protein interactions with other transcription factors, such as AP-1 or Sp1; (C) non-genomic activity involves other signal transducers and causes rapid responses (adapted from Swedenborg et al. 2009)

1.3. Estrogen receptors

After establishment that estrogens may contribute to the development of breast cancer, attention has been paid to estrogen receptors to their potential as targets for

breast cancer treatment. For almost 40 years estrogen receptor alfa (ERα), which had been known since early 1960s, was considered as the only estrogen receptor (Bai and Gust). But in 1996 another estrogen receptor was discovered in rat prostate, the ERβ (Bai and Gust 2009). Both of them have high affinity to E_2 and are encoded by separate genes, the ESR1 and ESR2, respectively (Chen et al. 2008).

ERs are members of steroid hormone receptors superfamily and contain some of its typical structural and functional domains. They have six functional domains (A – F) (Fig 4). The first domain A/B is found in the amino terminal of the protein, mediates transcriptional activation and is the binding region for co-activator proteins. The main differences between nuclear receptors are localized in this domain. The C domain is the sequence, through which ER bind DNA. D-domain connect C- and E- domain and is very flexible. E-domain provides ligand binding and dimerization. The F-domain is located in the C - terminal and has complex regulatory effect. D- and E-domain provide a nuclear localization signal (Swedenborg et al. 2009).

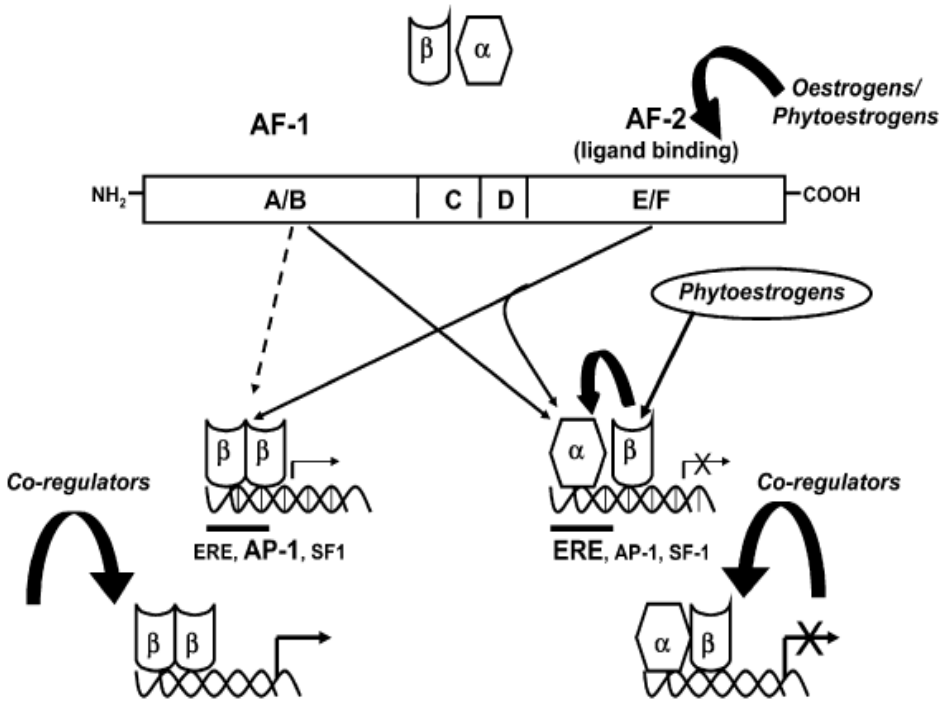


Fig. 4 Estrogen receptor´s functional and structural domains (adapted from Rice and Whitehead 2008)

ERα and ERβ share a high degree of sequence identity within their DNA-binding domains (DBDs) (Matthews and Gustafsson 2003), but in the E-domain they show just 56% homology (Swedenborg et al. 2009).

These two receptors are frequently distributed in different cells and organs (Hartman et al. 2009). ERα is expressed primarily in the uterus, kidney, heart and liver. ERβ can be found mostly in the ovary, lung, gastrointestinal tract, bladder, prostate and hematopoietic and central nervous system (Mathews and Gustafsson 2003). Both of them may be coexpressed in the same tissue as the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain (Mathews and Gustafsson 2003). Though, this does not mean that they have the same effects. ERs are involved in the promotion and proliferation of some tumors. Breast, gynecologic and endocrine glands cancer (including adrenocortical, ovarian, pancreatic, prostate and thyroid) are associated to the activity of ERα, whereas lung and digestive cancer (including colorectal, esophageal, liver and pancreatic) are associated to ERβ activation. Nevertheless, it appears that proliferation of one group of tumors inhibit the proliferation of the other (Chen et al. 2008).

There is also a difference between expression of ERs in normal and cancer mammary tissue. Whereas in 70% to 80% of breast cancer ERα is overexpressed (Lesniewska et al. 2009, Hartman et al. 2009), in normal mammary gland ERβ plays a dominant role. Though, not clarified, it is suggested that this could be related to hypermethylation of ERβ promoter in breast cancer cells. In fact, a demethylating agent reactivates ERβ expression in breast cancer cells, which results in an anti-proliferative effect (Warner et al. 2010, Hartman et al. 2009). Nevertheless, trials with ERβ-agonists were controversy. Two of them, that were effective, showed that tumour necrosis factor, secreted by natural killer cells acted on the tumour cells expressing ERβ. The other two ERβ-agonists did not show any effect (Warner et al. 2010). However, the presence of ERβ in breast cancer cells has not been clarified (Lesniewska et al. 2009). The remaining 20% to 30% of breast cancer cases are ERα negative. These present a more aggressive behaviour and the prognosis is generally worse than in the cases of ERα-positive (Lesniewska et al. 2009).

1.4. Aromatase

Aromatase belongs to the cytochrome P450 super family, which contain 74 families, where aromatase is a member of family 19 (Simpson et al. 2002). There is 13% to 20% of sequence homology with other CYP450 isozymes. A 3-D model of aromatase (Fig 5) showed that this enzyme contains four beta-sheets and fifteen alfa-helices, which create together two main domains (Hong et al. 2009).

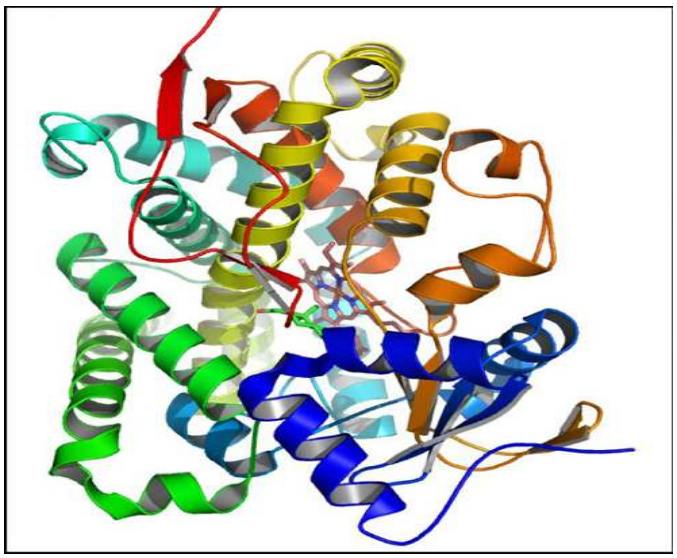


Fig.5 The crystal structure of human placental aromatase cytochrome P450 complexed with androstenedione (adapted from Ghosh et. al 2009)

As mentioned above, aromatase is a very important enzyme that catalyses the last step of estrogen biosynthesis, converting androgens to estrogens (Fig. 6). Aromatase is able to convert androstendione into estrone (E_1), as well as testosterone to estradiol (E_2), by catalyzing three consecutive hydroxylations. (Simpson et al. 2002). Aromatase needs NADPH as cofactor to catalyse this reaction.

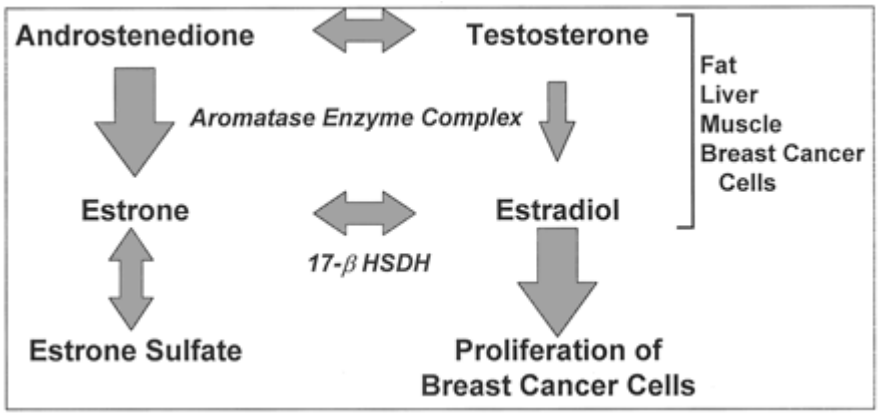


Fig.6 Enzyme pathway for estrogen synthesis from androgens via aromatase inhibitors (adapted from Buzdar and Howell 2001)

Aromatase could be found in ovary, testis, adipose tissue, skin, hypothalamus, placenta, chondrocytes and osteoblasts and in the vasculature smooth muscle. Aromatase is mainly expressed in the ovaries of premenopausal women and in postmenopausal women its expression is mainly in adipose tissue, breast tissue and skin. In addition, it may be overexpressed in breast tumour tissues, contributing to the

maintenance and tumour growth (Bulun et al. 2009). Its expression is not only controlled by different tissue-specific promoters (Simpson et al. 2002), but also by other factors, like cytokines (IL-6 and TNF α) and prostaglandins (PGE_2) (Simpson et al. 2002). All these facts led to consider aromatase as a good target for breast cancer treatment.

1.5. Phytoestrogens

Phytoestrogens are natural substances occurring in plants that have structural similarities to the endogenous steroid hormone, estradiol (Fig 7). They have weak estrogenic activity and bind to ER. Their family include isoflavone (genistein, daidzein, biochain A), lignans (enterodiol and enterolactone), stilbenes and coumestans. Soybeans are the main source of isoflavones, but they can also be found in other legumes, fruits or vegetables. The composition and total amount of these compounds depend on soya beans treatment, as fermentation or boiling may reduce the content in isoflavones up to 50% (Duffy et al. 2007, Knight and Eden 1995, Bingham et al. 1998).

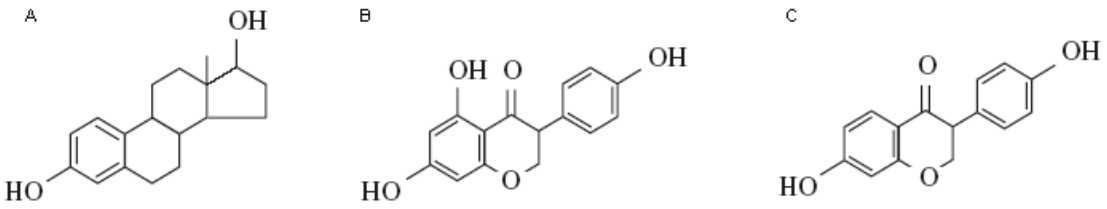


Fig. 7 Structural comparison of estrogens. (A) 17β-estradiol, (B) genistein, (C) daidzein (adapted from Limer and Speirs 2004)

It was shown that the daily intake of phytoestrogens in Asia was 20 to 50 mg phytoestrogens per day, whilst in the United States it reached only 0.15 – 3 mg. The average consumption in Europe is even lower than in United States (0.49 – 1 mg) (Mense et al. 2008).

As soya intake is high in Asian countries and epidemiologic studies showed that this population has lower risk and incidence of breast cancer, isoflavones have received much attention over the last 20 years. Since then, thousands of articles about soya food and phytoestrogens have been published. Nevertheless, the results of these studies are controversial and it has not yet been clarified the exact cellular mechanism responsible for the anti-cancer properties of soya isoflavones.

Interestingly, it was suggested that only high soya intake during adolescence

could be useful for protection against breast cancer, because breast tissue is more susceptible to carcinogens during this period of life-time (Velentzis et al 2008, Messina and Loprinzi 2001). Furthermore, Asian women that live in Western countries and adopt local diet have higher incidence of breast cancer (Probst-Hensch et al. 2000), showing that the low breast cancer incidence in Asian women was not due to genetic factors.

Phytoestrogens are not only important as anti-cancer agents (Fig. 8); they present other properties like antiviral, bactericidal, antifungal activities. Other studies showed that soya extract reduce the risk of cardiovascular diseases. Soya consumption was reported to decrease low-density lipoprotein concentrations, triglycerides, C-reactive protein, homocystein, oxidized LDL, lipoprotein(a) and blood pressure, and increase high-density lipoproteins, which have a protective effect. But it must be pointed out, that these have only been associated to soybean and not to isoflavone intake (Song et al. 2007). Moreover, it has been referred that isoflavones also can act as antiproliferative, antioxidant, anti-inflammatory and antihypertensive agents (Knight and Eden 1995).

Menopausal symptoms and bone density beneficial effect of soya has not been significantly proved. Some studies reported reduction of hot flash severity (50 mg isoflavones/day) and in maintenance or even an improvement of bone mineral density (35-54 mg of aglycone/day). In contrast to cardiovascular disease studies, the menopausal symptoms and bone density related studies have shown greater effect for isoflavones than for soya intake (Song et al. 2007).

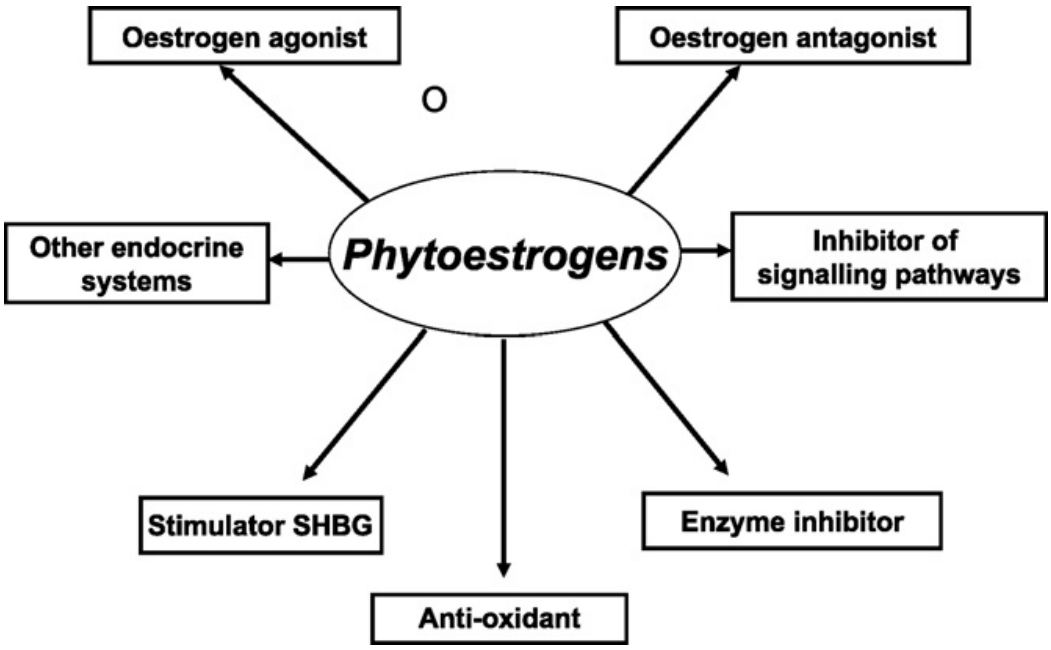


Fig. 8 Effects of phytoestrogens (adapted from Rice and Whitehead 2008)

1.5.1. Genistein and daidzein

As mentioned above, soybean is the main source of the isoflavones in the form of glycosidic conjugates. Only after deconjugation, by intestinal bacteria glucosidases, is obtained the active compounds of soya, genistein and daidzein. Then, these compounds can be further metabolized by bacteria to p-ethylphenol (Duffy et al. 2007) and equol or/and O-desmethyl-angolensin, respectively. Daidzein metabolization to equol or ODMA depends on individual. Only about 30% of population seem to be able to convert daidzein to equol (Sathyamoorthy and Wang 1997). Around 90% of genistein in blood undergo metabolic inactivation in liver where it is conjugated with sulfate or glucuronic acid, reaching free genistein only nanomolar concentrations.

People with Western life style have their plasmatic concentrations of genistein 100 to 1000 fold lower than regular consumers of soya food. Furthermore, and in spite of the same soya intake, final concentration of isoflavones may differ from individual to individual due to specific metabolism, depending on intestinal bacteria, age, gender and also exposure to antibiotics during a life-time (Pavese et al. 2010, Velentzis et al. 2008, Duffy et al. 2007, Knight and Eden 1995).

Genistein was isolated in 1899 by Perkin and Newbury and begun to be investigated because of its potential anti-cancer properties. Nevertheless, it has many other benefits such as lowering the incidence of cardiovascular diseases, preventing osteoporosis and other problems related to menopause (Pavese et al. 2010, Banerjee et

al. 2008).

Genistein and daidzein are described as natural selective estrogen receptor modulators (SERM) (Oseni a kol. 2008) that bind to both ERα and β, but with approximately 20-fold higher affinity to ERβ (Pavese et al. 2010). Moreover, it was shown, in breast cancer cell lines, that genistein induced up regulation of ERβ inhibiting, in that way, estogen-induced cell growth. Depending on concentration, these isoflavones can act as ER-agonists or ER-antagonists. Generally, genistein and daidzein on ER^+ cells, between 10 nM and 1 μM, induced cell growth, whilst, at higher concentrations (1-10 μM or above), these compounds caused growth inhibition of ER^+ and ER^- cells (Yang et al. 2010). On the contrary, other study showed that genistein, within the same range of concentrations, promoted cell proliferation in MCF-7 cells, though for higher concentrations (50 μM) an anti-proliferative effect was observed. All these facts led to the hypothesis that genistein can cause proliferation via the ER, whereas its anti-proliferative effect was ER-independent. The same study found that daidzein was ineffective against MCF-7 and BT20 cell line (Theil et al. 2010). On the other hand, Jin et al. (2010), using a breast cancer cell line, showed that daidzein significantly decreased proliferation in a dose- and time-dependent manner. These authors also demonstrated that daidzein promoted apoptosis through the mitochondrial pathway due to the generation of ROS.

Genistein is a known inhibitor of protein-tyrosine kinase, DNA topoisomerase I, II and 5α-reductase (Banerjee et al. 2008) as well as a modulator of the epidermal growth factor receptor (EGFR) (Yang et al. 2010) that may contribute to its anti-proliferative and pro-apoptotic effects. This isoflavone has also been found to have antioxidative properties, due to the existence of phenol groups in its structure, which are able to scavenge free radicals, contributing to the prevention of oxidative DNA damage (Pavese et al. 2010, Bingham et al. 1998).

Genistein and daidzein were described as inhibitors of 17β-HSD, the enzyme that converts the conversion of estrone to estradiol, in human placental microsomes, genital skin fibroblasts and granulosa luteal cells (Brooks and Thompson 2005). In addition, the former was also found to be a modulator of expression and activity of 17β-HSD but not of aromatase in human MCF–7 (Brueggemeier et al. 2001). However, the inhibition of 17β-HSD by genistein appears to be cell-dependent (Brooks and Thompson 2005). These studies also suggested that modulation of enzyme activity could be one possible mechanism by which genistein protected against breast cancer. Nevertheless, some studies reported genistein as an inhibitor of enzymes, 17β-HSD and

aromatase, in human placental microsomes (Le Bail et al. 1998).

Finally, genistein can also induce hormonal or metabolic changes, like alterations in insulin, thyroid hormone, leptin, adrenocorticotropic hormones, corticosterone and cortisol as well as changes in the expression of genes involved in lipid metabolism (Szkudelska and Nogowski 2007).

Several studies also showed that genistein could promote cell cycle arrest in different cancer cell lines (Dampier et al. 2001), but the exact mechanism has not yet been clarified. One possible mechanism is to arrest the cell cycle in phase G_2/M through inactivation of Cdk1 and reduction of cyclin B1, which are the main regulators of the G_2/M point (Pavese et al. 2010). Other mechanism that may be responsible for the inhibition of cell proliferation is the promotion of apoptosis. The most important apoptotic regulators belong to the Bcl-2 family. A reduction in Bcl-xL, an anti-apoptotic protein, and an increase in Bax, a pro-apoptotic factor, was observed in breast cancer cells after genistein treatment (Pavese et al. 2010). Furthermore, genistein can also induce activation of caspases (Banerjee et al. 2008). Like daidzein, genistein also showed the ability to lower the Bcl-2/Bax ratio, which is also a marker for apoptosis (Sakamoto et al. 2010, Pavese et al. 2010). Moreover, increased cytochrome c release was observed together with activation of caspase-9 in result of reactive oxygen species (ROS) production.

According to other study, the induction of apoptosis seems to be ER-independent (Sakamoto et al. 2010). This has also been supported by the fact that ERα-silencing did not cause any alteration in the Bcl-2/Bax ratio (Sakamoto et al. 2010). To explain these observations Sakamoto et al. (2010) investigated p53 and NF-κB-dependent transcriptional activity. After genistein treatment, they found not only an increase in p53 but also in NF-κB-dependent transcriptional activity. These results are not consistent with other study that described genistein as a NF-κB inhibitor (Pavese et al. 2010).

Beyond the above mentioned effects, genistein can also modify cell adhesion, decrease the cell migration, matrix metalloproteinase expression and cell invasion, contributing, in that way, to a decrease of cancer cell invasion and metastasis (Pavese et al. 2010).

Although genistein has all these properties, to reach these effects it is necessary higher levels than those found in the blood of people with high soya intake (Banerjee et al. 2008). In comparison to genistein, daidzein has not been so throughly investigated. In fact most studies showed that this isoflavone was less potent than genistein.

In spite of all, several studies suggested as mentioned above, that some

concentrations of genistein and daidzein may also promote proliferation of breast tumors. On the other hand, it is still unclear if these compounds are beneficial or dangerous to women that survived breast cancer, though, in general, isoflavones consumption is not recommended to these women (Akhtar et al. 2010). All these facts show that isoflavones need to be more thoroughly investigated to clarify the mechanism underlying the anti-cancer activity.

2. THE AIM OF STUDY

Breast cancer belongs to the most frequently diagnosed cancers in women. The majority of these cancers, in post-menopausal women, are hormone-dependent. Epidemiological studies suggested that there is a low incidence of breast cancer in countries with high soya intake. Moreover, the two major isoflavones in soya, genistein and daidzein, are thought to be effective in breast cancer prevention, presenting an anti-proliferative activity. Recently, we have shown that a soya extract derived from the enzymatic action of *p*-glucosidase of *Aspergillus Awamori* has the capacity to inhibit aromatase. However, the mechanism associated to the anti-cancer properties has not yet been totally clarified. In addition, as this soya preparation has better parental absorption it was raised the hypothesis that this formulation could provide a better protection to women regarding menopausal breast cancer. In that way, the propose of this study is to evaluate and compare the biological effects of the soya extract and of the major isoflavones, genistein and daidzein, on MCF-7aro, an estrogen-dependent breast cancer cell line stably transfected with the aromatase gene, mainly on their effects on cell viability and proliferation. Moreover, since several flavonoids and this soya extract demonstrated anti-aromatase activities, lowering, in that way, estrogen biosynthesis, the other aim of this study is to investigate if genistein and daidzein are able to inhibit aromatase activity, on placental microsomes.

3. MATERIALS AND METHODS

3.1. Materials

The soya extract biotransformed by *Aspergillus Awamori* was from the laboratory of Prof. Maria José Vieira Fonseca and Prof. Maria Regine Torqueti of Faculty of Pharmacy of Ribeirão Preto, University of São Paulo in Brazil. Eagle´s minimum essential medium (MEM), L-glutamine, fetal bovine serum, antibiotics penicillin-streptomycin-amphotericin B, Geneticin (G418) and trypsin were obtained from Gibco Invitrogen Co. (Paisley, Scotland, UK). The standards of genistein and daidzein, sodium pyruvate, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) ethylenediaminetetracetic acid (EDTA), trypan blue, testosterone, dimethylsulfoxide (DMSO), Hoechst 33258, dextran and charcoal were supplied by Sigma-Aldrich Co. (Saint Louis, USA). Vectashield mounting medium was from Vector (Burlingame, CA, USA). Giemsa´s azur eosine methylene blue solution was from Merck KGaA (Damnstadt, Germany). DPX was supplied by Pancreac Quimica Sau (Barcelona, Spain). [^{3}H]-thymidine was obtained from Amersham (Amersham International, Amersham, UK). Tritiated androstenedione was purchased from Perkin-Elmer (Boston, USA) Liquid scintillation cocktail Universol was supllied by ICN Radiochemicals (Irvine, CA, USA) and dye reagent for Bio-Rad protein assay was supplied by Bio-Rad (München, Germany). MCF-7aro, the ER-positive human breast cancer cell line stably transfected with human placental aromatase gene was supplied by Prof. Shiuan Chen from the Beckman Research Institute (City of Hope, Duarte, CA, USA).

3.2. Methods

3.2.1. Preparation of soya biotransformed extract

The biotransformed soya extract was prepared by Prof. Maria Regine Torqueti, from the Faculty of Pharmacy of Ribeirão Preto, University of São Paulo, Brazil. It contained a mixture of the two main isoflavones, genistein (G) and daidzein (D) in different concentrations. Extract was dissolved in 500 µl of DMSO and 500 µl red phenol free medium with 5% charcoal-stripped heat inactivated fetal bovine serum (FBS). This stock solution was stored at -20°C. For further dilutions an appropriate

medium or buffer was used.

3.2.2. Preparation of charcoal pellets

A suspension of 5% charcoal and 0.5% dextran in PBS was prepared and 1 ml of this suspension was put into each eppendorf tube and centrifuged for 10 min at 14000 g. The supernatant was removed and the pellets were dried into an oven.

3.2.3. Preparation of placental microsomes

Aromatase inhibition studies were performed using human placental microsomes that contain a high level of aromatase. Placental microsomes were obtained as described by Yoshida and Osawa (1991), with some modifications. Human placentas delivered from a local hospital were placed in cold 67 mM potassium phosphate buffer (pH 7.4) that contained 1% KCl. All procedures were performed at 0-5 ºC. The cotyledon tissue was separated and homogenized in a Polytron homogenizer with 67 mM KH_2PO_4 (pH 7.4) containing 0.5 mM dithiotreitol (DTT, 1:1, w/v) and 0.25 M sucrose. The homogenate underwent a differential centrifugation. First it was centrifugated at 5000 g for 30 min. Then the supernatant was taken and centrifugated twice at 20 000 g for 30 min and at 54 000 g for 45 min to obtain the microsomal pellet. The microsomes were washed and resuspended in 67 mM potassium phosphate buffer (pH 7,4), which contained 0.25 M sucrose, 20% glycerol and 0.5 mM DTT and stored at -80 ºC. The protein concentration was determined by Bradford protein assay using bovine serum albumin as a standard.

3.2.4. Aromatase assay

This assay was carried according to Thompson and Siiteri (1974) to evaluate aromatase activity. Aromatase assay evaluates the amount of tritiated water released from radiolabeled androstenedione, the substrate of aromatase, during aromatisation activity.

Standards of daidzein and genistein and mixture of these two standards were investigated at concentrations of 2 µM, respectively. Potassium phosphate buffer (67 mM, pH 4) was used for all dilutions and for completing the final volume of 1 ml per tube and also for control. Each tube contained microsomes that corresponded to 20 µg of microsomal proteins, tested compounds, 150 µM of NADPH, which is the coenzyme for aromatase reaction, 40 nM of 1β-[^{3}H]-androstendione and KH_2PO_4 at 67 mM to complete the final volume of 1 ml. This mixture was vortexed and incubated in a

shaking water bath for 15 min. The reaction was stopped with 250 µl of 20% TCA. The mixture was then transferred to eppendorf tubes with the above mentioned charcoal pellets, vortexed and incubated for 1 h at room temperature. After this the mixture was centrifugated at 14000 g for 10 min, the supernatant was transferred to new tubes with charcoal pellets, vortexed, incubated for 10 min and centrifuged under the same conditions. The supernatant was transferred to eppendorf tubes without charcoal pellets and centrifugated immediately under the same conditions to avoid the presence of charcoal in the samples. Then, 600 µl of supernatant were mixed with 3 ml of scintillation cocktail and a scintillation counter was used to measure the amount of the tritiated water in the samples. This experiment was carried out in triplicate and 0.5 µM formestane was used as a positive control. The blank did not contain genistein or daidzein.

3.2.5. Preparation of charcoal dextran heat-inactivated fetal bovine serum

The fetal bovine serum (FBS) was inactivated at 56 ºC, for 1 hour. Then, 500 ml of heat-inactivated FBS were treated with 8 g of activated charcoal at room temperature during 24 h and then centrifuged 5-6 times at 4000 g for 15 min and passed through a 0.22 µm filter.

3.2.6. Cell Culture

MCF-7aro is an ER-positive breast cancer cell line stably transfected with the aromatase gene. The maintaining medium for these cells was standard MEM medium, containing Earle´s salt, 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 1% penicillin, streptomycin, amphotericin B and 70 nM geneticin (G418). The cells were kept in 75 cm^2 flask at the temperature of 37 °C under an atmosphere of 5% CO_2. The medium was changed every 3 days and when the cells achieved 80 – 90% of confluence, the medium was removed, washed with PBS, 2 ml of 0.25% trypsine with 1 mM EDTA were added and cells were incubated for 2 minutes under the conditions mentioned above. After this time, cells were washed with medium containing FBS to stop trypsin action. The detached cells were taken out and centrifugated at 400 g, at 4 °C for 6 min. The pellet was resuspended and seeded into new flask with red phenol free medium with 5% charcoal-treated heat-inactivated FBS, with 2 mM L-glutamine and without geneticin. After three days, cells were trypsinised and counted after staining with vital dye Trypan blue 0.1% in a Neubauer chamber. Cells were then seeded into the red phenol free medium containing 5% charcoal-

stripped FBS with 1 nM of testosterone and incubated at temperature of 37 °C under an atmosphere of 5% CO_2 for 24 h. After that, different concentrations of soya extract (0.74; 1.25; 1.5 and 2.3 μM) or of genistein and daidzein (1, 5, 10, 25 μM) were added.

3.2.7. Giemsa staining

To study cell morphology the Giemsa staining was used. Cells were seeded into 24-well plate with cover slips at density of $2x10^5$ cells/ml. After 48 h of incubation with different concentrations of extract or of standards, the medium was removed and cells were washed with PBS. Then cells were fixed with 1 ml of 4% solution of paraformaldehyde (in PBS) or methanol for 30 min at 4 °C. After that, cells were washed twice with PBS and incubated with Giemsa stain (diluted 1:10 in distilled water) for 30 min. After incubation cells were washed with tap water to remove the excess of Giemsa stain. As final step the cover slips were taken out of the wells dehydrated and mounted in DPX.

3.2.8. Hoechst staining

This staining is used for nuclear morphological evaluation. After treatment and incubation time of 48 hours under the same conditions as described for thymidine incorporation assay in 24-wells plate with cover slips and at density of cells of $2x10^5$ cells/ml, the medium was taken out and cells were washed with PBS. The cells were fixed with 4% solution of paraformaldehyd for 30 min at 4 °C. After this time cells were washed with PBS and incubated with Hoechst 33258 at 0.5 μg/ml (in PBS) for 20 min at room temperature, covered with aluminium foil. After incubation cells were washed with PBS and mounted with vectashield. When cells were not observed immediately they were covered with aluminium foil and frozen. Samples stained with Hoechst were observed under fluorescence microscope (Eclipse E400, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm and processed by Nikon ACT-2U image software.

3.2.9. Acridine orange

The cells were seeded at a density of $2x10^5$ cells/ml in 24-wells plate with cover slips. After treatment for 48 h under the same condition as for Hoechst staining, cells were incubated with acridine orange in a final concentration of 0.1 μg/ml per well for 15 min at the temperature of 37 °C, under an atmophere of 5% CO_2. After that time, cells were washed twice with PBS and the cover slips were put on slides covered with

PBS to keep wet the cover slips and observed under the fluorescence microscope.

3.2.10. Thymidine assay

To evaluate DNA synthesis the thymidine incorporation assay was used. Cells were seeded into 96-well plate at density of $2.5x10^4$ cells/ml and treated with tested compounds in the complete red phenol free medium with 1 nM testosterone. Cells were incubated at temperature of 37 °C under an atmosphere of 5% CO_2 for 48 h. [^{3}H]-thymidine (0.5 µCi) was added 8 h before the end of the assay. To lyse the cells a cycle of freezing/defrosting was carried out. Then, liquid was sucked out using a semi-automated cell harvester (Scaton Instruments, Norway) and DNA was retained in a special filter. The filter circles were put into tubes and 1 ml of scintillation solution was added to each tube to determine the DNA incorporated [^{3}H]-thymidine, using scintillation counter (LS 6500, Beckman Coulter). The blank did not contain the extract, genistein or daidzein. Assays were carried out in quadruplicate and are representative of at least three independent experiments.

3.2.11. MTT assay

To evaluate cell viability the MTT assay was performed. 96-well plate with a cell density of $2.5x10^4$ cells/ml was used for this experiment. Incubation had the same condition as described for thymidine incorporation assay. After incubation time, 25 µl were taken out and put into another 96-well plate, which was later used for LDH assay. MTT solution (5mg/ml) was added into each well (17.5 µl) to the remaining supernatant, to obtain a dilution of 1:10. Then the plate was incubated for 2 h and 30 min at the temperature of 37°C under atmosphere of 5% CO_2. After this time the supernatant was removed and 200 µl of a mixture of DMSO and isopropanol (3:1) was added. The plate was mixed on a shaker plate for 15 min at the room temperature. The absorbance was measured at the wavelength of 540 nm. The experiments were done in quadruplicate and are representative of at least three independent experiments.

3.2.12. Cell cycle analysis

Cell cycle analysis was performed by flow cytometry. Cells at the density of $7x10^5$ cells/ml were seeded in 6-well plate and treated with compounds for 48 h. After this incubation time, medium was taken out and put into a tube, cells were trypsinised, as referred above, removed and mixed in the tube with cells that were not attached. All cells were washed twice in PBS and resuspended in a final volume of 0.5 ml in PBS.

The cell suspension was fixed with cold 70% ethanol and kept at 4 °C for at least 2 h.

The ethanol-suspended cells were centrifuged and the pellets were washed in PBS. Fixed cells were finally resuspended in 0.5 ml DNA staining solution (5 µg/ml PI, 0.1% Triton X-100 and 200µg/ml DNase free RNase A in PBS) and kept 30 min at room temperature.

Flow cytometric analysis of DNA content was based on the acquisition of 20000 events in a Becton Dickinson FACSCalibur (San Jose, CA, U.S.A.) equipped with CELLQuest Pro software. Debris, cell doublets and aggregates were gated out using a two parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. Detectors for forward (FSC) and side (SSC) light scatter and the three fluorescence channels (FL-1, FL-2 and FL-3) were set on a linear scale. The effect was indicated by the percentage of cells in G_0/G_1, S and G_2/M phase of the cell cycle. Assays were performed in triplicate in three independent experiments.

3.2.13. Statistical Analysis

The data presented are expressed as the mean ± SE. Statistical analysis of data was performed using analysis of variance (ANOVA) followed by Dunnett pos-hoc test. Values of $p<0.05$ were considered statistically significant.

4. RESULTS

4.1. Effects on aromatase activity

In a previous work it was shown that the biotransformed soya extract (SBE) presented a strong inhibitory aromatase activity. In this study aromatase assay was carried out to investigate if the two main isoflavones present in the extract were responsible for the anti-aromatase activity. After treatment with 2 µM of genistein (G) or daidzein (D) it was obtained an inhibition of aromatase activity of only 34% and of 17.5%, respectively (Fig 9). The mixture of both isoflavones presented an anti-aromatase activity similar to the observed for the isolated G. Formestane (0.5 µM), used as a positive control, caused a reduction in aromatase activity of more than 97%.

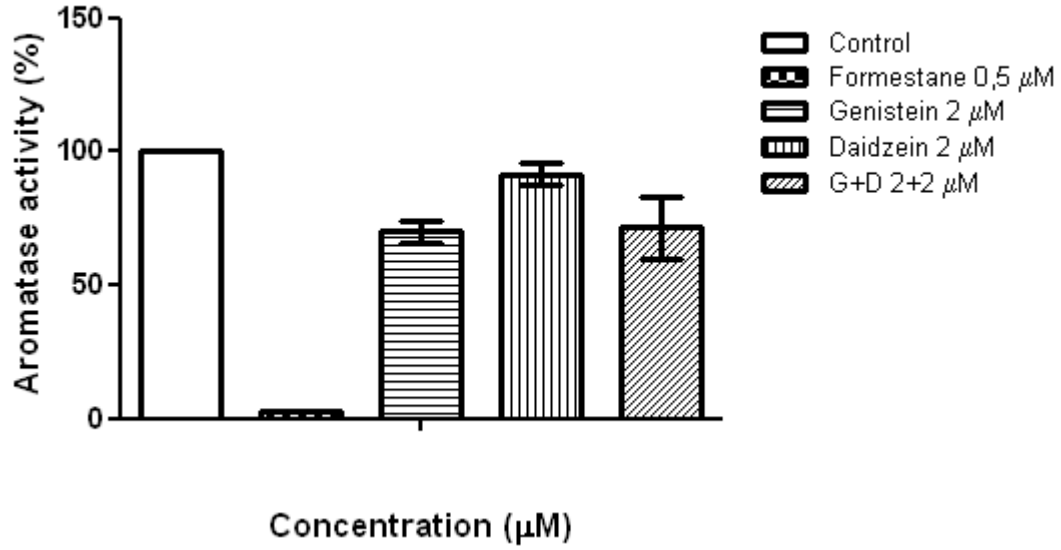


Fig. 9 Effects of isoflavones on aromatase activity in placental microsomes. Activity in microsomes treated with testosterone (1 nM) was considered as 100% of aromatase activity. Microsomes treated with 0.5 µM of formestane were used as a positive control. Results are presented as percentage of aromatase activity and are the mean ± SE of three independent experiments performed in triplicate.

4.2. Morphological studies

To evaluate the morphological alterations induced by the biotransformed soya extract and by the isolated isoflavones, MCF-7aro cells were cultured with or without the compounds and examined by phase-contrast microscopy, Giemsa and Hoechst staining.

The untreated cells (only with 1 nM testosterone) did not show any morphological changes during the incubation period. After 48h of incubation with SBE,

G or mixture of D and G, it was observed some round cells showing membrane blebbing (black arrows in Fig. 10, 11 and 12). The number of blebbings was dose-dependent. The cells treated with D did not show any morphological alterations (Fig. 13).

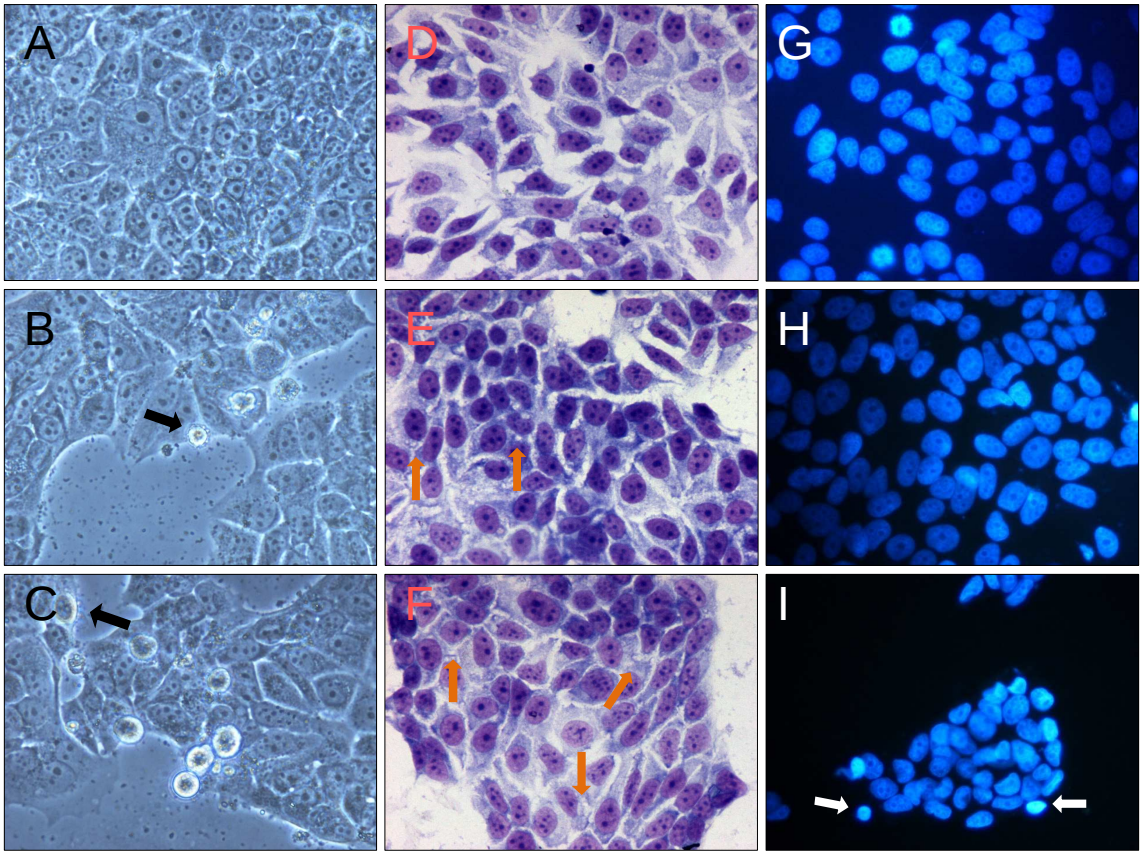


Fig. 10 Effect of soya biotransformed extract on MCF-7aro cell morphology by phase contrast (A, B, C), Giemsa (D, E, F) and Hoechst (G, H, I) staining. Cultured MCF-7aro cells were examined in the absence (A, D, G) or in the presence of 0.74 µM (B, E, H) and 1.5 µM (C, F, I) of SBE for 48 hours. Black arrows indicate membrane blebbings. Orange arrows indicate vacuolisation. White arrows show chromatin condensation.

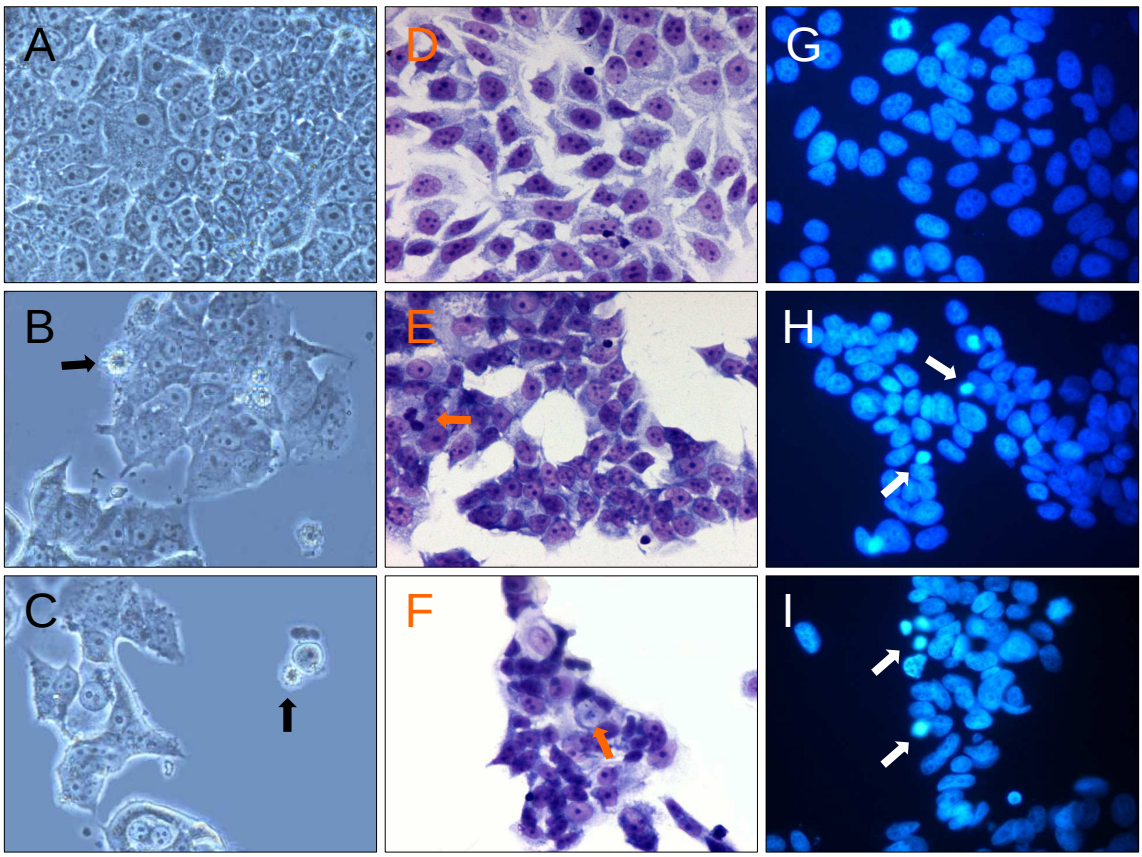


Fig. 11 Morphological changes of MCF-7aro cells treated with genistein. Cultured MCF-7aro cells were examined by phase contrast phase microscopy (A, B, C), Giemsa (D, E, F) or Hoeschst (G, H, I) staining, respectively, in the absence (A, D, G) or in the presence of 10 µM (B, E, H) or 25 µM (C, F, I) of genistein for 48 hours. Black arrows indicate membrane blebbings. Orange and white arrows show chromatin condensation.

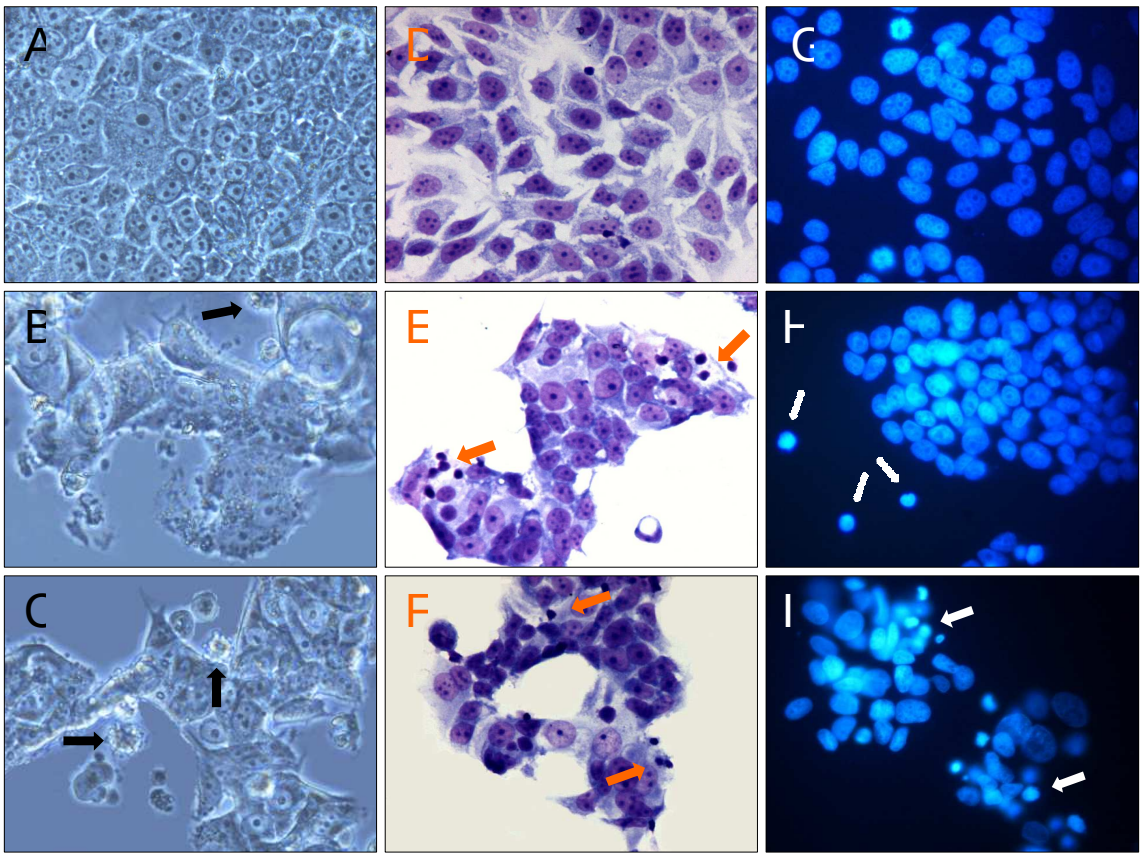


Fig. 12 Morphological changes of MCF-7aro cells treated with the mixture of dadzein and ginestein. Cultured MCF-7aro cells were examined by phase contrast microscopy (A, B, C), Giemsa (D, E, F) or Hoeschst (G, H, I) staining, respectively, in the absence (A, D, G) or in the presence of 10 μM (B, E, H) or 25 μM (C, F, I) of G and D for 48 hours. Black arrows indicate membrane blebbings. Orange and white arrows show chromatin condensation.

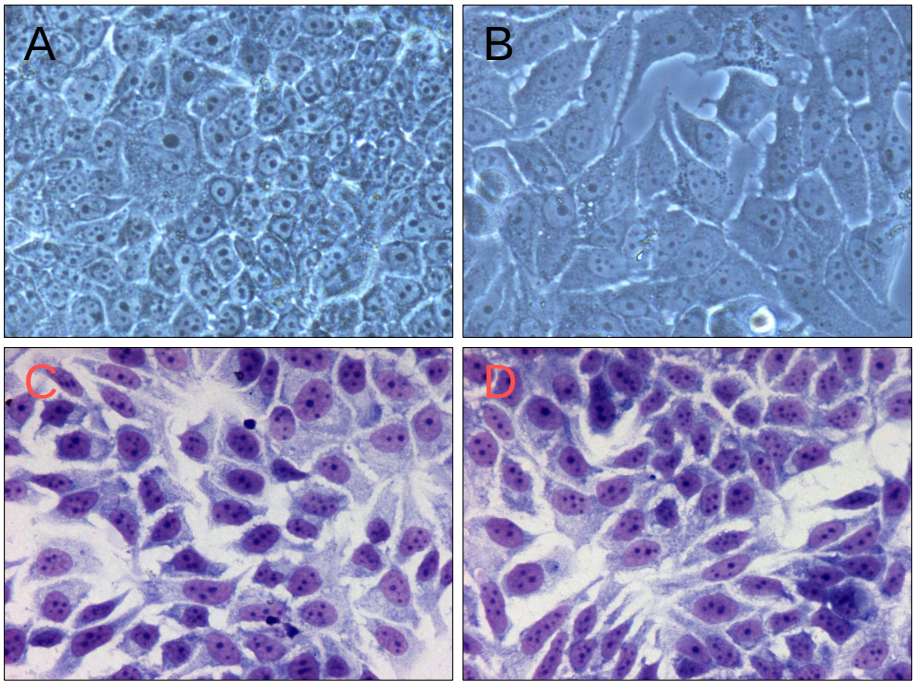


Fig. 13 Morphological changes on MCF-7aro cells treated with daidzein. Cultured MCF-7aro cells were examined by phase contrast microscopy (A, B,), Giemsa (C, D) staining, respectively, in the absence (A, C) or in the presence of 25 µM (B, D) of D for 48 hours.

Interestingly, the effect of SBE in the higher concentration, which corresponded to 1.28 µM of daidzein and 1 µM of genistein, was more intense than the treatment with the isolated genistein in a concentration of 1 µM (Fig. 14).

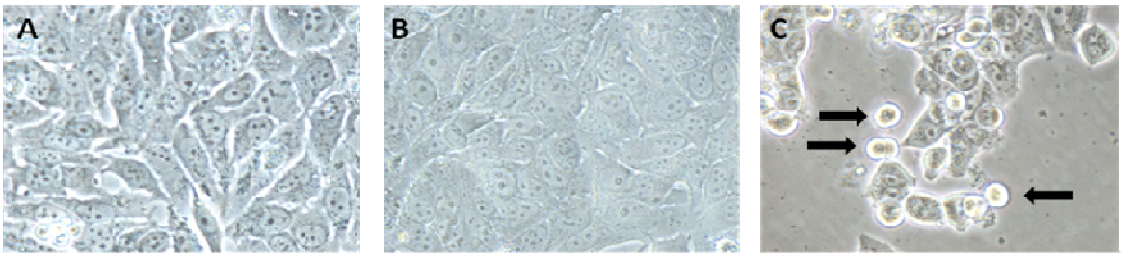


Fig. 14 Effect of genistein and SBE on MCF-7aro on cell morphology using phase contrast. Cultured MCF-7aro cells were examined in the absence (A) or in the presence of 1 µM of G (B) or of 2.3 µM of SBE (corresponding to 1 µM of G) (C) for 48 hours. Black arrows indicate dead cells.

By Giemsa staining it was observed chromatin condensation in the higher concentrations of SBE, G and mixture of G and D. These results were confirmed by Hoechst staining. A reduction in cell density was also observed and was dose-dependent (Fig. 10, ,11, 12). The highest concentration of SBE (2.3 µM) caused

massive cell death when compared to control and other concentrations (Fig. 14). The other SBE doses caused some vacuolation in the cytosol (Fig. 10). On the other hand as in phase contrast microscopy, even after treatment with the highest concentration, daidzein did not induce marked alterations in cell morphology (Fig. 13).

The appearance of vacuoles was further studied by acridine orange staining. Acridine orange is a cell-permeable fluorescent dye that stains DNA and cytoplasm bright green. It can also enter acidic compartments, such as lysosomes or autolysosomes, where it becomes protonated and sequestered. At low pH acridine orange emits red fluorescence with intensity proportional to the degree of acidity. An increase in acidic vesicular organelles was observed in cells treated with SBE in the concentrations of 0.74 µM and 1.5 µM as well as with 10 µM G or with the mixture of both isoflavones (Fig. 15, 16)

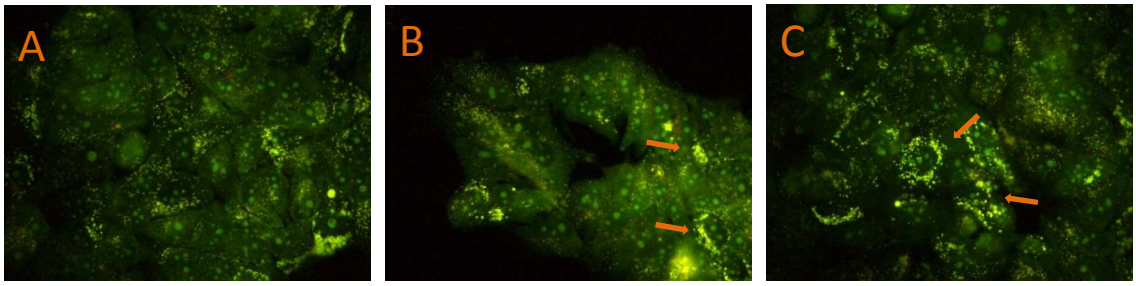


Fig. 15 Effect of soya biotransformed extract on MCF-7aro cell morphology using acridine orange. Cultured MCF-7aro cells were cultured in the absence (A) or in the presence of 0.74 µM (B) and 1.5 µM (C) of SBE for 48 hours. Orange arrows indicate acidic vesicular organelles.

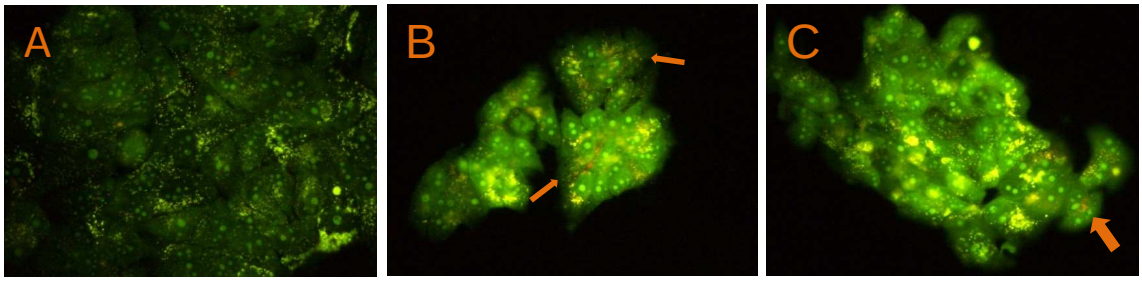


Fig. 16 Effect of isoflavones on MCF-7aro cell morphology using acridine orange staining. Cultured MCF-7aro cells were examined in the absence (A) or in the presence of 10 µM of G (B) , or both, G and D (C) for 48 hours. Orange arrows indicate acidic vesicular organelles.

4.3. Effects on MCF-7aro cell proliferation

To investigate the effect of the biotransformed soya extract and of the main isoflavones, G and D on cell proliferation, thymidine incorporation assay was performed. The MCF-7aro cells were treated with four different concentrations of SBE:

0.74 µM (corresponding to 0.415 µM of D and 0.325 µM of G); 1.25 µM (corresponding to 0.702 µM of D and 0.548 µM of G); 1.5 µM (0.960 µM of D and 0.750 µM of G) and 2.3 µM (1.28 µM of D and 1 µM ofG), with four concentrations of the isolated G and D (1, 5, 10 and 25 µM) and with the mixture of these isoflavones (1+1, 5+5, 10+10, 25+25 µM), for 48 hours. As shown in Figure 17, SBE induced a reduction in DNA synthesis, which was dose-dependent. For the highest concentration of SBE that corresponded to 1.28 µM of D and 1 µM of G was obtained a reduction of 63.44% in DNA synthesis.

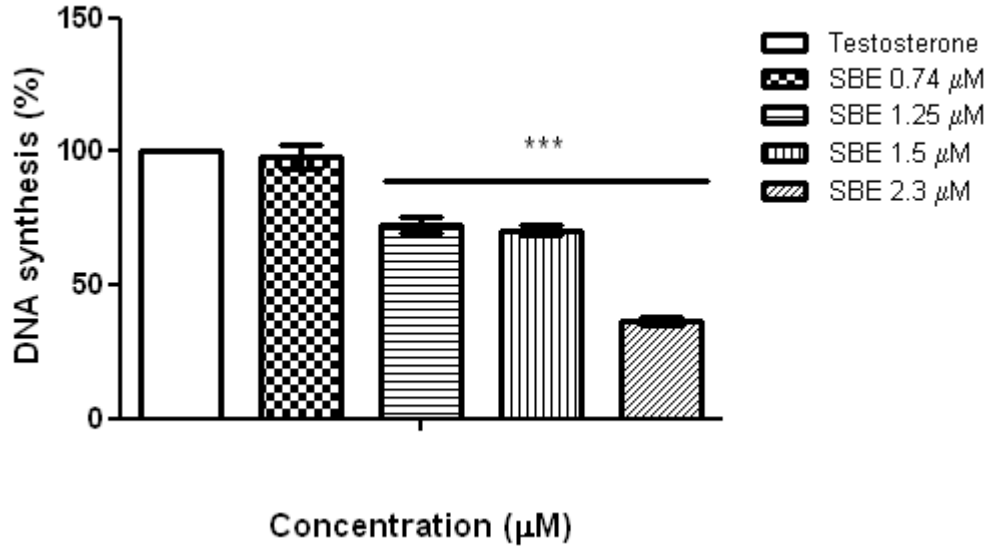


Fig. 17 Effects of SBE on rate of DNA synthesis, estimated by the thymidine incorporation assay. Cells were treated with four concentrations of SBE for 48 hours. Cells cultured with testosterone (1nM) were used as control and represented the maximum of DNA synthesis. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control and treated cells are denoted by ***($p<0.001$).

In relation to the effect of the pure compounds, G was the most potent isoflavone reducing cell proliferation in a dose-dependent manner. It was observed a significant decrease in DNA synthesis ($p<0.001$) of 28.69%, 31.32%, 71.73%, 94.91% for the concentrations of 1, 5, 10 and 25 µM, respectively (Fig. 18). Daidzein treatment did not show any significant changes, it induced only an inhibition of 34.83% at the highest concentration (Fig. 18). The results obtained with the mixture of isoflavones were similar to the ones observed for the isolated G. The highest concentration of SBE that contained approximately 1 µM of G caused a higher decrease in DNA synthesis than the same concentration of the isolated isoflavone (63.44% vs 28.69%)

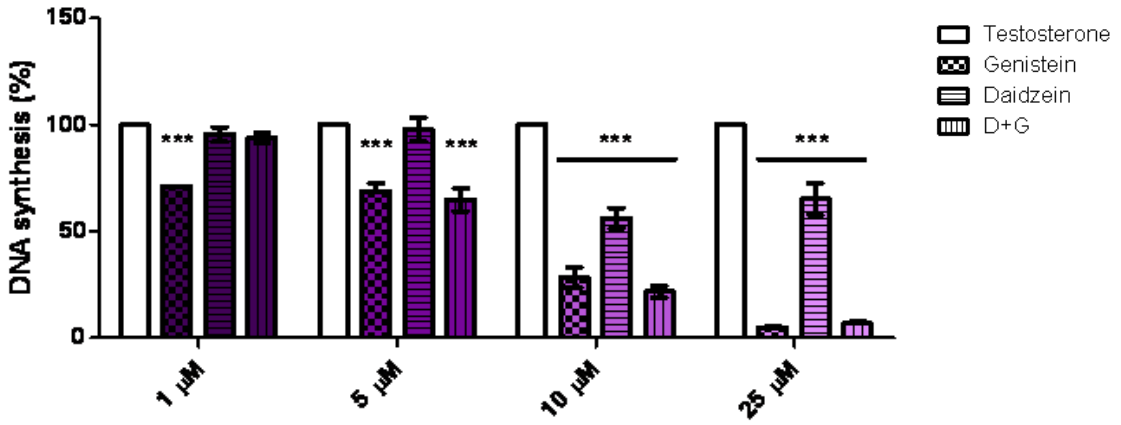


Fig. 18 Effects of genistein and daidzein on the rate of DNA synthesis, estimated by the thymidine incorporation assay. Cells were treated with four concentrations of G, D or both standards for 48 hours. Cells cultured with testosterone (1nM) were used as control and represented the maximum of DNA synthesis. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control and treated cells are denoted by ***($p<0.001$).

4.4. Effects on MCF-7aro cells viability

Evaluation of cell viability was provided by MTT assay. MTT is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and is used to measure mitochondrial dehydrogenase activity, which forms purple formazan salt crystals.

Cells were treated with the same compounds and concentrations as for the thymidine incorporation assay. SBE and G reduced cell viability in a dose-dependent manner, being the maximum reduction of approximately 50% for the highest concentration of SBE and of G (46.67% for SBE and 47.77% for genistein, respectively) as shown in Fig. 19 and 20.

On the other hand, treatment with daidzein itself did not show any significant changes, as the decrease in cell viability was of only 8% at the highest concentration. The mixture of G and D caused a similar reduction in cell viability when compared to pure G in all the concentrations used.

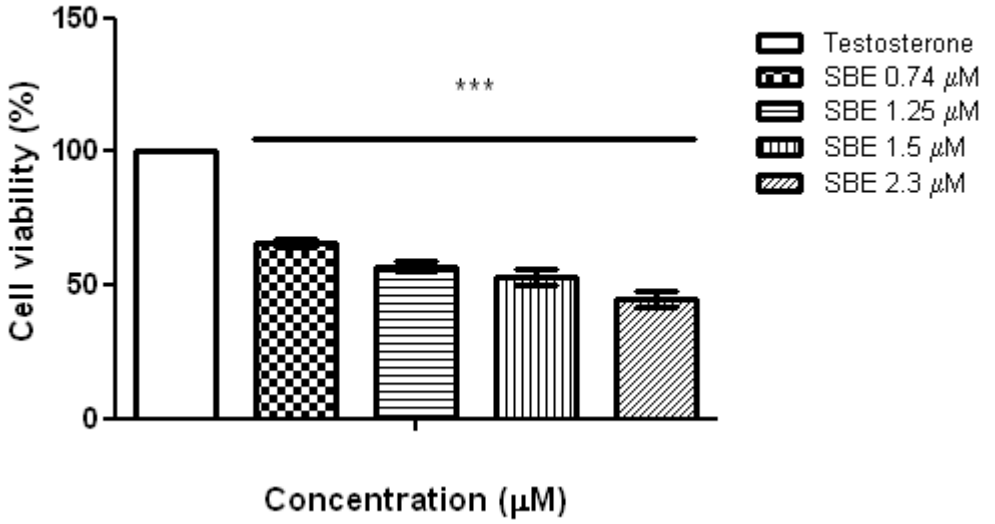


Fig. 19 Effect of SBE on cell viability, estimated by MTT assay Cells were treated with four concentrations of SBE for 48 hours. Cells cultured with testosterone (1 nM) were used as control and represented the maximum of cell viability. Results are the mean ± SE of three independent experiments performed in triplicate. Significant differences between the control and treated cells are denoted by ***($p<0.001$).

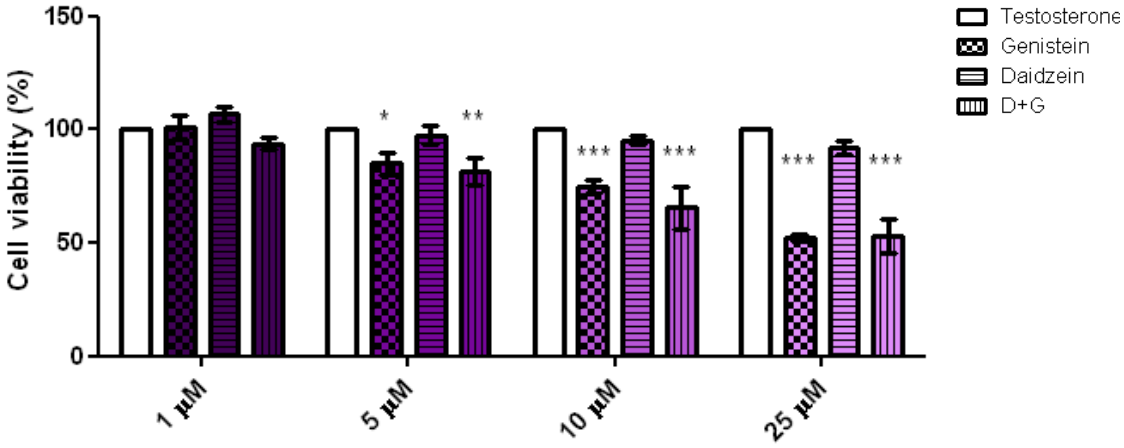


Fig. 20 Effect of isoflavones on cell viability, estimated by MTT assay. Cells were treated with four concentrations of G, D or both compounds for 48 hours. Cells cultured with testosterone (1 nM) were used as control and represented the maximum of cell viability. Results are the mean ± SE of three independent experiments performed in triplicate. Significant differences between the control and treated cells are denoted by *($p<0.05$), **($p<0.01$) and ***($p<0.001$).

4.5. Cell cycle analysis

To investigate the mechanisms underlying the antiproliferative effect of the extract and of genistein on MCF-7aro cells, discrimination of cells in G_0/G_1 *versus* S and G_2/M phases of cell cycle was carried out, by measuring the DNA content by flow

cytometry. Cells were treated with two concentrations of SBE (1.5 µM and 2.3 µM) and of G (10 µM and 25 µM) and stained, after 24 and 48 h, with PI.

After 48 hours, SBE treatment did not induce any significant alterations at the concentration of 1.5 µM. However, a reduction of the percentage of MCF-7aro cells in G_0/G_1 phase (from 74% to 66%) and an increase in the fraction of cells in G_2/M cell cycle phase (from 15.78% to 23.83%) was observed, after treatment with 2.3 µM SBE (Fig. 21). Interestingly, cells treated with genistein for 48 hours demonstrated an accumulation of cells in G_0/G_1 phase for G at 10 µM (80%) in comparison to control cells (74.5%) and a decrease in S and G_2/M phase. For the higher concentration it was only observed a decrease in S phase (3.27%) in comparison to the control (6.35%) (Fig. 21 and Table 1).

Table 1 Effects of biotransformed soya extract and of genistein on cell cycle distribuition on human breast cancer MCF-7aro cells

	G_0/G_1	**S**	**G_2/M**
Testosterone	74.57 ± 0.54	6.35 ± 0.40	15.78 ± 0.60
SBE 1,5 µM	73.83 ± 0.89	6.2 ± 0.29	16.36 ± 0.61
SBE 2,3 µM	66.75 ± 0.67**	5.26 ± 0.10	23.83 ± 0.18**
G 10 µM	80.09 ± 0.10*	4.90 ± 0.16	12.85 ± 0.10
G 25 µM	76.40 ± 1.4	3.27 ± 0.5**	16.29 ± 1.2

Cells were treated with different concentrations of the compounds for 48 h. Treated cells were harvested, fixed and their DNA content was evaluated by PI labeling followed by flow cytometry analysis. Data are presented as single cell events in G_0/G1, S and G_2/M phases of the cell cycle. The data represents the mean and ± SE of triplicates and are representative of three independent experiments. Significant differences between control and treated cells with different concentrations of extract (SBE) or G are indicated by *($p<0.05$) and **($p<0.001$).

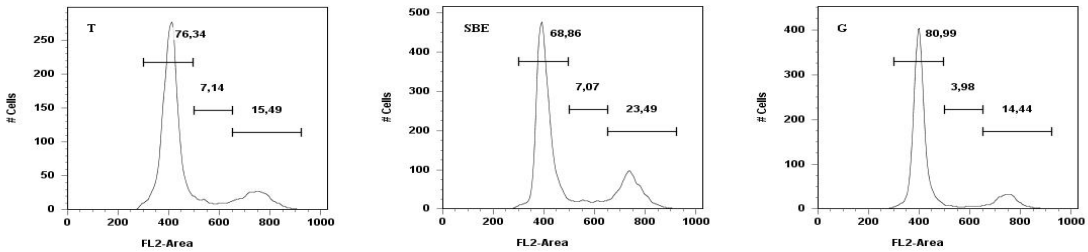
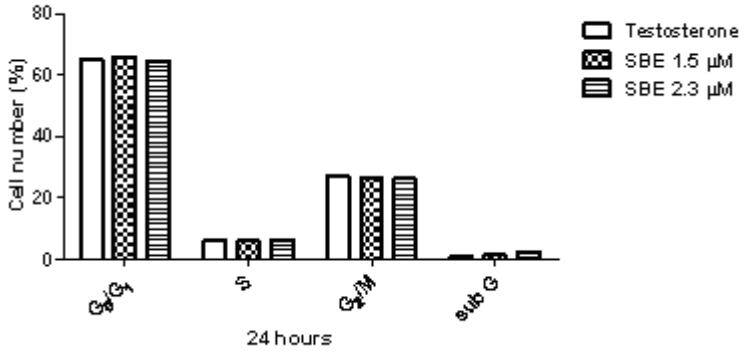


Fig 21. Effect of biotransformed soya extract and of genistein on cell cycle distribution. Cells were treated without (T) or with SBE (2.3 µM) and G (25 µM) for 48 h, and subjected to flow cytometry after PI staining. Histograms were analysed with FlowJo Software (Tree Star, Inc). Data are a representative of three independent experiments performed in triplicate.

A



B

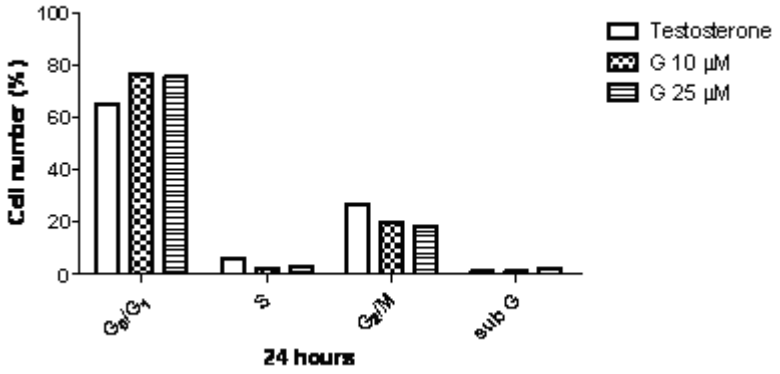


Fig. 22 Effect of biotransformed soya extract and of genistein on cell cycle distribuition. Cells were treated with two concentrations of (A) SBE (1.5 and 2.3 µM) and (B) G (10 and 25 µM) for 24 h, and subjected to flow cytometry after PI staining. Cells cultured with testosterone (1 nM) were used as control. Results are presented as percentage of cells being in a given phase of cell cycle.

Though preliminary, the results obtained after 24 hours of treatment showed that SBE did not cause any alterations, whereas both concentrations of G induced an arrest

in G_0/G_1 phase (Fig. 22). In testosterone treated cells (control cells) there was 65.5% of cells in G_0/G_1 and 27.2% in G_2/M. In treated cells with 10 µM of G the percentage of cells arrested in G_0/G_1 phase was 76.8%, while in G_2/M was 19.8%. Cells treated with 25 µM of G reached 75.9% and 18.7%, respectively, in G_0/G_1 and G_2/M phases. There was also noticed a decrease in S phase from 6.25% (control) to 2.3% and 3.2% respectively for 10 µM and 25 µM of G (Fig. 22).

5. DISCUSSION AND CONCLUSION

Breast cancer is the major hormone-dependent cancer in women. Thus, prevention and treatment is an important issue that is under intense investigation. Epidemiological studies have evidenced that soya rich diets contribute to lower rates of breast cancer in Asian countries when compared to western population. The main soya isoflavones, genistein and daidzein, though, exhibiting estrogen-like properties also induce tumor cell death in a variety of cancer types (Jemal el al. 2002). In addition, recent results from our laboratory demonstrated that the extract of biotransformed soya, derived from the enzymatic action of p-glucosidase of *Aspergillus awamori* front soya flour carefully selected and processed, was able to inhibit aromatase activity in placental microsomes.

Aromatase is a key enzyme responsible for the estrogen biosynthesis that is expressed in higher levels in breast cancer cells and surrounding stromal adipose tissue than in non-cancerous cells. Inhibition of aromatase is one of the main approaches for breast cancer treatment in post-menopausal women. In order to clarify if the anti-aromatase activity observed for the SBE was due to the main soya isoflavones, it was determined the effect of the pure compounds, G and D, on the aromatase activity, using placental microsomes. The results indicated that only G presented anti-aromatase activity. The inhibitory activity in the concentrations used was only moderate, as it was observed a decrease of 34% in the enzyme activity after incubation with 2 μM of G. This observation is in agreement with other authors that showed that this isoflavone, at 10 μM, was able to inhibit aromatase in MCF-7 cell line (Brooks and Thompson, 2005). However, this is in contrast to other studies that demonstrated that G did not present anti-aromatase activity in placental microsomes (Le Bail et al. 1998, 2000). Nevertheless, the soya extract, in the highest concentration that corresponded to 1 μM of G, was much more potent than the isolated compound. Thus, it cannot be discarded the possibility that the extract may contain other bioactive molecules that either present anti-aromatase activity or, at least, induce a synergistic effect to the action of G.

In order to further explore the *in vitro* effects of the soya extract and of its principal isoflavones, G and D, cell growth, cell cycle progression and cell death was studied using the MCF-7aro cell line, an ER-positive breast cancer cell line stably transfected with the aromatase gene.

All the experiments carried out with D showed that this isoflavone did not have any

significant effect on aromatase activity and cell viability, in the range of concentrations used. These results are in accordance to Theil et al. (2010) that obtained similar effects on cell viability. By contrast, our results in the concentration of 10 and 25 μM of D induced a significant decrease in cell proliferation. Other authors (Jin et al. 2010; Choi and Kim, 2008), demonstrated also that D, ranging from 25 to 100 μM significantly reduced proliferation of MCF-7 cells in a dose- and time-dependent manner. Furthermore, it was demonstrated that D caused breast cancer cell apoptosis through the mitochondrial pathway, being ROS crucial to induce apoptosis and acting as signalling molecules to initiate cell death (Jin et al. 2010).

The total extract induced a significant decrease in cell viability and cell proliferation in a dose-dependent manner. Interestingly, the highest dose of extract, which corresponded to the lowest concentration of the isolated isoflavones tested, was more potent than the pure compounds.

There are some evidences, from *in vitro* and *in vivo* studies, showing that the anticancer activity of isoflavones is due to apoptosis and cell cycle arrest. In fact, the results obtained from the MTT and the thymidine incorporation assays may be explained by a cell cycle progression arrest or cell death. By phase contrast microscopy and Giemsa staining it was detected the appearance of membrane blebbings and chromatin condensation, features of the apoptotic process, for the highest concentration of extract, G or mixture of isoflavones. These results are consistent with studies that reported that G induced apoptosis in various cancer cell lines (Banerjee et al. 2008; Gu et al. 2009) by activation of caspases via several pathways, namely by up-regulation of Bax/Bcl2. Though, it cannot be forgotten that MCF-7aro cells do not contain caspase-3 and other mechanisms can be involved. It is generally accepted that G can cause G_2/M cell cycle arrest in breast cancer that was explained by the reduction in the expression of cyclin B, which plays a crucial role in the positive regulation of CDK activity (Banerjee et al. 2008). However, other report showed that this isoflavone could also arrest cell cycle in G_0/G_1 phase (Kuzumaki et al.1998). Interestingly, our results demonstrated that while the extract induced cell cycle arrest in G_2/M phase, G caused an arrest in G_0/G_1 phase. This different behaviour may be explained by the different concentrations used. These effects were more prominent at 48 h for the extract, though preliminary results, for the pure compound were more significant at 24 h. The extract contained 1 μM of G whereas the pure compound was used at 10 and 25 μM. This dual effect should be further explored.

Giemsa and acridine orange staining showed that treated cells with extract, G or mixture of isoflavones presented an increase in acidic vesicular organelles. These type of structures can be due to the occurrence of an autophagic process responsible for cell survival or, when exacerbated, for cell death.

In summary, this study showed that, on contrary to the biotransformed soya extract, G presented only a moderate anti-aromatase activity and D did not have any activity. The latter isoflavone only induced a significant decrease in cell proliferation. It was also demonstrated that the extract and G reduced cell viability and proliferation. These effects can be explained by cell cycle arrest and/or cell death. Surprisingly, while the extract induced cell cycle arrest in G_2/M, G caused only an arrest in G_0/G_1. This dual effect may be due to the difference in concentrations used in relation to G, though further studies are necessary to confirm this hypothesis. In addition, the type of cell death caused by G and the extract are in accordance to an apoptotic and/or autophagic cell death. Future studies will be needed to assess if autophagy is associated to apoptosis, because it is known that apoptosis and autophagy are not mutually exclusive pathways. In fact they have been shown to act in synergy and also to counter each other. Moreover**,** although genistein seems to play a key role on proliferation and viability of ER-positive breast cancer cells, it must be considered that soya biotransformed extract is a natural product that may contain other bioactive molecules responsible by the potent anti-aromatase activity and may also contribute to the other cellular effects observed. Further studies about genistein and other potential factors must be performed in order to find out exactly, what is responsible for all soya properties and if it can be used in the prevention and/or treatment of breast cancer.

6. ABREVIATIONS

17β-HSD	17β-hydroxysteroid dehydrogenase
AI	aromatase inhibitors
AO	acridine orange
AP-1	activator protein 1
AVO	acidic vesicular organelle
CYP	cytochrome P
D	daidzein
DBD	DNA-binding domain
DMSO	dimethylsulfoxide
E_1	estrone
E_2	17β-estradiol
E_3	estriol
EDTA	ethylenediaminetetracetic acid
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen responsive element
FBS	fetal bovine serum
G	genistein
LDH	lactate dehydrogenase
LDL	low density lipoprotein
MEM	minimum essential medium
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
PGE	prostaglandine E
SP-1	specificity protein 1
TNF	tumor necrosis factor
SBE	soya biotransformed extract
SERD	selective estrogen receptor down regulator
SERM	selective estrogen receptor modulator
T	testosterone

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Soya isoflavones, genistein and daidzein: Biological effects on a ER–positive breast cancer cell line.

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Introduction

In recent years, the relationship between soya foods and breast cancer has become controversial due to the oestrogen-like properties of soya-derived isoflavones. In contrast, epidemiological studies suggested that there is a low incidence of breast cancer in countries with high soya intake, and Japanese breast cancer patients, in comparison to Western women, exhibit better survival rates. Soya contains the isoflavones **daidzein** and **genistein**, which are responsible for the protective properties, although their exact effects have not yet been clarified. Some of them are related to apoptosis and inhibition of cell growth such as reduction of Bcl-2/Bax ratio, activation of caspases and inhibition of tyrosine kinase [1]. **Daidzein** and **genistein** are described as selective estrogen receptor modulators (SERM). They act as estrogen receptor agonists at lower concentrations, but as antagonists at higher concentrations [2].

In this study we evaluated and compared the effects of a **biotransformed soya extract** and standards of the major isoflavones, **genistein** (G) and **daidzein** (D) on the cell viability and proliferation in a ER-positive breast cancer cell line stably transfected with aromatase gene, MCF-7aro. We also evaluated the biological effects of these compounds on MCF-7aro cell morphology.

Materials and Methods

The **soya extract biotransformed** by fungi *Aspergillus awamori* was obtained from the laboratory of Dr. M.J.V. Fonseca and Dr. M.R.T. Toloi of Faculty of Pharmacy of Ribeirão Preto, University of São Paulo in Brazil. The **biotransformed soya extract (SBE)** contained a mixture of two isoflavones, **genistein** and **daidzein** in different concentrations. Standards of genistein and daidzein were obtained from Aldrich Co. (Saint Louis, USA).

For the biological evaluation of **soya extract** in MCF-7aro cell line, three days before the experiments, MCF-7aro cells were cultured in steroid-free medium treated with 5% charcoal–stripped fetal bovine serum (CFBS). After this period, cells were treated with testosterone (1nM) and different concentrations of soya extract (0.11, 0.74, 1.25 and 1.5 µM), standards of genistein and daidzein (1, 5, 10 and 25 µM) and with mixture of both isoflavones (1+1, 5+5, 10+10, 25+25 µM) for 48 hours. MTT assay was used to find out the effect on cell viability. Cell proliferation was evaluated by thymidine incorporation assay and the morphological alterations in MCF-7aro were evaluated by phase contrast microscopy, Giemsa and Hoechst staining .

Results

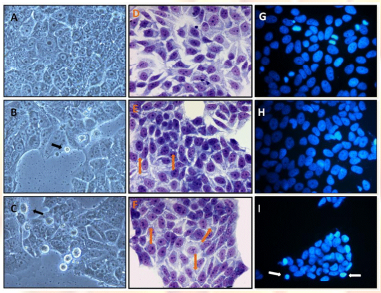

Fig. 1 Effect of *Soya* biotransformed extract on MCF-7aro on cell morphology by phase contrast (A, B, C), Giemsa (D, E, F) and Hoechst (G, H, I) staining. Cultured MCF-7aro cells were examined in the absence (A, D, G) or in the presence of 0,74µM (B, E, H) and 1,5µM (C, F, I) of SBE for 48 hours.

Cell viabilty and proliferation

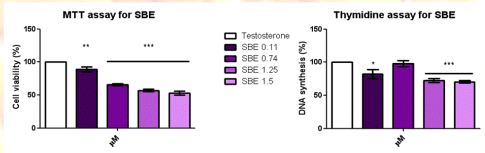

Fig. 2 Effect of SBE on cell viability and proliferation of MCF-7aro cells estimated by MTT and thymidine incorporation assay, respectively. Cells were treated with four concentration of SBE for 48 hours. Cells cultured with testosterone (1nM) were used as control. Significant differences between the control and treated cells are denoted by *(p<0,05), **(p<0,01) and ***(p<0,001).

Cells treated with SBE showed 'blebbings'(black arrows), chromatin condensation (white arrows) and vacuolisation in cytoplasm (orange arrows). SBE induces a decrease in cell viability in dose-dependent manner and reduces cell proliferation at higher concentrations.

Cell viabilty and proliferation

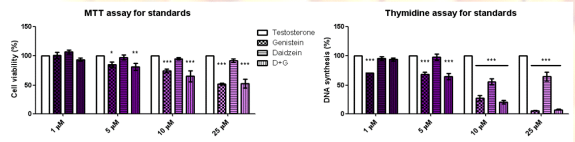

Fig. 3 Effect of isoflavones on cell viability and proliferation of MCF-7aro cells estimated by MTT and thymidine incorporation assay, respectively. Cells were treated with four concentration of G, D or both standards for 48 hours. Cells cultured with testosterone (1nM) were used as control. Significant differences between the control and treated cells are denoted by *(p<0,05), **(p<0,01) and ***(p<0,001).

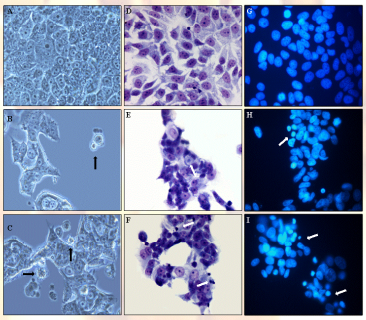

Fig. 4 Effect of soya isoflavones on cell morphology by phase contrast (A, B, C), Giemsa (D, E, F) and Hoechst (G, H, I) staining. Cultured MCF-7aro cells were examined in the absence (A, D, G) or in the presence of 25µM of genistein (B, E, H) or 25 µM of genistein and daidzein(C, F, I) for 48 hours .

Genistein and mixture of genistein and daidzein are able to inhibit viability and proliferation of MCF-7aro cells in dose-dependent manner. Cells treated with G 25µM and G+D (25+25µM) present 'blebbings' (black arrows), chromatin condensation (white arrows) in Giemsa and Hoechst staining.

Conclusion

The soya biotransformed with the fungi *Aspergillus awamori* and isoflavone, genistein, decreased cell viability and inhibited proliferation of MCF-7aro cell line in a dose-dependent manner. Further, morphological alterations, after 48 hours of treatment, as chromatin condensation, DNA fragmentation and a few vacuoles in the cytoplasm, specially for SBE, were observed. A decrease in cell density was observed in cells treated with genistein and mixture of the isolated compounds at the concentration of 25 µM. Interestingly, though with lower content of isoflavones, the SBE was more effective in relation to the reduction at cell viability and proliferation. The effect of daidzein was not significant for the majority of used concentrations. Although, the results suggest that genistein may be responsible for the SBE effects, it cannot be discarded that other substances may be present in SBE. So further studies are required to clarify the effects of SBE in these breast cancer cells.

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