Bowman−Birk Inhibitor and Genistein among Soy Compounds That Synergistically Inhibit Nitric Oxide and Prostaglandin E₂ Pathways in Lipopolysaccharide-Induced Macrophages

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Inflammation has an important role in the development of chronic diseases. In this study, we evaluated the anti-inflammatory properties of eight soybean bioactive compounds using lipopolysaccharide-induced RAW 264.7 macrophages. Genistein, daidzein, a mix of isoflavone glucosides, saponin A group glycosides (saponin A), saponin B group glycosides (saponin B), sapogenol B, Bowman−Birk inhibitor (BBI), lunasin, and pepsin−pancreatin glycinin hydrolysates were tested by measuring their ability to inhibit cyclooxygenase-2/prostaglandin E₂ (PGE_2) and inducible nitric oxide synthase (iNOS)/ nitric oxide (NO) inflammatory pathways. Of the eight soy bioactive compounds (SBCs) tested, BBI and sapogenol B resulted in the highest inhibition of pro-inflammatory responses at a concentration 10 times lower than the one used for the other compounds. Also, sapogenol B and genistein (molar ratio 1:1) synergistically inhibited NO and additively inhibited PGE_2. Saponin A group glycosides showed inhibition of the iNOS/NO pathway only, while pepsin−pancreatin glycinin hydrolysates enhanced induction and production of the four inflammatory responses. For the first time, synergistic interactions were observed between BBI and genistein inhibiting NO (92.7%) and PGE_2 (95.6%) production. An antagonistic interaction was observed between the saponin B group glycosides and sapogenol B. All interactions were further confirmed by isobolographic analysis. These findings demonstrated that some SBCs possess anti-inflammatory properties and therefore are important in modulating mammalian inflammation pathways which may lead to inhibition of some types of chronic disease. Furthermore, through their interaction they can modulate the inflammatory process.

KEYWORDS: Inflammation; synergistic interaction; antagonistic interaction; isobolographic analysis; NO/ iNOS; PGE_2/COX-2; soybean bioactive compounds

INTRODUCTION

Soybean (*Glycine max*) is an ancient legume traditionally used in the preparation of fermented and nonfermented foods and a staple dietary component among Asian populations (*1*). Substantial epidemiological evidence suggests that Asian populations consuming a high amount of soybean foods have a lower risk of certain chronic diseases such as cardiovascular disease and cancer (*2−5*). This functional property of soybean is attributed to biologically active components, either natural or derived by fermentation or enzymatic hydrolysis. Soybean contains several biologically active compounds such as isoflavones, saponins, peptides, and proteins. Genistein is the isoflavone present in high concentration in soybean (*6*) and is proposed to be the most biologically active (*7*). The biological properties of isoflavones are associated with its capability to prevent osteoporosis, cancer, and cardiovascular disease (*1, 8*). Soy saponins contain a triterpenoid aglycon (sapogenol A or sapogenol B) with a number of different carbohydrate moieties which are linked through an ether linkage at one or more glycosylation sites (*9*). Mechanistic studies indicate that saponins regulate apoptosis pathways leading to programmed cell death of cancer cells (*10, 11*). Soybean also contains peptides and proteins that possess certain biological activities such as Bowman−Birk inhibitor (BBI), Kunitz inhibitor, and lunasin. Furthermore, fermentation and enzymatic hydrolysis of soybean proteins result in the generation of small biologically active peptides (*12, 13*). Soybean peptides have shown interesting biological activities such as suppression of cholesterol absorption (*14, 15*), weight management (*16*), anticancer (*17*) and antihypertensive (*18*) properties, and immunostimulation (*19*).

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Inflammation is part of the host response to either internal or external stimuli (*20*). It participates importantly in host defenses against infectious agents and injury, but it also contributes to the pathophysiology of many chronic diseases (*21*). It acts as an adaptive host defense against infection or injury; however, inadequate resolution of inflammatory responses often may lead to various chronic ailments including cancer (*22, 23*). Several studies have shown that organ-specific carcinogenesis is partly associated with a persistent local inflammatory state (*24−27*). Macrophages are ubiquitous cells that are involved in various physiological processes such as pathogen destruction, inflammation, tissue repair, and remodeling (*28*). When stimulated with lipopolysaccharide (LPS), they can secrete pro-inflammatory responses such as prostaglandin E_2 (PGE$_2$) and nitric oxide (NO), which in turn can cause sepsis, sepsis shock, and systemic inflammatory response syndrome (*29*). Nitric oxide is a molecule that plays a key role in the pathogenesis of inflammation and is considered as a pro-inflammatory mediator (*30*). It is synthesized from the amino acid arginine by nitric oxide synthase and induces tissue injury at the inflammatory site (*31*). Also, it has been shown that NO plays an important role in inflammation-associated carcinogenesis by direct modification of DNA and inactivation of DNA repair enzymes (*32*). On the other hand, an elevated prostaglandin level is exhibited in many human cancers due to upregulation of cyclooxygenase-2 (COX-2) (*33*). PGE$_2$ promotes cell proliferation and favors tumor growth by inhibiting cell death (*34*). Also, some studies demonstrated that PGE$_2$ is capable of promoting mouse skin and colon carcinogenesis (*35, 36*).

The objective of this study was to evaluate in vitro the anti-inflammatory properties of soy bioactive compounds (SBCs) and their interactions by determining the inhibitory effect of isoflavones, saponins, and peptides on the production of nitric oxide and prostaglandin E_2 and induction of cyclooxygenase-2 and inducible nitric oxide synthase by lipopolysaccharide-stimulated RAW 264.7 macrophages.

MATERIALS AND METHODS

Materials. Soybean isoflavones (genistein aglycon, daidzein aglycon, and a mix of soy isoflavone glycosides) and saponins (saponin A group glycosides, saponin B group glycosides, and sapogenol B aglycon) were isolated in our laboratory from soybean and soybean extracts with >95% purity (*37, 38*). Bowman−Birk inhibitor (80% purity) was purchased from Sigma (St. Louis, MO). Pepsin−pancreatin glycinin hydrolysate was prepared in our laboratory following a previously reported protocol (*39*). Lunasin was purified following the procedure by Dia et al. (*40*). The macrophage RAW 264.7 cell line and Dulbecco's modified Eagle's medium (DMEM) with L-glutamine were purchased from the American Type Culture Collection (Manassas, VA). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY). Sodium nitrite, sulfanilamide, *N*-1-(naphthyl)ethylenediamine dihydrochloride, and LPS from *Escherichia coli* O55:B5 were purchased from Sigma (St. Louis, MO). COX-2 and inducible nitric oxide synthase (iNOS) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antimouse IgG horseradish peroxidase secondary antibody was purchased from GE Healthcare (Buckinghamshire, U.K.).

Cell Culture, Treatment, and Cell Viability. A macrophage cell line, RAW 264.7, was cultured in growth medium containing DMEM, 1% penicillin/streptomycin, 1% sodium pyruvate, and 10% fetal bovine serum at 37 °C in 5% CO$_2$/95% air.

Cell treatment was conducted by seeding 2×10^5 cells in a six-well plate, and the cells were allowed to grow for 48 h at 37 °C in 5% CO$_2$/95% air. The cells were treated with SBCs (1−300 μM) and 1 μg/mL LPS for 24 h. **Table 1** presents the combinations and respective concentrations of SBCs tested for potential interactions, and **Figure 1** presents their chemical structures. The ratio of SBCs tested was

Table 1. Combinations of Purified Soy Bioactive Compounds and Their Respective Concentrations[a]

combination	concn, μM
BBI/genistein	18/15
BBI/sapogenol B	18/15
BBI/saponin B	18/40
BBI/mix of isoflavones	18/30
lunasin/sapogenol B	2/15
lunasin/BBI	2/18
lunasin/genistein	2/15
lunasin/mix of isoflavones	2/30
lunasin/saponin B	2/40
sapogenol B/genistein	15/15
saponin B/genistein	40/15
saponin B/sapogenol B	40/15

[a] BBI = Bowman−Birk inhibitor.

approximately equal to the relative weight at which these compounds occur naturally in soybean (i.e., 1.2:1 molar ratio for BBI/genistein).

Cell viability was determined using the CellTiter 96 AQueous One Solution proliferation assay using the novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES) (Promega Corp., Madison, WI). Briefly 5×10^4 cells were seeded in a 96-well plate, and the total volume was adjusted to 200 μL with growth medium. The cells were allowed to grow for 24 h at 37 °C in 5% CO$_2$/95% air. After treatment, the growth medium was replaced by 100 μL of fresh growth medium, and 20 μL of MTS/PES was added to each well. The plate was incubated for 2 h at 37 °C, and the absorbance was read at 515 nm. The percentage of viable cells was calculated with respect to cells treated with phosphate-buffered saline (BBI and glycinin hydrolysates), 0.3% DMSO (isoflavones), and 0.3% 1:1 DMSO/methanol (saponins) and their combinations. Solvents used at these concentrations showed no cytotoxicity to the cells.

Nitrite Measurement. Nitrite was measured as an indicator of NO production after 24 h of treatment and LPS induction. A 100 μL sample of the culture supernatant was plated in a 96-well plate, and an equal amount of Griess reagent (1% sulfanilamide and 0.1% *N*-1-(naphthyl)ethylenediamine dihydrochloride in 2.5% H$_3$PO$_4$) was added. The plate was then incubated for 5 min and the absorbance measured at 550 nm. The amount of NO was calculated using the sodium nitrite standard curve ($y = 0.1402x + 0.04$, $R^2 = 0.99$).

Prostaglandin E$_2$ Measurement. After 24 h of treatment and LPS induction, the culture supernatant was collected. Prostaglandin E$_2$ (PGE-2) was measured using a PGE$_2$ EIA monoclonal kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Briefly, 50 μL of diluted cell supernatant was plated in a 96-well goat antimouse IgG coated plate and incubated for 18 h at 4 °C. After incubation, the plate was washed using the provided wash buffer, and the color was developed by adding 200 μL of Ellman's reagent and shaking the plate for 60−90 min in the dark. The amount of prostaglandin E$_2$ was calculated using the PGE$_2$ standard curve ($y = -37.285x + 108.45$, $R^2 = 0.98$).

Interaction Study and Isobolographic Analysis. Possible interactions between SBCs on inhibiting NO and PGE$_2$ production were investigated. Calculated and experimental inhibition values by various combinations were compared to determine the interactions. Experimental values that were significantly different from the calculated value at the 95% confidence interval were defined as synergistic or "more than additive" interactions (*41*). To further understand the interactions, isobolographic analysis was performed (*41*). IC$_{35}$ values for these compounds were used in constructing the isobolograms. A straight line connecting the IC$_{35}$ values of different SBCs predicted the IC$_{35}$ value of an additive effect. When combination of these compounds deviated significantly to the left of the additivity line with a 95% confidence interval, the interaction was confirmed as synergistic, when it deviated to the right, the interaction was antagonistic, and when it fell within the confidence limit for the additivity line, the interaction was considered to be additive (*41*). The IC$_{35}$ values were calculated by linear regression using Prism 4.0 (GraphPad Software, San Diego, CA).
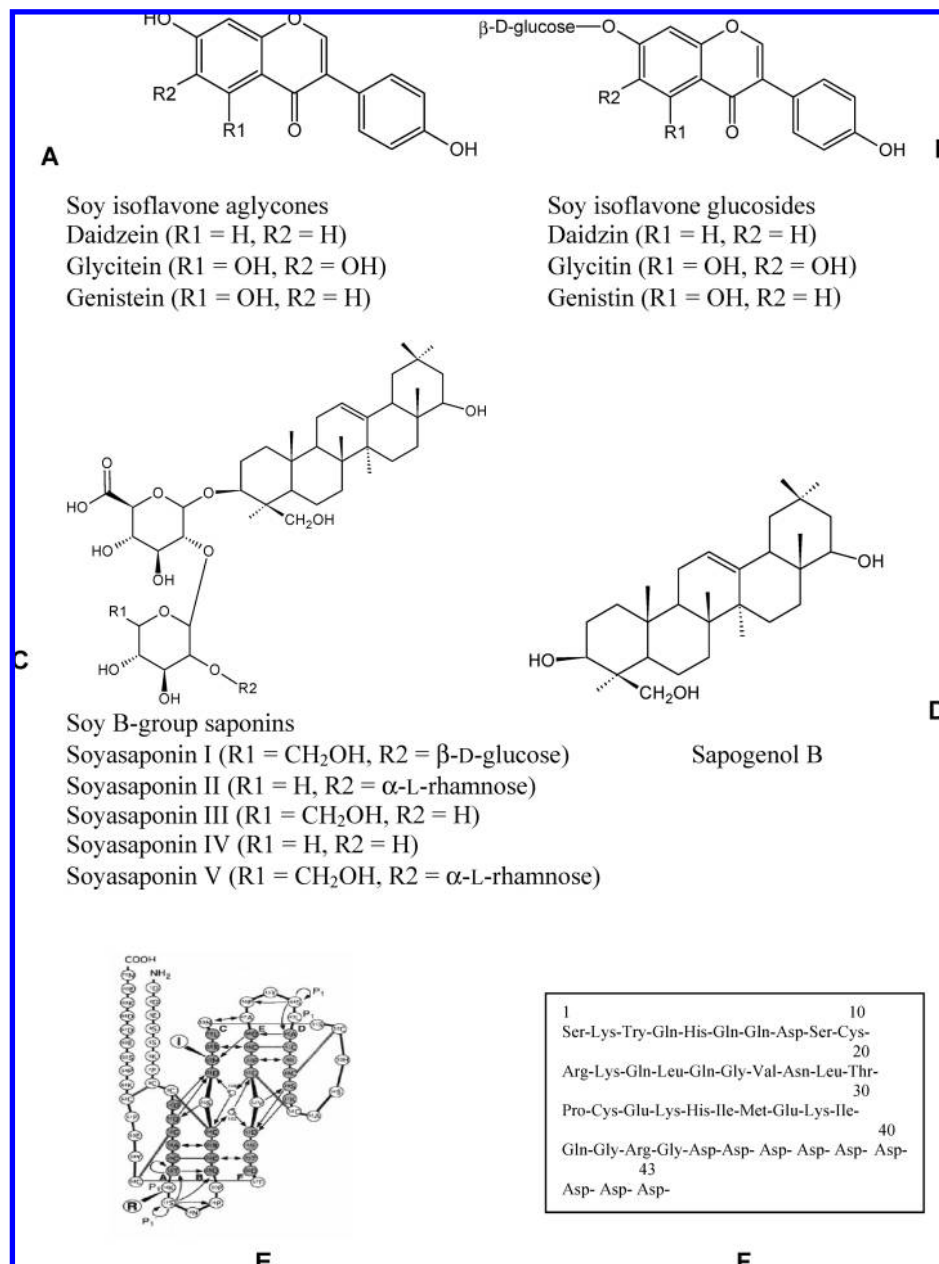
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Figure 1. Chemical structures of (**A**) soy isoflavone aglycons, (**B**) soy isoflavone glucosides, (**C**) soy B group saponins, (**D**) sapogenol B, (**E**) BBI (*65*), and (**F**) lunasin (*17*).

Measurement of iNOS and COX-2 Protein Expressions. iNOS and COX-2 enzyme expressions were determined in cell lysates. Briefly, treated cells were washed with ice cold DMEM and ice cold phosphate-buffered saline before treatment with 200 μL of Laemmli buffer (Biorad Laboratories, Hercules, CA) with 5% β-mercaptoethanol as the lysing buffer. The cell lysates were boiled for 5 min, and approximately 25 μg of protein was loaded in 4−20% Tris−HCl ready gels (Biorad Laboratories, Hercules, CA) for protein separation. Separated proteins were transferred to a PVDF membrane and blocked with 5% nonfat dry milk in 0.1% Tris-buffered saline Tween 20 (TBST) for 1 h at 4 °C. The membranes were washed with 0.1% TBST (five times, 5 min each) and incubated with either COX-2 or iNOS mouse monoclonal antibody (1:1000) at 4 °C overnight. The membrane was washed again and incubated with antimouse IgG horseradish peroxidase conjugate secondary antibody for 3−4 h at room temperature. After incubation and repeated washings, the expression of COX-2 and iNOS was visualized using chemiluminescent reagent (GE Healthcare, Bucking-hamshire, U.K.) following the manufacturer's instructions. The membrane picture was taken with a Kodak Image Station 440 CF (Eastman Kodak Co., New Haven, CT).

Statistical Analysis. Experiments were repeated at least three times with consistent results. Unless otherwise stated, the data are expressed as the mean $\pm$ SD. The data obtained were analyzed using one-way ANOVA to compare experimental to control values, while comparisons between multiple groups were performed using LSD mean separation, and differences were considered significant at $p < 0.05$. The concentration to inhibit 35% (IC_{35}) of NO and PGE_2 production was determined by nonlinear regression (curve fit) using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Cell Viability of RAW 264.7 Macrophages. No effect on the viability of RAW 264.7 macrophages was observed with any of the concentrations of SBCs used in this study, indicating no cellular toxicity (data not shown).

Effect of Different SBCs on NO Production and iNOS Expression. Figure 2 shows the effect of different concentrations of SBCs on nitrite concentrations (indicative of NO
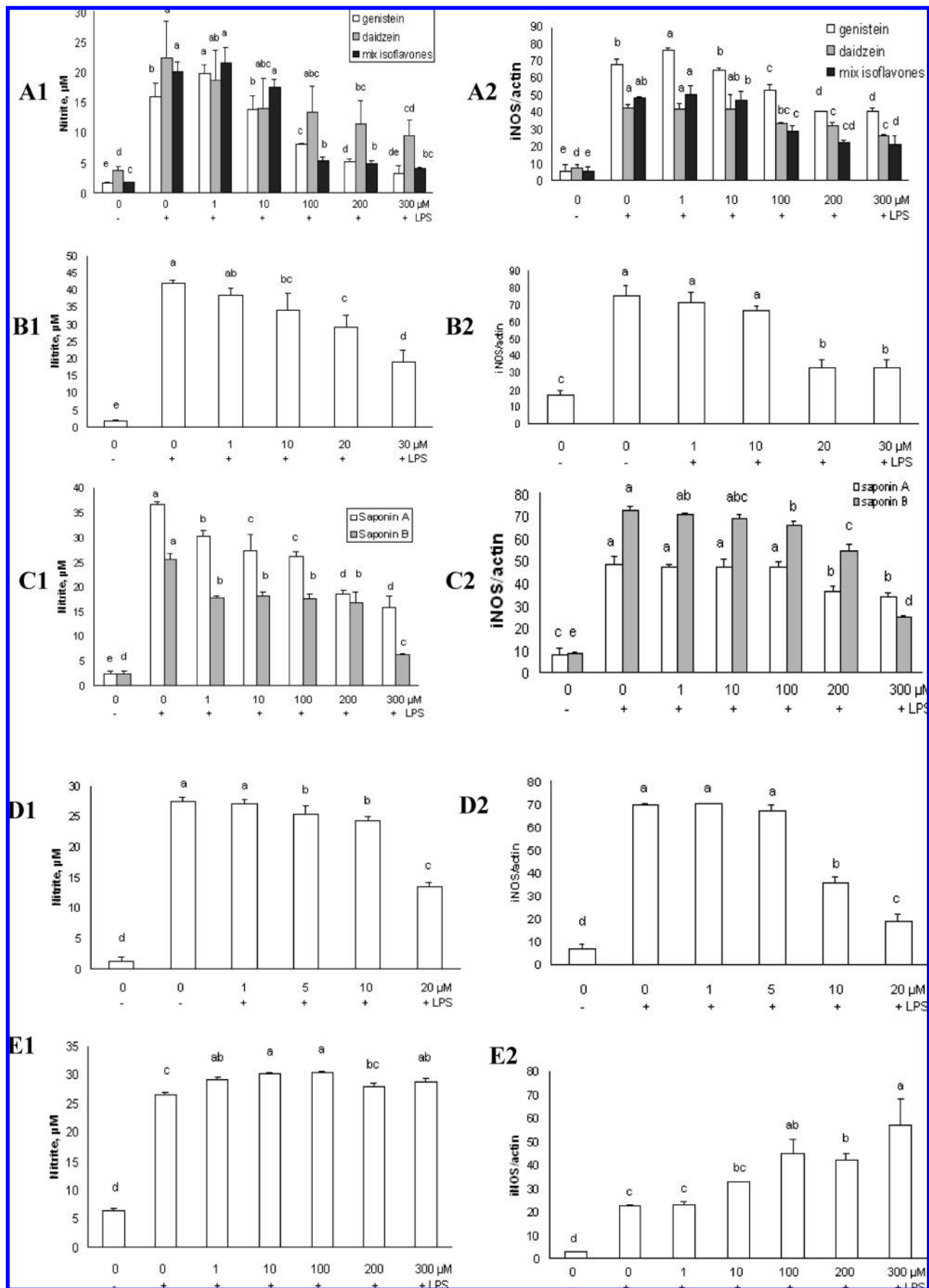
Figure 2. Effect of SBCs on NO (**1**) and iNOS (**2**) in LPS-induced RAW 264.7 macrophages: (**A**) soybean isoflavones, (**B**) sapogenol B, (**C**) soybean saponin glycosides, (**D**) BBI, (**E**) pepsin–pancreatin glycinin hydrolysates. The data represent the mean ± SD from three independent experiments. Different letters indicate significant differences, $p < 0.05$.

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production) (panels **A1−E1**) and iNOS expression (panels **A2−E2**) of LPS-stimulated RAW 264.7 macrophages. After an initial slight increase in NO production, genistein decreased significantly the NO and iNOS levels in a dose-dependent manner. Soy isoflavone glycosides (approximately 84% genistein) and daidzein showed the same behavior, although less marked (**Figure 2A1**). Sapogenol B (**Figure 2B1,B2**) potently inhibited NO production and iNOS followed by saponin B group glycosides and saponin A group glycosides (**Figure 2C1,C2**). BBI, at low concentration (5 μM), showed a significant reduction in NO production and iNOS by LPS-induced RAW 264.7 cells (**Figure 2D1,D2**). On the other hand, pepsin−pancreatin glycinin hydrolysates showed a significant increase in NO production and iNOS expression (**Figure 2E1,E2**).

Different SBCs affected iNOS expression in LPS-induced RAW 264.7 cells. Genistein significantly decreased expression of iNOS at 10 μM, while the other two isoflavones resulted in a significant reduction of iNOS expression at 100 μM. Treatment of LPS-induced RAW 264.7 cells with soybean saponins resulted in a statistically significant reduction of iNOS expression at 200 μM for the saponin A group glycosides, 100 μM for the saponin B group glycosides, and 20 μM for sapogenol B. BBI significantly reduced iNOS expression at 10 μM, while pepsin−pancreatin glycinin hydrolysates resulted in increased expression starting at 100 μM.

Also, it must be noted that lower concentrations of SBCs were needed to inhibit NO production than iNOS expression.

Effect of Different SBCs on PGE$_2$ Production and COX-2 Expression by LPS-Induced RAW 264.7 Cells. Figure 3 shows the effect of different concentrations of SBCs on PGE$_2$ production (panels **A1−E1**) and COX-2 expression (panels **A2−E2**) of RAW 264.7 macrophages induced by LPS. Cells treated with at least 1 μM genistein, daidzein, mix of soy isoflavone glycosides (**Figure 3A1**), sapogenol B (**Figure 3B1**), saponin B group glycosides (**Figure 3C1**), and BBI (**Figure 3D1**) significantly suppressed PGE-2 production when compared to the positive control. At 1 μM, BBI and sapogenol B exhibited the highest inhibition of PGE$_2$ with values of 31.3 ± 1.8% and 31.4 ± 3.3%, respectively. At the same concentration, the mix of soy isoflavone glycosides inhibited 28.3 ± 0.8%, saponin B group glycosides 27.7 ± 5.0%, genistein 27.3 ± 5.0%, and daidzein 21.0 ± 1.6%. On the other hand, saponin A group glycosides had no effect on PGE$_2$ production, indicating that it is a poor inhibitor of COX-2. Also, treatment of RAW 264.7 with 1−300 μM pepsin−pancreatin glycinin hydrolysates resulted in increased production of PGE$_2$ (**Figure 3E1**). COX-2 expression was significantly decreased by genistein and the mix of soy isoflavone glycosides at 100 μM, while daidzein required 300 μM for its significant reduction (**Figure 3A2**). Sapogenol B at 10 μM (**Figure 3B2**) exhibited the most potent inhibition of COX-2 expression among soybean saponins. On the other hand, saponin A group glycosides had no effect on COX-2 expression, while saponin B group glycosides decreased expression of COX-2 at 100 μM (**Figure 3C2**). BBI (5 μM) showed very potent inhibition of COX-2 expression (**Figure 3D2**, while glycinin hydrolyzed by pepsin and pancreatin (300 μM) increased COX-2 expression (**Figure 3E2**).

Table 2 presents the potency of each of the seven SBCs tested for production of NO and PGE$_2$ and expression of iNOS and COX-2. BBI showed the highest inhibitory capacity for NO production with an IC$_{35}$ of 16.2 μM, followed by sapogenol B (16.2 μM), the mix isoflavone glycosides (18.0 μM), genistein (32.9 μM), saponin B group glycosides (97.6 μM), saponin A group glycosides (116.3 μM), and daidzein (137.1 μM). Among

the SBCs tested, BBI showed the highest inhibition of iNOS expression (IC$_{35}$ = 9.1 μM), followed by sapogenol B (15.8 μM), the mix isoflavones glycosides (76.5 μM), genistein (134.8 μM), saponin B group glycosides (191.7 μM), daidzein (286.5 μM), and saponin A glycosides (>300 μM). This trend is similar to that of inhibition of the production of NO, indicating a strong correlation between inhibition of NO production and iNOS expression.

Among the SBCs tested, BBI showed the highest inhibition of COX-2 expression (IC$_{35}$ = 1.3 μM), followed by sapogenol B (5.4 μM), genistein (44.5 μM), saponin B group glycosides (63.3 μM), the mix of isoflavone glycosides (73.6 μM), and daidzein (155.2 μM).

BBI and sapogenol B exhibited the lowest IC$_{35}$ values for each of the parameters, indicating that they are the most potent bioactive compounds present in soybean in preventing inflammation through NO/iNOS and PGE$_2$/COX-2 pathways.

Interaction Study and Isobolographic Analysis. Figures 4 and **5** present the effect of different combinations of SBCs on the production of NO and PGE$_2$, respectively. BBI and genistein combination at a molar ratio of 1.2:1 resulted in an inhibitory synergistic or "more than additive" effect for both NO (92.7 ± 2.1%) and PGE-2 (95.6 ± 0.7%) (**Figures 4A** and **5A**). Isobolographic analysis of this mixture confirmed their synergistic interaction (**Figures 6A** and **7A**). Combinations of BBI (18 μM)/mix of soy isoflavone glucosides (30 μM) (**Figure 4A**), BBI (18 μM)/saponin B group glycosides (40 μM) (**Figure 4B**), genistein aglycon (15 μM)/saponin B group glycosides (40 μM) (**Figure 4E**), and genistein aglycon (15 μM)/sapogenol B aglycon (15 μM) (**Figure 4E**) resulted also in synergistic interactions in inhibiting NO production. These interactions were further confirmed by isobolographic analysis as shown in **Figure 6B−E**.

Additive effects in inhibiting NO production, but not potentiating, synergistic, or antagonistic interactions, were observed for BBI (18 μM)/sapogenol B aglycon (15 μM) (**Figure 4B**), lunasin (2 μM)/genistein aglycon (15 μM) (**Figure 4C**), lunasin (2 μM)/mix of soy isoflavone glucosides (30 μM) (**Figure 4C**), lunasin (2 μM)/saponin B (40 μM), lunasin (2 μM)/sapogenol B aglycon (15 μM) (**Figure 4D**), and lunasin (2 μM)/BBI (18 μM) (**Figure 4F**). On the other hand, combination of saponin B group glycosides (40 μM)/sapogenol B aglycon (15 μM) resulted in the increased production of NO (**Figure 4F**) and PGE$_2$ (**Figure 5F**) by LPS-induced RAW 264.7 macrophages. This was classified as an antagonistic interaction as further confirmed by isobolographic analysis (**Figures 6F** and **7B**). All the interactions in the production of PGE-2 were additive (**Figure 5**), except for those already presented for BBI/genistein (synergistic) and saponin/sapogenol B (antagonistic).

DISCUSSION

Nitric oxide, prostaglandins, cyclooxygenase-2, and inducible nitric oxide synthase are considered to be major molecular participants in the inflammation-to-cancer axis. The results of this study show the capability of different SBCs to inhibit NO, PGE$_2$, iNOS, and COX-2 pro-inflammatory responses in LPS-induced RAW 264.7 macrophages. iNOS and COX-2 are inducible forms of nitric oxide synthase and cyclooxygenase enzymes that are overexpressed during inflammation and can lead to carcinogenesis. Some reports indicate that there may be cross-talk between the COX-2 and iNOS genes in macrophages and that their relationship is complex and cell type specific (*42*). NO is a signaling molecule important in the pathogenesis of inflammation and is considered as a pro-inflammatory mediator
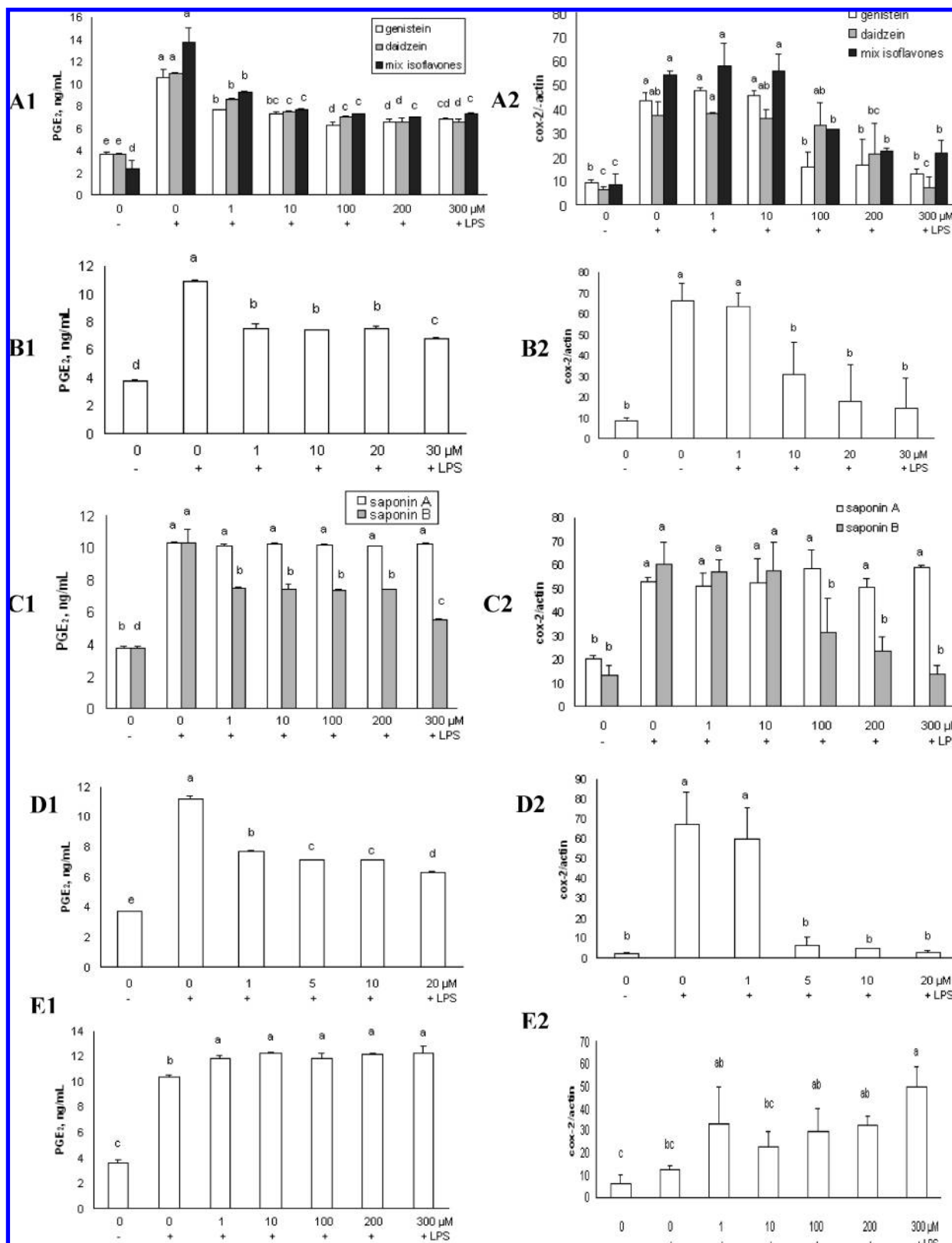
Figure 3. Effect of SBCs on PGE$_2$ (**1**) and COX-2 (**2**) in LPS-induced RAW 264.7 macrophages: (**A**) soybean isoflavones, (**B**) sapogenol B, (**C**) soybean saponin glycosides, (**D**) BBI, (**E**) pepsin−pancreatin glycinin hydrolysates. The data represent the mean ± SD from three independent experiments. Different letters indicate significant differences, *p* < 0.05.

that induces inflammation due to its overproduction in abnormal situations (*30*). It is produced by three isomers of nitric oxide synthase (NOS), constitutive NOS (cNOS), neuronal or brain NOS (bNOS), and inducible NOS. Inducible NOS is not present in most tissues but can be induced by bacterial LPS, and once induced, iNOS can produce >1000-fold NO for longer periods

as compared to either cNOS or bNOS (*43*). Furthermore, NO can induce modification of DNA either by altering DNA through reactive nitrogen oxide species (RNOS) or indirectly by inhibiting various repair processes. In addition, it has been shown that high concentrations of NO can cause inhibition of DNA ligase, which can amplify the genotoxicity resulting from

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Table 2. $IC_{35}{}^a$ of Individual SBCs against Pro-Inflammatory Response Production and Expression[b]

SBC	NO	PGE_2	iNOS	COX-2
BBI	16.2 ± 0.2	5.3 ± 0.14	9.1 ± 1.4	1.3 ± 0.3
sapogenol B	16.2 ± 1.0	27.6 ± 0.9	15.8 ± 3.1	5.4 ± 3.1
mix of ISFs	18.0 ± 1.2	3.2 ± 0.1	76.5 ± 2.2	73.6 ± 20.8
genistein	32.9 ± 0.7	29.8 ± 0.9	134.8 ± 11.4	44.5 ± 0.6
daidzein	137.1 ± 1.4	25.8 ± 10.2	286.5 ± 13.8	155.2 ± 3.5
saponin B	97.6 ± 10.8	189.9 ± 5.2	191.7 ± 16.3	63.3 ± 1.8
saponin A	116.3 ± 0.7	NS	>300	NS

^{a} IC_{35} is the concentration (μM) that resulted in 35% reduction of production/expression of pro-inflammatory responses ($n \geq 2$, mean $\pm$ SD). ^{b} Abbreviations: SBCs, soybean bioactive compounds; NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; BBI, Bowman–Birk inhibitor; ISFs, isoflavones; NS, no significant inhibition observed.

either RNOS or reactive oxygen species and in the presence of superoxide and peroxide can lead to single- and double-strand breaks in DNA (44). On the other hand, elevated prostaglandin levels are exhibited in many human cancers due to up-regulation of COX-2 (33). Aberrant activation of cyclooxygenase/prostaglandin signaling is widespread in human neoplasia (45, 46). Also, elevated prostaglandin levels have been found in mucosal and rectal dialysates of patients with inflammatory bowel disease (47), and many tumors secrete high levels of pro-inflammatory cytokines, including PGE_2 (48, 49) and thus can induce pro-inflammatory microenvironments.

The results presented here suggest that different SBCs have different capabilities in inhibiting inflammation through iNOS/NO and COX-2/PGE_2 pathways. Soybean isoflavones inhibited the production and expression of pro-inflammatory mediators differently. The mix of soy isoflavone glycosides exhibited the highest potency among the three isoflavones tested followed by either genistein or daidzein alone. The mix of soy isoflavone glycosides used was composed of 84% genistein glucoside, ~16% daidzein glucoside, and 0.3% glycitein glucoside. The presence of other isoflavones in the mixture resulted in higher potency as compared to individual isoflavones, suggesting a possible synergistic and/or additive effect. Genistein performed better in inhibiting COX-2/PGE_2 and iNOS/NO pathways than daidzein possibly due to the difference in the structure of the two isoflavones, i.e., the presence of one more hydroxyl group in genistein. Several reports have investigated the possible role of soybean isoflavones in mediating inflammation. Kao et al. (50) demonstrated that isoflavone powder produced from soybean cake inhibited LPS-induced inflammation in BALB/c mice by lowering the secretions of interleukin-1β, interleukin-6, NO, and PGE_2. These same authors also found that genistein resulted in a slight initial increase of NO production in peritoneal exudate fluid, which agrees with the findings in the present research. The role of soybean isoflavones in inhibiting inflammation via activation of PPAR-γ has been assumed, which may reduce expression of pro-inflammatory responses, thereby retarding tumor cell proliferation (51, 52). Another study showed that genistein inhibited LPS-induced microglia activation and production of tumor necrosis factor-α, NO, and superoxide in mesencephalic neuron–glia cultures and microglia-enriched cultures, suggesting that genistein may protect dopaminergic neurons from LPS-induced injury (53).

Saponins were another group of SBCs evaluated in this study. Our results showed that saponin A group glycosides inhibited inflammation through an iNOS/NO pathway and not by a COX-2/PGE_2 pathway. It was also seen that saponin B group glycosides significantly decreased NO production (1 μM) and inhibited iNOS expression (100 μM) at a lower concentration

than saponin A. On the other hand, saponin B group glycosides inhibited inflammation through both COX-2/PGE_2 and iNOS/NO pathways, indicating that it is a more potent anti-inflammatory agent than saponin A. Of the three saponin fractions tested, sapogenol B exhibited the highest potency against inflammation. At a concentration 10 times lower, sapogenol B inhibited the two pathways at the same rate as saponin A and saponin B group glycosides. This can be explained by the different affinities of these molecules with cellular membranes, which are influenced by their respective hydrophobicity. The presence of sugar moieties in the saponin A and B groups make their hydrophobicity low, and as such, they will have difficulty traversing cell membranes, while sapogenol B, which is relatively hydrophobic, may be able to enter cells easily, having a more pronounced effect against inflammation. Kim et al. (54) showed that crude soy saponin extract dose-dependently decreased cell growth of HT-29 colon cancer cell. Furthermore, they demonstrated the capability of crude soy saponin to modulate the expression of IκBα, protein kinase C, and COX-2 in human colon cancer cells, thus supporting their hypothesis that soy saponins can reduce the risk of colon tumorigenesis possibly by suppressing inflammatory responses.

BBI is a soybean peptide that inhibits trypsin-like and chymotrypsin-like serine proteases. The results of our study indicate that BBI is a very potent inhibitor of inflammation via COX-2/PGE_2 and iNOS/NO pathways. At a low concentration (20 μM), BBI inhibited NO production in LPS-induced RAW 264.7 macrophages by almost 50% while PGE_2 production was inhibited by 45%. BBI can suppress the release of superoxide anion radicals from human polynuclear leukocytes (55), and BBI concentrate added to the food of carcinogen-treated animals resulted in suppression of cancer and inflammation (56). Also, BBI concentrate reduced colon inflammation in mice treated with dextran sulfate sodium-induced ulcerative colitis when given during and after treatment (57). On the other hand, treatment of LPS-induced RAW 264.7 cells with pepsin–pancreatin glycinin hydrolysates resulted in induction of COX-2/PGE_2 and iNOS/NO pathways when compared to positive controls. This observation on the pro-inflammatory property of glycinin hydrolyzed by gastrointestinal enzymes deserves further study to explain the importance of the type of soy protein in food ingredients.

To better understand the role of soybean in inflammation, we also tested the possible interactions between SBCs in inhibiting NO and PGE_2 production. Our results showed that BBI and genistein, combined at a molar ratio of 1.2:1, synergistically inhibited the production of NO and PGE_2 more than the sum of their individual inhibition values. The straight, solid line drawn between the IC_{35} values with BBI alone and genistein alone predicted the IC_{35} values of all combinations as an additive effect (**Figures 6A** and **7A**). The observed IC_{35} from BBI and genistein combination deviated toward the lower left portion of the additive line, confirming their synergistic interaction in inhibiting NO and PGE_2 production. BBI can prevent the release of oxygen free radicals, which is assumed to be related to its potent anti-inflammatory activity (58). BBI has a direct and potent inhibitory effect on the catalytic activities of major proteases involved in inflammatory processes, such as cathepsin G (59), elastase (59), and chymase (60). It has been reported that genistein can reduce reactive oxygen species (ROS) by attenuating the expression of ROS-producing enzymes (61), which might contribute to its anti-inflammatory activity. On the other hand, combination of saponin B group glycosides and sapogenol B aglycon resulted in an antagonistic inhibition of
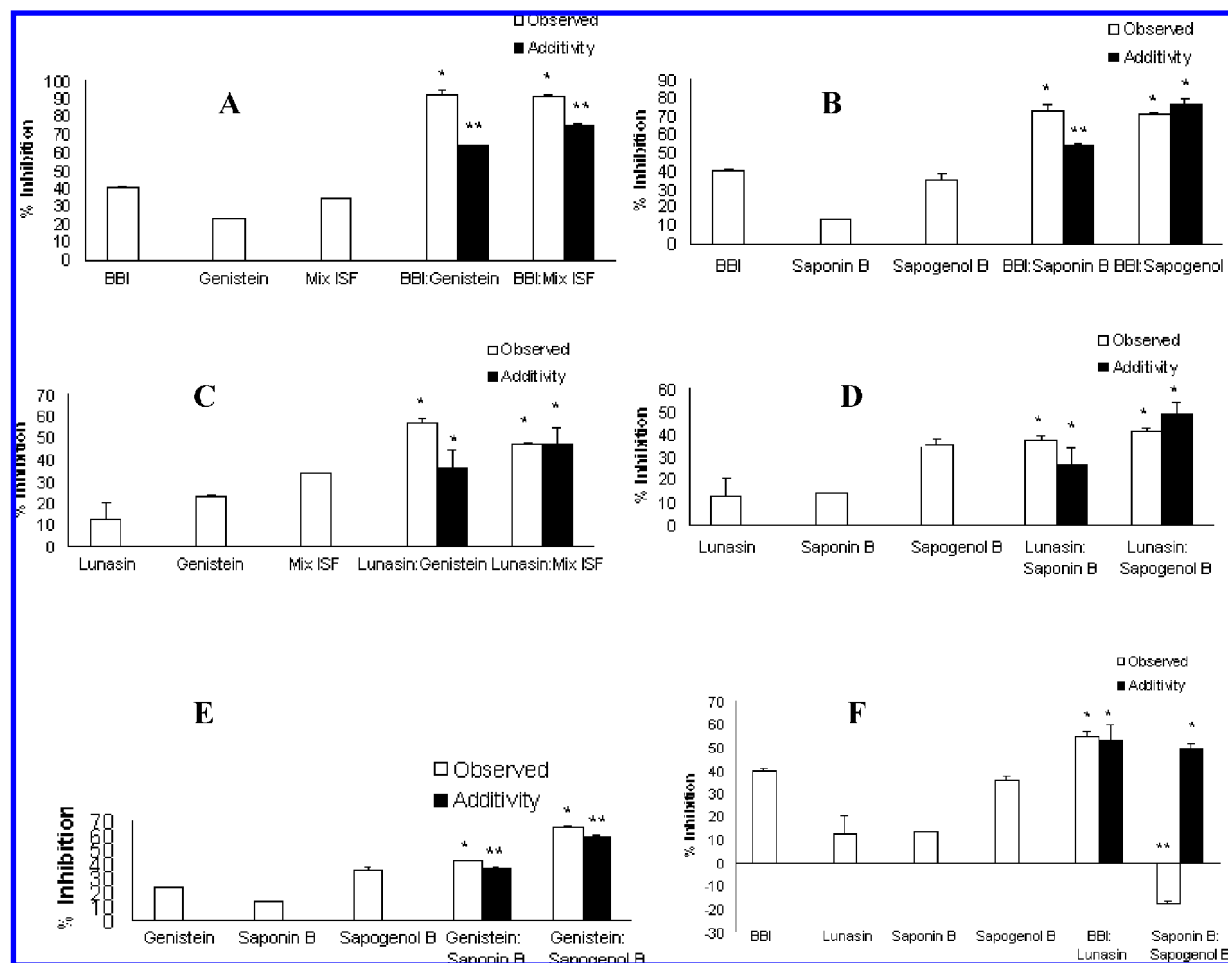
Figure 4. Inhibition of NO (%) production by LPS-induced RAW 264.7 macrophages after treatment with (**A**) BBI (18 μm), genistein (15 μM), and the mix of soy isoflavone glucosides (30 μM) and their combinations (BBI, 18 μM, and genistein, 15 μM; BBI, 18 μM, and the mix of soy isoflavone glucosides, 30 μM), (**B**) BBI (18 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (BBI, 18 μM, and saponin B, 40 μM; BBI, 18 μM, and sapogenol B, 15 μM), (**C**) lunasin (2 μM), genistein (15 μM), and the mix of soy isoflavone glucosides (30 μM) and their combinations (lunasin, 2 μM, and genistein, 15 μM; lunasin, 2 μM, and the mix of soy isoflavones glucosides, 30 μM), (**D**) lunasin (2 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (lunasin, 2 μM, and saponin B, 40 μM; lunasin, 2 μM, and sapogenol B, 15 μM), (**E**) genistein (15 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (genistein, 15 μM, and saponin B, 40 μM; genistein, 15 μM, and sapogenol B, 15 μM), and (**F**) BBI (18 μM), lunasin (2 μM), saponin B (40 μM), sapogenol B (15 μM), a combination of BBI (18 μM) and lunasin (2 μM), and a combination of saponin B (40 μM) and sapogenol B (15 μM). The data represent the mean $\pm$ SD ($n \geq 2$). Significant differences between observed values and additive interaction are reported at $p < 0.05$.

NO (**Figure 4F**) and PGE$_2$ (**Figure 5F**) production. The observed IC$_{35}$ values in the isobolograms for this combination shifted toward the right of the additivity line, thereby confirming the antagonistic interaction between saponin B and sapogenol B. Sapogenol B is the aglycon form of saponin B; it is then possible that a conjugation reaction between them happened and that this conjugation reaction resulted in the loss of biological activity. Previously, Su et al. (*62*) showed that combination of a 20 μg/mL concentration each of genistein, biochanin A, and daidzein resulted in synergistic interaction on cell growth inhibition, apoptosis induction, and antiapoptotic protein expression in human hepatoma cells. Also, they showed that combination of these isoflavones resulted in a more significant tumor-suppressive effect in nude mice. Murakami et al. (*63*) showed that genistein in combination with indomethacin and aspirin resulted in synergistic inhibition of PGE$_2$ while genistein in combination with nimesulide had an antagonistic effect. Also, they showed that combination of genistein and epigallocatechin gallate, at 10 μM, resulted in synergistic inhibition of COX-2 expression.

It is uncertain whether the combination of compounds tested in the present investigation could induce COX-1. However, Hooshmand et al. (*64*) have demonstrated that genistein, at a concentration of 100 μM, resulted in the reduction of COX-2 protein expression in LPS-stimulated human chondrocytes but not COX-1. The ability of genistein to suppress COX-2 but not COX-1 is advantageous since suppressing COX-2 leads to suppression of production of pro-inflammatory molecules. On the other hand, COX-1 is responsible for releasing prostaglandins in cellular house-keeping functions such as maintenance of gastrointestinal integrity and vascular homeostasis.

Data suggested that, even though individual SBCs resulted in inhibition of pro-inflammatory responses, combination of two SBCs has a more beneficial effect in preventing inflammation than individual SBCs alone. This is important as many soy product supplements contain mixtures of SBCs at various proportions.

It can be concluded that BBI and sapogenol B were the most potent inhibitors of inflammation when compared to other SBCs used in this study. Other soybean bioactive compounds such as
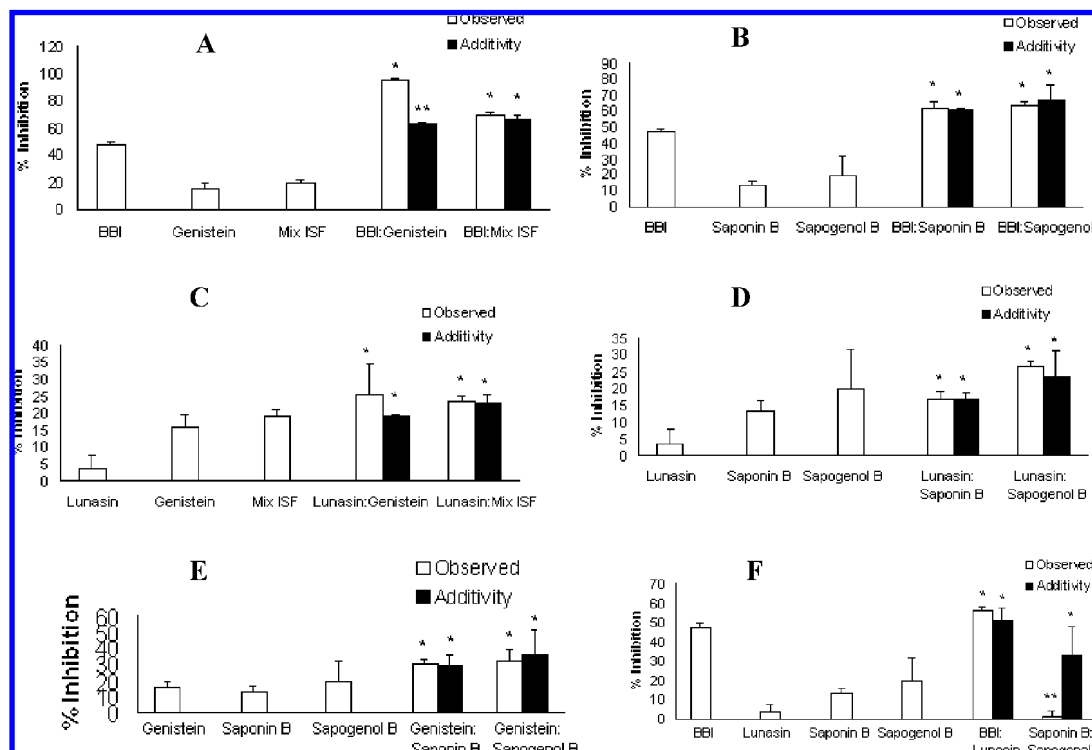
Figure 5. Inhibition of PGE$_2$ (%) production by LPS-induced RAW 264.7 macrophages after treatment with (**A**) BBI (18 μm), genistein (15 μM), and the mix of soy isoflavones glucosides (30 μM) and their combinations (BBI, 18 μM, and genistein, 15 μM; BBI, 18 μM, and the mix of soy isoflavone glucosides, 30 μM), (**B**) BBI (18 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (BBI, 18 μM, and saponin B, 40 μM; BBI, 18 μM, and sapogenol B, 15 μM), (**C**) lunasin (2 μM), genistein (15 μM), and the mix of soy isoflavones glucosides (30 μM) and their combinations (lunasin, 2 μM, and genistein, 15 μM; lunasin, 2 μM, and the mix of soy isoflavone glucosides, 30 μM), (**D**) lunasin (2 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (lunasin, 2 μM, and saponin B, 40 μM; lunasin, 2 μM, and sapogenol B, 15 μM), (**E**) genistein (15 μM), saponin B (40 μM), and sapogenol B (15 μM) and their combinations (genistein, 15 μM, and saponin B, 40 μM; genistein, 15 μM, and sapogenol B, 15 μM), and (**F**) BBI (18 μM), lunasin (2 μM), saponin B (40 μM), sapogenol B (15 μM), a combination of BBI (18 μM) and lunasin (2 μM), and a combination of saponin B (40 μM) and sapogenol B (15 μM). The data represent the mean $\pm$ SD ($n \geq 2$). Significant differences between observed values and additive interaction are reported at $p < 0.05$.
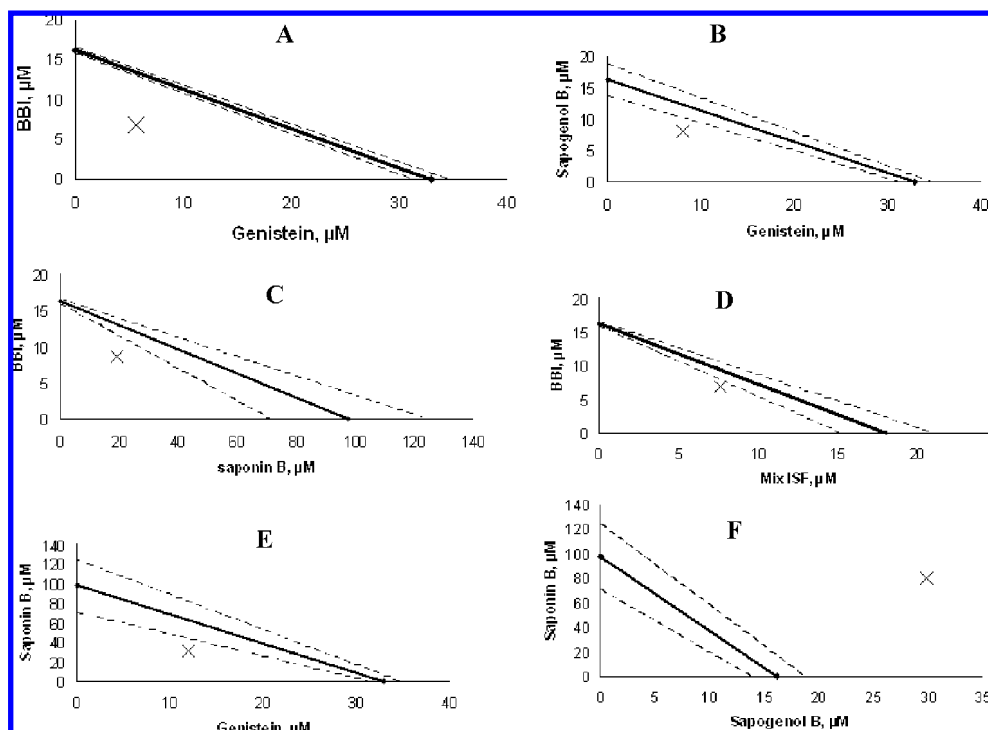

Figure 6. IC$_{35}$ isobologram for the synergistic interaction of (**A**) BBI and genistein, (**B**) sapogenol B and genistein, (**C**) BBI and saponin B, (**D**) BBI and the mix of isoflavones, and (**E**) saponin B and genistein and antagonistic interaction of (**F**) saponin B and sapogenol B with respect to inhibition of NO (%) production by LPS-induced RAW 264.7 macrophages. The line of additivity (solid line) is shown with its 95% confidence interval (dotted lines).
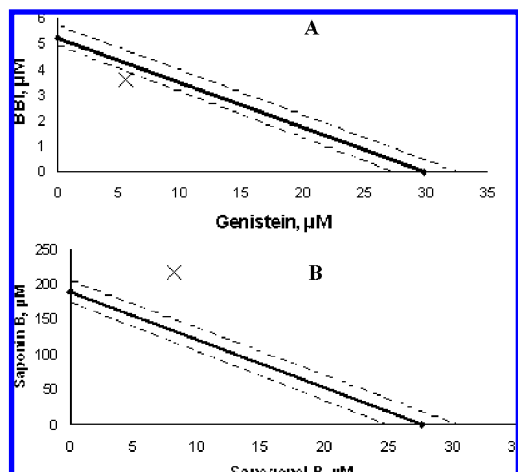
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Figure 7. IC$_{35}$ isobologram for the synergistic interaction between (**A**) BBI and genistein and for the antagonistic interaction between (**B**) saponin B and sapogenol B with respect to inhibition of PGE$_2$ (%) production by LPS-induced RAW 264.7 macrophages. The line of additivity (solid line) is shown with its 95% confidence interval (dotted lines).

isoflavones and saponin B group glycosides exhibited anti-inflammatory properties at relatively higher concentrations than BBI and sapogenol B, while pepsin and pancreatin glycinin hydrolysates resulted in the induction of such pro-inflammatory responses. The results indicate that some SBCs suppressed LPS-induced production of NO and PGE$_2$ through the down-regulation of protein expressions of COX-2 and iNOS. We also showed for the first time that combinations of two SBCs, such as BBI and genistein, at concentrations naturally present in soy resulted in interactions that can synergistically inhibit NO and PGE$_2$ production in LPS-induced macrophages. Our results are important as they give information on what type of SBC warrants further in vivo study.

ABBREVIATIONS USED

LPS, lipopolysaccharide; BBI, Bowman–Birk inhibitor; NO, nitric oxide; PGE$_2$, prostaglandin E$_2$; ISFs, isoflavones; SBCs, soybean bioactive compounds; PPGHs, pepsin–pancreatin glycinin hydrolysates.

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