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# Soybean extracts enriched with free isoflavones promote nitric oxide synthesis and affect the proliferation of breast adenocarcinoma cells

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*Article*

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**Abstract:** Although soybean isoflavones naturally accumulate in their conjugated forms, the beneficial effects on human health of soybean-containing foods have been credited to their aglycone forms. In the present study we analyzed the effects of a chemical agent, sodium nitroprusside (SNP), in eliciting the exudation of non-conjugated isoflavones from intact soybean seeds, embrionary axes and cotyledons. The isoflavones in the exudates were determined by high performance liquid chromatography and mass spectrometry. The effect of the exudates on the emission of nitric oxide (NO) and on the proliferation of breast carcinoma cells (MCF-7) was also evaluated. SNP elicitation increased the production of the aglycone forms dose- and time-dependently. Exudates of embrionary axes and cotyledons stimulated NO emission and showed biphasic effects on viability of MCF-7 cells. At lower concentrations both extracts presented proliferative effects (10-25%), and at higher concentrations inhibited (15%) cell proliferation. The biphasic effect might be due to the action of isoflavone aglycones in activating estrogen receptors which in turn stimulate the production of NO. Overall, the results suggest that soybean extracts enriched in isoflavone aglycones by elicitation with SNP could be exploited as a functional ingredient in the food industry.

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## Introduction

The potential of functional foods to provide health benefits has been increasingly recognized. Epidemiological evidence has supported the proposal that isoflavones are the agents in soybean which account for the apparent association between the increase in soy consumption and the reduction in the incidence of various chronic diseases (Adlercreutz & Mazur, 1997; Brouns, 2002). Several reports have described the positive effect of isoflavones on the prevention of hormone-dependent cancers, cardiovascular diseases, osteoporosis, adverse menopausal manifestations and accelerated cognitive decline (Pilšáková et al., 2010). Other studies have demonstrated a significant correlation between the consumption of isoflavonoid-rich foods by people in East Asia and a lower incidence of breast, prostate and large intestine carcinomas and cardiovascular disease, the so-called "diseases of western countries" (Adlercreutz & Mazur, 1997).

Isoflavones are found as aglycones, β-glucosides and β-glucosides conjugated with malonyl and acetyl acids in nature. Although predominantly found in their conjugated forms (Liu, 1997), several evidences indicate that the free forms of soybean-derived isoflavones (the aglycones daidzein, glycitein and genistein) exhibit the highest biological activities (Brown & Setchell, 2001). These activities have been attributed to their antioxidant action (Yen & Lai, 2003) and structural similarity to the estradiol hormone (Pilšáková et al., 2010). There are claims that, acting as estradiol, isoflavones aglycones may modulate immunological responses, cancer proliferation and prevent cardiovascular diseases and osteoporosis (Messina & Messina, 1991; Brouns, 2002; Taku et al., 2011). At least part of these effects is due to nitric oxide synthase (NOS) modulation by estradiol

(Townsend et al., 2011). In cancer, at low concentrations, NO is proangiogenic and may promote tumor growth; however, at higher concentrations, NO can be cytostatic and cytotoxic, thus inhibiting tumor growth (Jenkins et al., 1995).

Despite the controversial data concerning the bioavailability of isoflavones, it is accepted that only aglycones forms are absorbed in the stomach (Piskula et al., 1999), and are absorbed faster and in greater amounts than their glucoside forms in the intestine (Izumi et al., 2000). In fact, isoflavone absorption was shown to require initial hydrolysis by intestinal β-glucosidases (Setchell et al., 2002). These observations suggest that biotechnological and biochemical processes that would induce the production of aglycones may provide both nutritional and economic benefits in diseases prevention.

Soybean isoflavones are synthesized as a branch of the phenylpropanoid pathway, a secondary biosynthetic route in plants that can be activated in response to a variety of biotic and abiotic stresses (Dixon et al., 2002). Nitric oxide has been identified as a key activator of this pathway by inducing transcriptional activation of genes encoding key enzymes in this route, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (Delledonne et al., 1998). In soybean cotyledons the NO donor sodium nitroprusside (SNP) was shown to induce large accumulation of free isoflavones (Modolo et al., 2002). This response was suggested to result from the stimulatory effect of SNP on the activity of PAL and, additionally, on the B-glicosidases, resulting in the release of pre-formed isoflavone conjugates (Kretzchmar et al., 2009). Taking into account these previous observations, in this study we present an improved technique to increase the yield of free isoflavones in soybean seeds using the NO donor molecule SNP, and evaluate the effects of the exudates produced in the proliferation of breast adenocarcinoma cells via NO production.

## Material and Methods

### *Plant material*

Soybean seeds [*Glycine max* (L.) Merril cv. IAC-18] were provided by Dr. Nelson R. Braga (Instituto Agronômico de Campinas, Campinas, São Paulo, Brazil) and kept at 4 °C until analysis.

### *Elicitation assay and exudates preparation*

Soybean seeds were sterilized with sodium hypochlorite 0.3%, seed coats were removed and detached embrionary axes or cotyledons (50 units/10 mL) were treated with an aqueous solution of sodium nitroprusside (SNP) at several concentrations and for different periods of time, as indicated in the figure legends. After the elicitation time, the samples were filtered and the exudate mixed with 10 mL methanol 80%. They were then extracted for 20 min on a rotary mixer model NT150 (Nova Técnica, Piracicaba, Brazil), dried, solubilized in 500 µL of methanol/formic acid 5% (1:1) and filtered through a 0.22 µm Millipore membrane (Billerica, MA) prior to HPLC analysis.

### *Determination of conjugated and aglycone isoflavones*

Analysis was carried out as previously described by Lin & Harnly (2007) in a HPLC model LC-20AD (Shimadzu, Kyoto, Japan) equipped with quaternary pumps, an on-line degasser, and an injection valve (Rheodyne LCC, Rohnert Park, USA) with a 20 µL loop. The equipment included, connected in series, a photodiode array detector (DAD) SPD-M20A (Shimadzu) and a mass spectrometer with an ion-trap analyzer (MS/MS) Esquire 4000 (Bruker Daltonics, Bremem, Germany) and an electron spray ESI ionization source. The isoflavones were identified according to Lin & Harnly (2007) and quantified using external calibration curves for daidzein and genistein obtained from Sigma-Aldrich Corp. (St. Louis, MO) and glycitein and their β-glucosides (daidzin, genistin and glycitin) from LC Labs (Woburn, MA). The 6"-*O*-malonyl-β-glucosides were quantified using the curve of the corresponding β-glucosides.

### *MCF-7 and 3T3 cell culture*

Human breast adenocarcinoma MCF-7, obtained from American Type Culture Collection (Manassas, USA), and Mouse embryo fibroblast 3T3, from National Institutes of Health (Bethesda, MD), cells were cultured in RPMI-1640 and DMEM medium, respectively, supplemented with FSB 10% and gentamicin 50 µg.mL-1 in a humidified atmosphere containing $CO_2$ 5%, according to specification of American Type Culture Collection. To assess the cell viability, 10 x $10^3$ cells were seeded in 96 well plates, and after 24 or 72 h of treatment with different concentrations of exudates 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, neutral red uptaken and nucleic acid content assays were performed as described *in vitro* cell viability assays section. For treatments, dry aliquots of exudates were solubilized in DMSO 100% and resuspended in culture medium (250 to 1000 µg/mL). Aliquots of 100 µL of these solutions were applied to each well plate containing 10 x $10^3$ cells. In all treatments DMSO concentration was kept at 0.25%.

In vitro *cell viability assays*

MTT reduction

Medium containing isoflavones was removed and 0.2 ml of MTT solution (0.5 mg of MTT/mL of culture medium) was added to each well. After incubation for 4 h at 37 °C, the medium was removed and intracellular formazan was released by solubilization in 0.2 mL of ethanol. Plate was shaken for 5 min on a plate shaker prior to measuring the absorbance at 570 nm (Mosmann, 1983).

Neutral red uptake

After 4 h incubation with serum-free medium containing 50 µg of neutral red/mL the cells were washed quickly with PBS and then 0.1 mL of a solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After shaking for 10 min on a plate shaker the absorbance was read at 540 nm (Borenfreund & Borrero, 1984).

Nucleic acid content

Cell monolayer was solubilized with 1 mL of 0.5 M NaOH at 37 °C for 1 h afterwards the absorbance of the solution in each well was measured at 260 nm and the results expressed as a percentage of the control $A_{260}$ (Cingi et al., 1991).

*Cell NO emission*

NO emission was determined using the DAF-2 fluorescent probe, as described by Hamuro et al. (2002). Briefly, the cells submitted to treatments were incubated in culture medium containing DAF-2 2.5 µmol.L$^{-1}$ for 1h at 37 °C. Culture medium was then collected and the fluorescence measured in a F-450 spectrofluorometer (Hitachi Instrument Co., Tokyo, Japan) at wavelengths of excitation at 488 nm and of emission at 530 nm. When indicated, L-NAME 1 mmol.L$^{-1}$ was added to culture medium to prevent NO production by NOS (Baylis et al., 1995).

*Statistical analysis*

Comparison of means was performed by two-way analysis of variance (ANOVA) followed by Tukey's test on Bioestat 5.0 software (Instituto de Desenvolvimento Sustentável Mamirauá, Tefé, Brazil).

## Results and Discussion

*Enriching the content of free isoflavones in soybean extracts*

Given the well-established pharmaco-therapeutic activity of free isoflavones, a methodology that is simple and efficient for the production of these compounds could be a great tool for the development of new products of commercial interest. To increase the proportion of the aglycone forms of isoflavones, soybean seeds were treated with the inducing agent sodium nitroprusside (SNP) which was previously proven to be very effective in activating the production of free isoflavones in cotyledons of soybean seedlings (Modolo et al., 2002; Kretzschmar et al., 2009). The

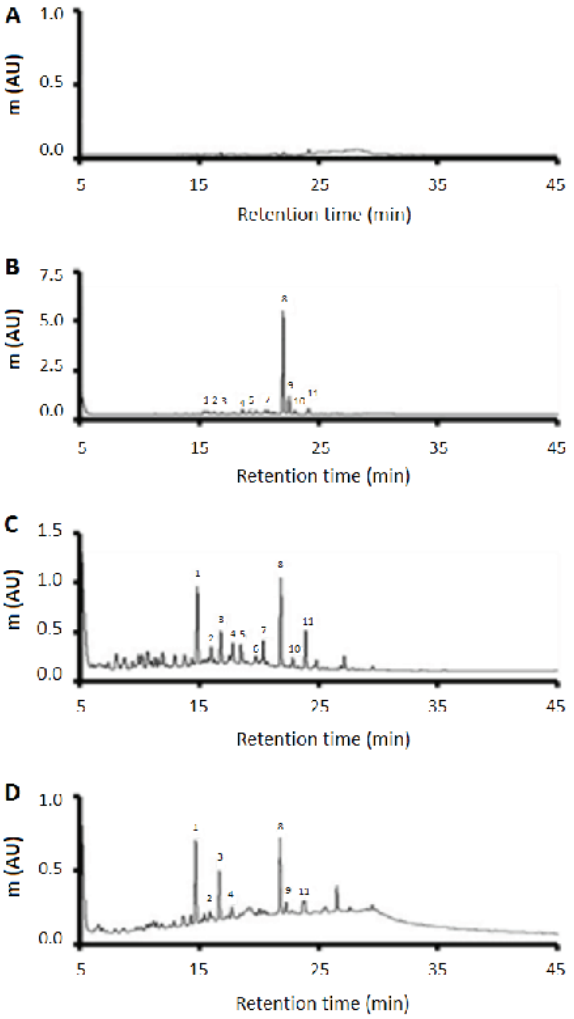


**Figure 1.** Chromatographic profile of isoflavones present in exudates of soybean seeds after treatment with 10 mM sodium nitroprusside (SNP) for 15 h. Extract of (A) untreated seeds and treated (B) embrionary axes, (C) cotyledons and (d) seeds. The identification of the numbered compounds is shown in Table 1.

efficacy of this treatment was then evaluated in intact and non-germinated seeds, as well as in their two main components (embrionary axis and cotyledon). To perform this evaluation, we compared the results of the identification and quantification by HPLC-DAD-MS of the main isoflavones produced. Figure 1 shows the chromatographic profile, and Table 1 shows the amounts of isoflavones identified in the exudates when intact soybean seeds, embrionary axes or cotyledons were treated with 10 mM SNP for 15 h.

To increase the reliability of the isoflavone identification in the samples and to assist in the identification of compounds β-conjugated to sugars and acids, the peaks detected by HPLC were also subjected to tandem mass spectrometry analysis and the results were consistent with the previously reported fragmentation profile of soybean isoflavones (Cuyckens & Claeys, 2004).

Figure 1 shows that after treatment with SNP, large amounts of isoflavones accumulated in the exudates of embrionary axes, cotyledons and seeds. The production of these compounds in the control was insignificant, thus eliminating the possibility that the imbibition of the seeds or their parts with water or the mechanical stress (shaking) on the tissues could account for the results observed. As shown in Table 1, the exudate of embrionary axes presented the best yield in terms of total isoflavone production (682±59.7 μg of isoflavone per g of dried exudate), when compared to that of cotyledons (101±13.0 $\mu g.g^{-1}$) and seeds (33±2.1 $\mu g.g^{-1}$). Additionally, it is remarkable that the treatment induced the exudation of a high proportion of aglycone forms (daidzein, genistein and glycitein) and much lower amounts of the glycosylated- and malonyl-conjugated forms in all treatments.

Among the aglycone forms, daidzein was the isoflavone that accumulated at the highest levels (approximately 50% of the total aglycone forms) in the exudates of the three SNP-treated tissues. The best induction of genistein was obtained in the exudates of elicited embrionary axes (32±3.5 $\mu g.g$ fresh $exudate^{-1}$).

The kinetics of isoflavonoid production as a function of the elicitation time and SNP concentration were evaluated in the exudates of embrionary axes. The production of isoflavones increased with incubation time (Figure 2A) and SNP concentration (Figure 2B), and the largest amount of aglycones was accumulated after 20 h of treatment with 200 $mmol.L^{-1}$of SNP. However, with this treatment there is an increase in the relative proportion of the conjugated forms, when compared with the samples treated with 100 $mmol.L^{-1}$ SNP (Figure 2C). As the relative levels of aglycones are important for our purpose, elicitation with 100 $mmol.L^{-1}$ SNP for 20 h produced the best response in terms of aglycone forms.

As observed with embrionary axes, the production of isoflavones was also stimulated as a function of SNP concentration and elicitation time in cotyledons. When cotyledons were exposed to 100 $mmol.L^{-1}$ SNP for 20 h total production of aglycones was 1,062±12 $\mu g.g^{-1}$, where 44% corresponded to daidzein, 44% to genistein and 12% to glycitein, indicating that this treatment can be used for the production of large amounts of genistein. It is important to mention that the analysis by HPLC and mass spectrometry did not reveal the presence of SNP or any degradation product of the

**Table 1.** Chromatographic parameters and concentrations of isoflavones identified in exudates of soybean seeds, cotyledons and embrionary axes treated with 10 mM sodium nitroprusside for 15 h.

| Peak | Compound | $R_t$ (min)[b] | $\lambda_{max}$ (nm)[c] | Concentration (μg.g dry $weight^{-1}$) | | |
|---|---|---|---|---|---|---|
| | | | | Embryo* | Cotyledon | Seed |
| 1 | not identified | 13.2 | 279, 308 | n.d. | n.d. | n.d. |
| 2 | daidzin | 14.1 | 260, 300 | 13±1.1[a] | 8±3.5[b] | 3±0.1[c] |
| 3 | glycitin | 15.1 | 260, 317 | 15±1.3[a] | 13±3.1[a] | 13±1.3[a] |
| 4 | genistin | 16.3 | 260, 330 | 10±1.7[a] | 5±0.4[b] | 3±0.1[c] |
| 5 | malonyl-daidzin[a] | 17.4 | 260, 300 | 60±1.5[a] | 1±0.1[b] | n.d. |
| 6 | malonyl-glycitin[a] | 18.3 | 262, 318 | 45±1.8[a] | 7±0.4[b] | n.d. |
| 7 | malonyl-genistin[a] | 19.2 | 261, 330 | 54±0.8[a] | 6±0.3[b] | n.d. |
| 8 | daidzein | 20.1 | 260, 302 | 243±37[a] | 29±1.3[b] | 8±0.2[c] |
| 9 | glycitein | 21.0 | 260, 319 | 210±11[a] | 14±2.2[b] | 3±0.1[c] |
| 10 | not identified | 21.7 | 288, 335 | – | | |
| 11 | genistein | 22.2 | 262, 330 | 32±3.5[a] | 18±1.7[b] | 3±0.4[c] |

[a]Concentrations were estimated using the calibration curves of compounds 2, 3 and 4; n.d.: not detected; $R_t$: retention time; [b]Eluate in the C18 column in oven at 32 ºC; [c]Linear gradient of methanol/5% formic acid. *Embrionary axis. Data are in μg isoflavone per g dry $weight\ extract^{-1}$. Samples were treated with 10 mM of SNP for 15 h (50 units/10 mL) and the exudates extracted for 20 min with 80% methanol and analyzed by HPLC-DAD-MS. Mean±SD of three independent analyses; different letters in the same line are statistically different ($p$<0.01).

elicitor agent in the exudates (results not shown).

In general, these results show that exudates from soybean tissues, mainly those of the embrionary axes, demonstrate good yields of aglycones when elicited by SNP.

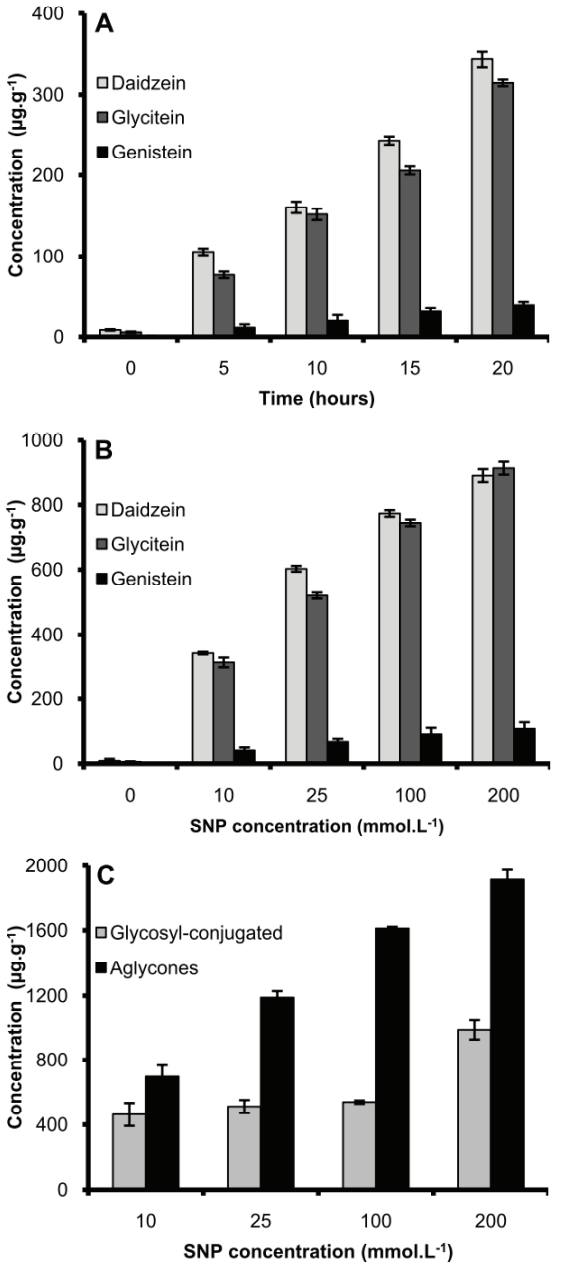


**Figure 2.** Effect of SNP concentration and time of elicitation in the accumulation of free and conjugated isoflavones. Embrionary axes were treated with SNP (50 units/10 mL) and the isoflavones produced determined in the exudates. (A) concentration of aglycones after the indicated periods of elicitation with SNP 10 mmol.$L^{-1}$; (B) concentration of aglycones and (C) aglycone and glycosyl-conjugates in response to different concentrations of SNP, as indicated, after 20 h of elicitation. Mean±SD of three independent experiments.

*Influence of soybean exudates in MCF-7 and 3T3 cells proliferation*

The antiproliferative activity of isoflavones has been well documented in various human tumor cell lines (Gacche et al., 2011). As the soybean exudates prepared by elicitation with 100 mmol.$L^{-1}$ SNP for 20 h produced the best response in terms of aglycone forms (Figure 2), their effects on MCF-7 and 3T3 cell lines were evaluated and compared with those of non-elicited extracts.

When MCF-7 cells were exposed to exudates from elicited embrionary axes (Figure 3A) and cotyledons (Figure 3B), a biphasic effect on cell proliferation was observed. The major effect in the cell viability was observed after treating the cells with 500 µg.$mL^{-1}$ of exudates from embrionary axes and cotyledons, which provoked an increase of approximately 20% of proliferation. When higher concentrations of those exudates were used, a tendency of cell proliferation decrease was observed (Figure 3A and B). In contrast, MCF-7 cells exposed to exudates of non-elicited embrionary axes (Figure 3C) and cotyledons (Figure 3D) showed a slight dose-dependent viability increase. For both exudates, the maximum observed effect in the viability was around 15% after exposure of cells to 1000 µg.$mL^{-1}$. These results show that soybean extracts enriched in free isoflavones show a biphasic effect on proliferation of MCF-7 cells. Differently, extracts from non-elicited tissues, which have low concentrations of aglycone isoflavones generally displayed a slightly effect on cell proliferation. Thus, the difference of action in cell viability between non-elicited and elicited exudates may be related to the content of free isoflavone aglycones.

Since MCF-7 cell line expresses estradiol receptor (Saceda et al 1988) it could explain the higher proliferation capacity of this cell towards lower isoflavones concentration. The proliferative effect of flavonoids has been attributed to isoflavones, especially genistein. This isoflavone is able to bind to the estrogen receptor, due to its structural similarity with estradiol, and promote cell growth (Wang et al., 1996). Exudates from elicited embrionary axes presented higher amounts of daidzein and glycitein that have lower estrogenic activity while those from elicited cotyledons presented higher content of the more potent genistein, what may explain their similar effectiveness towards MCF-7 cell proliferation.

The proliferative action of isoflavones was reported in low concentration of estradiol (Casanova et al. 1999). However, it has been reported their antiproliferative effect on tumor cells at high concentrations and this action seems to involve a receptor-independent pathway (Gacche et al., 2011). In agreement, mouse fibroblast cells (3T3) treated with exudates of elicited embrionary axes and cotyledons

did not display an expressive changing in their viability (results not shown).

*Emission of NO by MCF-7 and 3T3 treated with soybean exudates*

NO emission induced by treatment with soybean exudates enriched in isoflavone aglycones was evaluated in MCF-7 and 3T3 cells, since this radical is extensively involved in various cellular signals, including induction of cell death in tumor lines (Olson & Garbán, 2008).

The modulation of eNOS by estrogen has been demonstrated (Hisamoto & Bender, 2005). The eNOS is part of a family of enzymes responsible for synthesis of NO through the oxidation of L-arginine to L-citrulline (Pollock et al., 1991).

Figure 4 shows that MCF-7 cells, treated with exudates of elicited embrionary axes and cotyledons for 72 h, displayed emission of NO, which maximum effect observed at concentration of 1000 μg.mL$^{-1}$ exudates (39 and 25% for embryonic axis-elicited and cotyledons-elicited exudates, respectively). Interestingly, treatment of 3T3 cells with both exudates up to 1000 μg.mL$^{-1}$ did not induce significant changes in the emission of NO after 72 h. Accordingly, when MCF-7 cells were treated with exudates elicited up to a concentration of 1000 μg.mL$^{-1}$ and L-NAME, a selective NOS inhibitor (Baylis et al., 1995), the emission of NO was abolished.

Several evidences suggest the importance of NO in the development and establishment of cancer (Bing et al., 2001). However, other authors have reported that in high concentration NO presents cytotoxic effect and contribute for the reduction of tumor mass (Felly-Bosco, 1998). Our results are consistent with these observations, since at lower NO emission by tumor MCF-7 cells coincided with the proliferative effect of the exudates. However, when the emission of NO was higher, it was observed a tendency of cell proliferation inhibition. Our data suggest that the biphasic effect of exudates could be, at least in part, due to modulation of eNOS activity in MCF-7 cells.

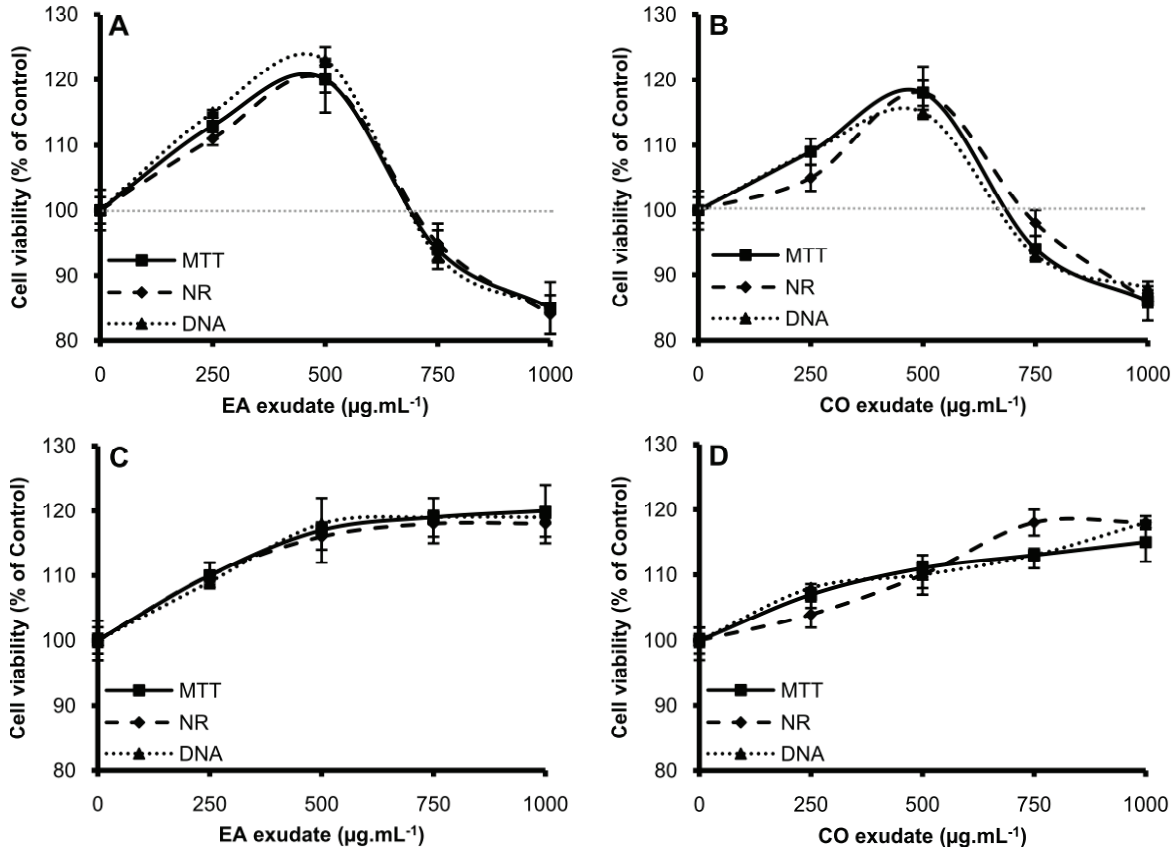


**Figure 3.** Effects of exudates in natura and elicited with SNP (100 mmol.L$^{-1}$) on MCF-7 cell viability. A, exudate of elicited embrionary axes (EA); B, exudate of elicited cotyledons (CO); C, exudate EA in natura; D, exudate CO in natura. Cells (10 x 10$^3$) were exposed to different exudates concentrations for 72 h. The results are expressed as percentage of control. Cellular viability was determined by MTT reduction, neutral red uptake (NR) and DNA content assay. Mean±SD of three independent experiments.

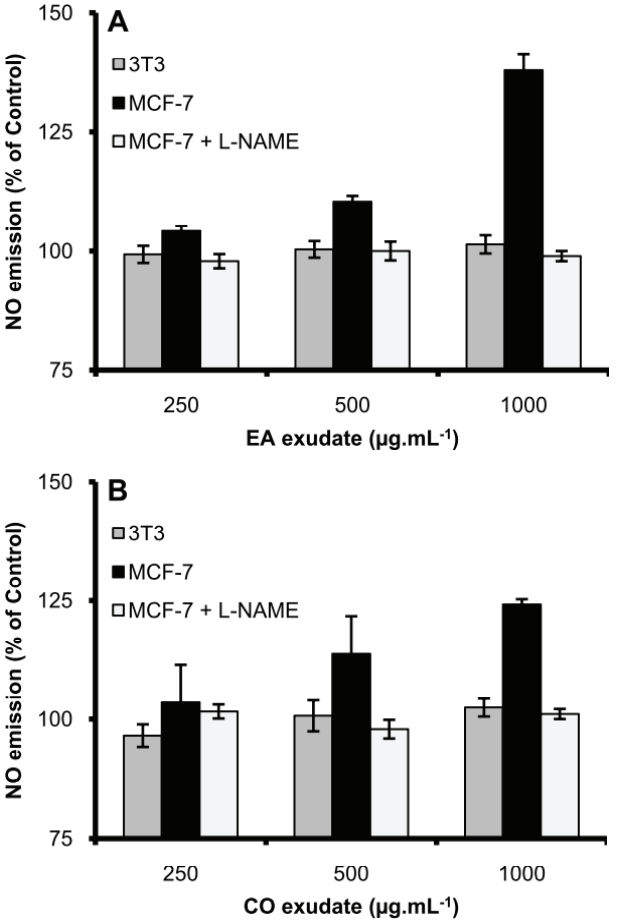


**Figure 4.** Nitric oxide (NO) emission by 3T3 and MCF-7 treated with SNP 100 mmol.L$^{-1}$ elicited exudates. A, embrionary axes (EA) exudates; B, cotyledons (CO) exudates. Cells (10 x 10$^3$) were exposed to different exudates concentrations for 72 h. L-NAME was used at 1 mM, where indicated. Mean±SD of three independent experiments.

## Conclusion

The results presented here demonstrate a rapid and easy method to enrich soybean extracts with isoflavones in their bioactive forms, the aglycones. Still, the enriched extracts show biphasic action on the proliferation of breast adenocarcinoma cells with proliferative action at lower concentrations and inhibitory effect at higher concentrations. It is likely that these effects are related to estrogenic activity of free isoflavones and their ability to modulate the activity of eNOS, and thus NO synthesis. Overall, our results suggest that elicitation with SNP could represent a simple method to produce exudates enriched in free isoflavones and could be exploited to produce functional foods or supplements to target health problems.

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