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Inhibition of Inducible Nitric Oxide Synthase Expression by an Acetonic Extract from *Feijoa sellowiana* Berg. Fruits

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Feijoa sellowiana Berg. fruits and especially the acetonic extract have been shown to possess biological activities, although the responsible compounds have never been identified. The present study was designed to evaluate the anti-inflammatory activity of an acetonic extract from *F. sellowiana* Berg. fruits on the nitric oxide (NO) pathway, which plays an important role in inflammation. To this aim the J774 cell line, which expresses inducible nitric oxide synthase (iNOS) following stimulation with lipopolysaccharide (LPS), has been utilized, and the effects of this extract and its fractions on NO production, iNOS protein expression, and signal pathways involved in its regulation have been evaluated. This study demonstrates that at least some part of the anti-inflammatory activity of the acetonic extract is due to the suppression of NO production by flavone and stearic acid. The mechanism of this inhibition seems to be related to an action on the expression of the enzyme iNOS through the attenuation of nuclear factor κB (NF-κB) and/or mitogen-activated protein kinase (MAPK) activation.

KEYWORDS: ***Feijoa sellowiana*; feijoa; flavone; fatty acid; macrophages; iNOS; NF-κB; ERK-1/2**

INTRODUCTION

Feijoa sellowiana Berg. (syn. *Acca sellowiana*, Myrtaceae) is an evergreen bush 5−8 m high with gray branches, elliptical buds, white and red flowers, and sweet-smelling leaves, originally native to South America. Presently, *F. sellowiana* grows throughout the Mediterranean area, where it was introduced at the end of the 19th century initially as an ornamental plant. The edible fruit (Feijoa), now widely used for human food, ripens in autumn as a spherical berry, 5−8 cm long, 20−30 g heavy. The pulp, which is granular and acidulous and has a sugary taste, contains large quantities of vitamin C (28 mg/100 g of fresh weight), hydrocarbons, minerals, and iodine (3 mg/100 g). Furthermore, it is rich in volatile substances including terpenoid compounds and methyl and ethyl benzoate, accounting for about 90% of the volatile fraction and responsible for the strong character "feijoa-like" typical of this fruit (*1*). Feijoa is also known to contain high amounts of vitamin P (P)-active polyphenols, such as catechins, leucoanthocyanins, flavonols, proanthocyanidins, naphthoquinones, and tannins (*2*), but generally phytochemical data are rough, and only the leaves have been well characterized (*3*). On the whole, the fresh feijoa fruit is well appreciated for its good nutritional characteristics (*4*, *5*) and for its pleasant flavor and aroma; for these reasons it is widely present on the market, especially in the Australian area, in the form of jam, paste, syrup, liqueur, and crystallized fruit. In the literature, various biological activities of different extracts from feijoa whole fruit or peel are described, although a complete characterization of the active principles is lacking and pharmaceutical studies on the constituents have barely been carried out. It has been reported that *F. sellowiana* shows potent antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi (*6*). Moreover, an antioxidant activity of an aqueous extract on oxidative burst of human whole blood phagocytes and on isolated polymorphonuclear leukocytes has been described (*7*). In addition, Feijoa peel contains promising levels of multidrug resistance-modulating and cytotoxic activity (*8*). Although these papers clearly show the therapeutic potential of feijoa, compounds responsible for the reported biological activities have never been identified.

Nitric oxide (NO) is known to participate in the physiological and pathological functions of many organs; in fact, it plays a

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major role in regulating vascular tone, neurotransmission, killing of microorganisms and tumor cells, and other homeostatic mechanisms. High levels of NO have been described in a variety of pathophysiological processes including various forms of circulatory shock (*9*), inflammation, and carcinogenesis (*10*). NO is synthesized by nitric oxide synthases (NOS), a family of enzymes that use tetrahydrobiopterin, flavin mononucleotide, and flavine adenine dinucleotide as cofactors to catalyze the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent oxidation of L-arginine to produce NO and L-citrulline. To date, three NOS isoforms have been identified: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) (*11*). The nNOS and eNOS are constitutively expressed in neuronal and endothelial cells, respectively, and are Ca^{2+}/calmodulin-dependent. The iNOS isoform is not constitutively present; it can be rapidly induced by inflammatory stimuli, including toxins such as lipopolysaccharide (LPS) and cytokines, and its activation is Ca^{2+}/calmodulin-independent (*12*). NO production by iNOS is mainly regulated at the transcriptional level (*13*). In macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which leads to the induction of the expression of many immediate early genes The presence of the cis-acting NF-κB element has been demonstrated in the 5′-flanking regions of iNOS genes (*13*). NF-κB exists mainly as a heterodimer, which is normally sequestered in an inactive cytoplasmic complex by binding to the inhibitory protein IκB. Exposure of cells to LPS causes rapid phosphorylation of inhibitor κB (IκB) by IκB kinase (IKK), with subsequent degradation by proteosomes. Dissociation of IκB from NF-κB allows the activated free dimer to translocate to the nucleus (*14*), where it induces the gene transcription through the cis-acting κB element. The NF-κB activation is regulated by mitogen-activated protein kinases (MAPK) (*15*). In fact, the extracellular signal-regulated kinase 1/2 (ERK-1/2) signaling pathway has been often implicated in NF-κB activation through phosphorylation and subsequent degradation of the inhibitory subunit IκB (*16*). Because the *Feijoa* fruit has antioxidant activity (*7*) and, in addition to radical oxygen species, overproduction of NO, due to expression of iNOS, also plays an important role in inflammation, in the present study we have evaluated the anti-inflammatory activity of an acetonic extract from *F. sellowiana* fruit. Particularly, we have investigated, in an *in vitro* model of inflammation, murine macrophages J774 cell line stimulated with LPS, the effects of this extract and its fractions on NO production, iNOS protein expression, and signal pathways involved in its regulation. To clarify the chemical structure of substances responsible for this effect, we have defined a fractionation procedure of the extract that allowed us to identify flavone and stearic acid as the active compounds of feijoa fruit.

MATERIALS AND METHODS

Plant Material. *F. sellowiana* Berg. (Myrtaceae) fruits were collected in the Botanical Garden of Naples (Italy). The identification was done by one of us (Prof. A. Basile, Section of Plant Biology, Department of Biological Sciences, University of Naples "Federico II"). A voucher specimen (NAP no. 96-125) is deposited at the Herbarium Neapolitanum (NAP), Department of Biological Sciences, University of Naples "Federico II" (Italy).

Materials. All solvents (analytical and deuterated grade) were purchased from Carlo Erba Reagenti, Milan, Italy. Merck silica gel (70−230 mesh) was used for column chromatography. Thin-layer chromatography (TLC) was performed on plates coated with silica gel 60 F_{254} Merck, 0.25 mm.

The protease inhibitor cocktail was purchased from Roche Applied Science. Mouse monoclonal antibody anti-iNOS was obtained from BD Transduction Laboratories; monoclonal antibodies anti-ERK-2, anti-IκBα, and anti-pERK-1/2 were obtained from Santa Cruz Biotechnology; and anti-mouse secondary antibodies were purchased from Dako Cytomation. All other reagents and compounds used were obtained from Sigma-Aldrich (Milan, Italy).

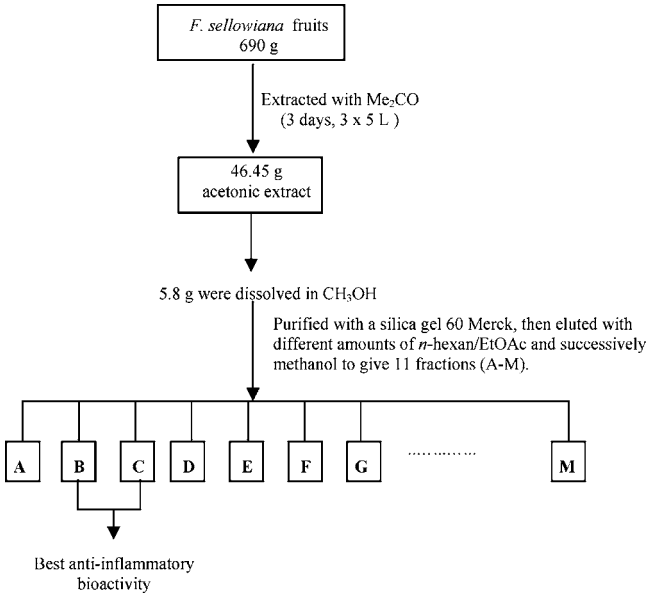

Figure 1. Preparation scheme of acetonic extract from *Feijoa sellowiana* fruits.

General Experimental Procedures. UV spectra were obtained on a Jasco 7800 UV−vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 MHz NMR spectrometer (^{1}H at 400.4 MHz, ^{13}C at 100.7 MHz), δ (ppm), J in hertz, using the residual solvent signal (δ 7.27 in ^{1}H and δ 77.0 in ^{13}C) as reference. ESIMS was obtained on Applied Biosystem API-2000 mass spectrometer.

Extraction and Fraction. Six hundred and ninety grams of whole fruit was blended, freeze-dried, and then extracted for 3 days with Me_2CO at room temperature (3 × 5 L). After filtration, the solvent was evaporated under reduced pressure and low temperature (35 °C) to give a gum (46.45 g); part of this acetonic extract was used for biological bioassays. Another amount of the extract (5.8 g) was dissolved in CH_3OH and then submitted to column chromatography on silica gel 60 Merck (4 × 100 cm, 300 g, 63−200 μm). Elution with *n*-hexane/EtOAc (from 100:0 to 0:100 stepwise gradient) and successively methanol afforded 11 fractions (A−M) of 100 mL each, gathered according to TLC analysis [eluent system *n*-hexane/EtOAc (1:1 vv), spray reagent $Ce(SO_4)_2$ in H_2SO_4], that were submitted for biological testing: fractions A (57 mg, eluted with *n*-hexane/EtOAc 80:20), B (35.6 mg, eluted with *n*-hexane/EtOAc 60:40), C (30.1 mg, eluted with *n*-hexane/EtOAc 50:50), D (28.4 mg, eluted with *n*-hexane/EtOAc 40:60), E (18.2 mg, eluted with *n*-hexane/EtOAc 30:70), F (22 mg, eluted with EtOAc 100%), G (31.4 mg, eluted with EtOAc/MeOH 90:10), H (457 mg, eluted with EtOAc/MeOH 70:30), I (1.4 g, eluted with EtOAc/MeOH 60:40), L (410 mg, eluted with EtOAc/MeOH 10:90), and M (58.2 mg, eluted with MeOH 100%). Sample preparation procedures are shown in **Figure 1**.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis. GC analyses were performed on a Perkin-Elmer Sigma-115 gas chromatograph equipped with a FID and a data handling processor. The separation was achieved using a HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The column temperature was 120 °C, with a 5 min initial hold, and then raised to 260 °C at 5 °C/min, where it was held for 15 min, using He as the carrier gas (1.0 mL/min). Injection mode was splitless (1 μL of a 1:1000 *n*-hexane solution). Injector and detector temperatures were 250 and 290 °C, respectively. Components' relative concentrations were obtained by peak area normalization. No response factors were calculated. GC-MS analyses were performed using an Agilent 6850 series II gas chromatograph linked on-line with an Agilent mass selective detector

MSD 5973Network. The column was a HP-5 fused-silica capillary column (30 m × 0.25 mm i.d.; 0.33 μm film thickness). The temperature conditions were the same as used for GC analysis. Other conditions were as follows: interface temperature, 295 °C; mass range, *m*/*z* 40−450; ionization energy, 70 eV; multiplier energy, 2000 V; scan time, 1 s; carrier gas, helium, at 1.0 mL/min. Peak identification was accomplished by comparison of their mass spectra with those stored on the GC-MS databases (NIST 02 and Wiley 275) and reported in the literature (*17*, *18*). The identification of the components was made also by co-injection with authentic substances.

Cell Culture. The murine monocyte/macrophage J774 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 25 mM Hepes, penicillin (100 units/mL), streptomycin (100 μg/mL), 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate.

Cells were plated to a seeding density of either 2×10^6 in P60 well plates or 2.5×10^5 in 24 multiwell plates. Cells were pretreated (for 2 h) with increasing concentration of test compounds and stimulated with LPS from *Escherichia coli*, serotype 0111:B4 (10 μg/mL). Treatment with test compounds and/or LPS was carried out under serum-free conditions. The concentrations of the acetonic extract utilized (50, 250, and 750 μg/mL) were chosen according to what was reported in the literature (*4*).

The tested concentrations of fraction B (0.30, 1.50, and 4.50 μg/mL) and fraction C (0.26, 1.30, and 3.90 μg/mL) corresponded to the percentage present in the whole acetonic extract.

Flavone was tested at the concentrations of 0.30, 1.50, and 4.50 μg/mL (corresponding to the percentage present in fraction B), stearic acid at the concentrations of 0.073, 0.365, and 1.095 μg/mL, and linoleic acid at the concentrations of 0.034, 0.170, and 0.510 μg/mL (corresponding to the percentage present in fraction C).

Cell Viability. Cell respiration, an indicator of cell viability, was assessed by the mithocondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After stimulation with LPS in the absence or presence of test compounds for 24 h, cells were incubated in 96-well plates with MTT (0.2 mg/mL), for 1 h. Culture medium was removed by aspiration, and the cells were dissolved in DMSO (0.1 mL). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}.

Nitrite Assay. Cells were pretreated with test compounds for 2 h and further incubated for 24 h with LPS (10 μg/mL). At the end of the incubation, the supernatants were collected for the nitrite measurement. The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μL of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100 μL samples. The optical density at 550 nm (OD_{550}) was measured using an ELISA microplate reader (SLT-Labinstruments, Salzburg, Austria). Nitrite concentration was calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Western Blot Analysis. The analyses of pERK-1/2, ERK-2, and iNOS in J774 macrophages were performed on whole cell lysates. After stimulation with LPS for 15 min (pERK-1/2, ERK-2) or 24 h (iNOS), cells were washed with cold PBS and lysed for 10 min at 4 °C with lysis buffer (50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.01% SDS) containing complete protease inhibitor cocktail. Lysates from adherent cells were collected by scraping and centrifuged at 12000*g* for 15 min at 4 °C. The supernatants were collected, and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad). The analysis of IκBα was performed on cytosolic extract. Thirty minutes after LPS stimulation, cells were washed with cold PBS, harvested in cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylethanesulfonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, 7 μg/mL pepstatin A, 5 μg/mL leupeptin, 0.1 mM benzamidine, 0.5 mM DTT), and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle five times, and the cytoplasmic fraction was then obtained by centrifugation at 13000*g* for 1 min at 4 °C. The supernatants were collected, and protein concentration was determined.

Equal amounts of protein (50 μg) were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/mL of bromophenol) in a ratio of 1:1, boiled for 3 min, and centrifuged at 10000*g* for 10 min. Each sample was loaded and electrophoresed on a 10% SDS−polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes (Hybond ECL Nitrocellulose, Amersham). The membranes were blocked with 0.1% PBS-Tween containing 5% nonfat dry milk for ERK-2, IκBα, iNOS, and β-actin, whereas they were blocked with 0.1% PBS-Tween containing 5% nonfat dry milk and 50 mM NaF for pERK-1/2. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4 °C. Mouse monoclonal antibodies anti ERK-2, IκBα, and anti iNOS were diluted 1:1000 in 0.1% PBS-Tween, 5% nonfat dry milk; mouse monoclonal antibody anti pERK-1/2 was diluted 1:1000 in 0.1% PBS-Tween, 5% nonfat dry milk, 50 mM NaF; mouse monoclonal antibody anti β-actin was diluted 1:10000 in 0.1% PBS-Tween, 5% BSA. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibodies diluted 1:1000 in 0.1% PBS-Tween containing 5% nonfat dry milk. The membranes were washed, and protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia). Densitometric analysis was performed with a Fluor S quantitative imaging system (Bio-Rad Laboratories).

Statistical Analysis. The results are expressed as mean ± standard error (SEM) of the mean of *n* observations, where *n* represents the number of experiments performed on different days. Triplicate wells were used for the various treatment conditions. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A p value of <0.05 was considered to be significant.

RESULTS

Cytotoxic Activity. To assess the cytotoxic activity of the *F. sellowiana* fruit acetonic extract, J774 cells were stimulated for 24 h with LPS after pretreatment with the extract (50, 250, and 750 μg/mL) and cell viability was tested using the MTT assay. Pretreatment with the extract did not affect cell viability at any of the concentrations tested (data not shown).

Effect of Acetonic Extract from *F. sellowiana* Fruit on LPS-Induced NO Pathway. To assess whether the acetonic extract from *F. sellowiana* fruits was able to reduce NO production, we utilized the Griess assay to measure nitrites, the stable end-products of NO, in supernatants of the murine macrophage cell line J774 stimulated with LPS (10 μg/mL) for 24 h. A marked increase of nitrite production in the cell medium was observed (63.70 ± 0.79 nmol/10^6 cells; $P < 0.001$, $n = 3$) with respect to unstimulated macrophages (2.95 ± 0.41 nmol/10^6 cells). When the acetonic extract from *F. sellowiana* fruits (50, 250, and 750 μg/mL) was added to J774 macrophages, 2 h before LPS stimulation, a concentration-dependent decrease of nitrite production in cell medium was observed (**Figure 2A**). In fact, the observed inhibition was 35.6, 75.8, and 92.5% at 50, 250, and 750 μg/mL, respectively ($P < 0.001$, $n = 3$). The IC_{50} was 86.62 μg/mL (95% IC 63.88 to 117.5 μg/mL; $r^2 = 0.9997$). The aminoguanidine (10 μg/mL), a well-known inhibitor of iNOS enzyme, inhibited the nitrite production by 84% ($P < 0.001$, $n = 3$). When added 24 h after LPS induction, the aminoguanidine inhibited the nitrite production by 66% ($P < 0.001$, $n = 3$), whereas the extract did not modify NO release (data not shown), suggesting an action at the level of iNOS protein expression. Therefore, we evaluated the levels of iNOS protein in whole lysate from J774 cells stimulated with LPS and pretreated with the extract, as described above. As shown in **Figure 2B,** LPS (10 μg/mL) caused, at 24 h, a significant increase of iNOS protein levels compared to unstimulated cells (control). When cells were pretreated with the acetonic extract from *F. sellowiana* fruit (50, 250, and 750 μg/mL) for 2 h before LPS stimulation, iNOS protein expression was

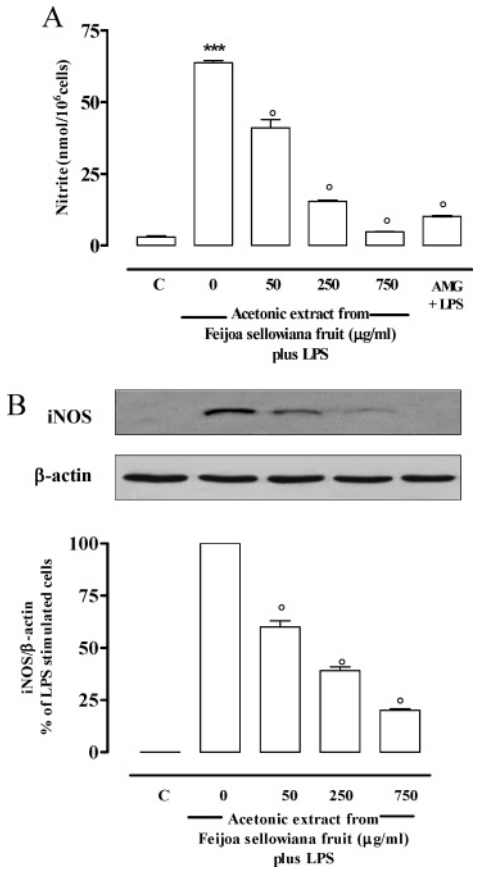

Figure 2. Effect of acetonic extract from *F. sellowiana* fruit on LPS-induced nitrite production (**A**) and iNOS expression (**B**). J774 cells were pretreated for 2 h with the extract (0, 50, 250, and 750 μg/mL), prior to LPS stimulation (10 μg/mL) for 24 h. (**A**) Nitrites, stable end-products of NO, were measured by the Griess reaction. Data are expressed as mean ± SEM from three separate experiments performed in triplicate. (**B**) Cell lysates and western blot analysis were performed as described (see Materials and Methods). The bands corresponding to iNOS protein were quantified by densitometric analysis, and the results are expressed as a percentage of LPS-stimulated cells. Densitometric analysis of iNOS protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin levels. ***, $P < 0.001$ versus unstimulated cells (C); °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

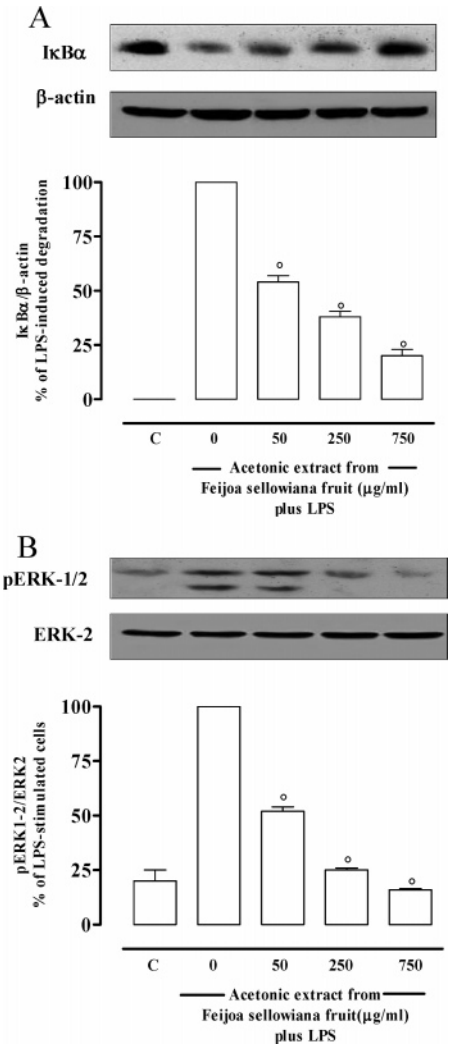

Figure 3. Effect of acetonic extract from *F. sellowiana* fruit on LPS-induced IκBα degradation (**A**) and ERK-1/2 activation (**B**). J774 cells, pretreated (for 2 h) with increasing concentrations of acetonic extract (0, 50, 250, and 750 μg/mL), were stimulated with LPS (10 μg/mL) for 30 min (IκBα) and 15 min (pERK-1/2). Cell lysates and western blot analysis were performed as described (see Materials and Methods). The bands were quantified by densitometric analysis, and the results are expressed as a percentage of LPS-induced degradation (IκBα) and as a percentage of LPS-stimulated cells (pERK-1/2). Densitometric analysis of protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin (IκBα) and ERK-2 (pERK-1/2) levels. °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

inhibited in a concentration-dependent manner with respect to LPS-stimulated cells. In fact, the observed inhibition was 40, 61, and 80% at 50, 250, and 750 μg/mL, respectively ($P < 0.001$, $n = 3$) (**Figure 2B**).

Effect of Acetonic Extract from *F. sellowiana* Fruits on IκBα Degradation. Because activation of NF-κB is critical for the induction of iNOS by LPS, we have investigated the action of the acetonic extract from *F. sellowiana* fruit on NF-κB activation in LPS-activated macrophages. It has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκBα, so the levels of this protein were measured as an index of NF-κB inhibition by test sample. As shown in **Figure 3A**, following cell stimulation with LPS for 30 min, a degradation of IκBα was observed, whereas pretreatment (2 h before LPS) with test compound (50, 250, and 750 μg/mL) prevented IκBα degradation in a concentration-dependent manner. The inhibition of IκBα degradation accounted for 46, 62, and 80% at 50, 250, and 750 μg/mL, respectively ($P < 0.001$, $n = 3$).

Effect of Acetonic Extract from *F. sellowiana* Fruits on LPS-Induced ERK-1/2 Activation. To investigate whether the inhibition of iNOS expression and IκBα degradation, observed in our experimental conditions, corresponded to a modulation of MAPK pathway, one of the signal transduction pathways involved in the regulation of iNOS expression, we analyzed ERK-1/2 activation 15 min after LPS treatment, in the presence or absence of the acetonic extract from *F. sellowiana* fruits (2 h before LPS). In fact, the ERK signaling pathway has been implicated in NF-κB activation through phosphorylation and subsequent degradation of the inhibitory subunit IκBα (*21*). As shown in **Figure 3B**, stimulation of the cells in the presence of LPS induced ERK-1/2 phosphorylation, whereas in the presence of increasing concentrations of the acetonic extract (50, 250, and 750 μg/mL) a concentration-dependent and significant

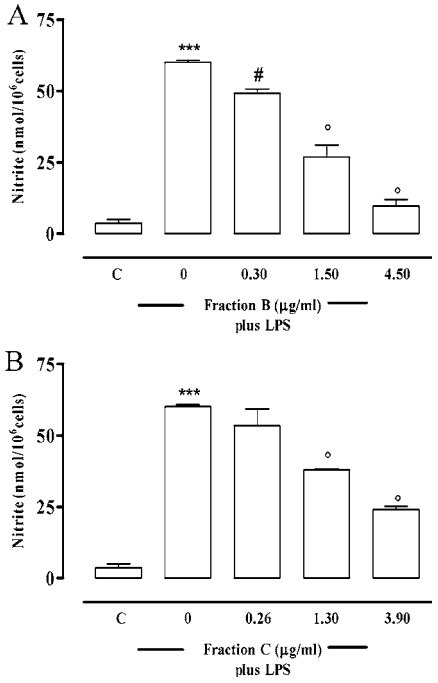

Figure 4. Effect of fractions B (**A**) and C (**B**) of the *F. sellowiana* acetonic extract on nitrite production. J774 cells were pretreated for 2 h with 0, 0.30, 1.50, and 4.50 μg/mL of fraction B (**A**) and 0, 0.26, 1.30, and 3.90 μg/mL of fraction C (**B**), amounts corresponding to the concentrations present in the acetonic extract, prior to LPS stimulation (10 μg/mL) for 24 h. Nitrites, stable end-products of NO, were measured by the Griess reaction. Data are expressed as mean ± SEM from three separate experiments performed in triplicate. ***, $P < 0.001$ versus unstimulated cells (C); #, $P < 0.05$; °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

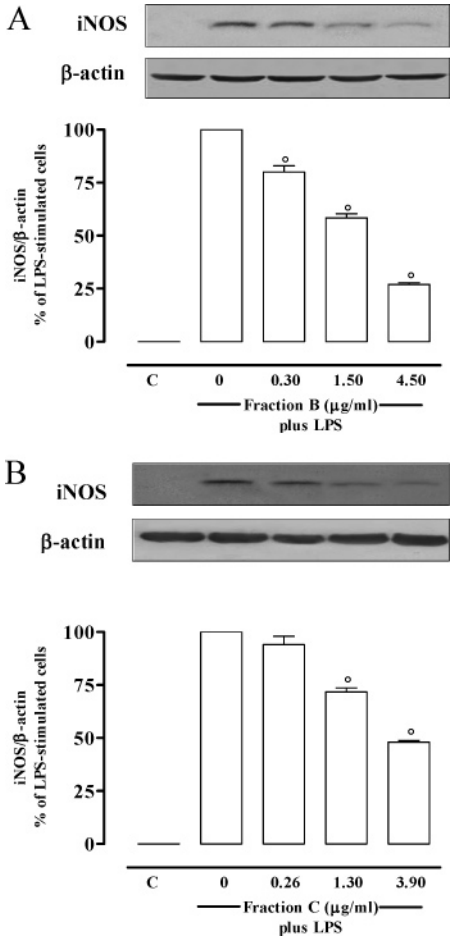

Figure 5. Effect of fractions B (**A**) and C (**B**) of the *F. sellowiana* acetonic extract on LPS-induced iNOS expression. J774 cells were pretreated for 2 h with 0, 0.30, 1.50, and 4.50 μg/mL of fraction B (**A**) and 0, 0.26, 1.30, and 3.90 μg/mL of fraction C (**B**), amounts corresponding to the concentrations present in the acetonic extract, prior to LPS stimulation (10 μg/mL) for 24 h. Cell lysates and western blot analysis were performed as described (see Materials and Methods). The bands corresponding to iNOS protein were quantified by densitometric analysis, and the results are expressed as a percentage of LPS-stimulated cells. Densitometric analysis of iNOS protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin levels. °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

inhibition of ERK-1/2 phosphorylation was observed. In fact, at 50, 250, and 750 μg/mL the observed inhibition was 48, 75, and 84%, respectively ($P < 0.001$, $n = 3$).

Effect of *F. sellowiana* Acetonic Extract Fractions on LPS-Induced Nitrite Production, iNOS Expression, IκBα Degradation, and ERK-1/2 Activation. To clarify the chemical structure of compounds responsible for the anti-inflammatory activity of the acetonic extract, we have defined a fractionation procedure that afforded 11 fractions (A−M, see Materials and Methods and **Figure 1**). The A−M fractions were subsequently tested for their anti-inflammatory activity. Pretreatment of the LPS-stimulated cells with increasing concentrations of each fraction showed that only fractions B and C were active. In fact, 0.30, 1.50, and 4.50 μg/mL of fraction B as well as 0.26, 1.30, and 3.90 μg/mL of fraction C, amounts corresponding to the concentrations present in the whole acetonic extract, inhibited nitrite production. Particularly, the observed inhibition accounted for 18, 55.3, and 84% at 0.30, 1.50, and 4.50 μg/mL, respectively, for fraction B ($P < 0.01$ at 0.30 μg/mL; $P < 0.001$ at 1.50 and 4.50 μg/mL, respectively $n = 3$) (**Figure 4A**). Fraction C significantly inhibited nitrite production, only from the concentration of 1.30 μg/mL. In fact, the inhibition was 12.1%, at the lowest concentration tested (0.26 μg/mL, $P > 0.05$, $n = 3$), and 36.8 and 60% at 1.30 μg/mL and 3.90 μg/mL, respectively ($P < 0.001$, $n = 3$) (**Figure 4B**). The IC_{50} was 1.20 μg/mL (95% IC = 0.52−2.74 μg/mL; $r^2 = 0.9975$) and 2.46 μg/mL (95% IC = 1.79−3.39 μg/mL; $r^2 = 0.9995$) for fractions B and C, respectively. Moreover, pretreatment of the LPS-stimulated cells with increasing concentrations of fraction B inhibited iNOS expression in a concentration-dependent manner (**Figure 5A**), the observed inhibition being 20, 41.6, and 73% at 0.30, 1.50, and 4.50 μg/mL, respectively ($P < 0.001$, $n = 3$). A good correlation between nitrite and iNOS expression inhibition was also observed for fraction C because iNOS expression was significantly inhibited only from the concentration of 1.30 μg/mL. The inhibition was 6%, at the lowest concentration tested (0.26 μg/mL, $P > 0.05$, $n = 3$) and 28.3 and 52% at 1.30 and 3.90 μg/mL, respectively ($P < 0.001$, $n = 3$) (**Figure 5B**).

Both fractions B (0.30, 1.50, and 4.50 μg/mL) and C (0.26, 1.30, and 3.90 μg/mL) prevented IκBα degradation (**Figure 6**) and inhibited ERK-1/2 phosphorylation (**Figure 7**). In particular, fraction B inhibited IκBα degradation by 23% at 0.30 μg/mL, by 42% at 1.50 μg/mL, and by 74% at 4.50 μg/mL (**Figure 6A**, $P < 0.001$, $n = 3$), whereas the inhibition of ERK-1/2 activation was 34% at 0.30 μg/mL, 51% at 1.50 μg/mL, and 77% at 4.50 μg/mL (**Figure 7A**, $P < 0.001$, $n = 3$). Fraction C prevented IκBα degradation and inhibited ERK-1/2 phosphorylation in a concentration-dependent manner, but only from

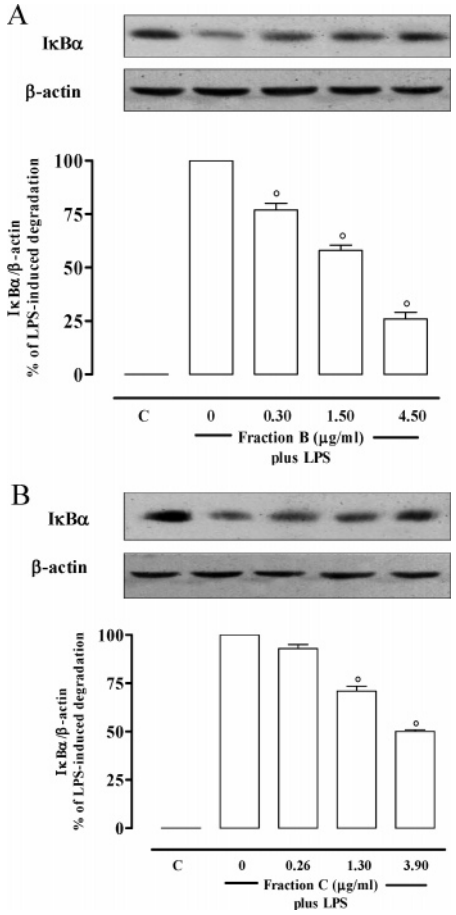

Figure 6. Effect of fractions B (**A**) and C (**B**) of the *F. sellowiana* acetonic extract on LPS-induced IκBα degradation. J774 cells, pretreated (for 2 h) with 0, 0.30, 1.50, and 4.50 μg/mL of fraction B (**A**) and 0, 0.26, 1.30, and 3.90 μg/mL of fraction C (**B**), amounts corresponding to the concentrations present in the acetonic extract), were stimulated with LPS (10 μg/mL) for 30 min. Cell lysates and western blot analysis were performed as described (see Materials and Methods). The bands were quantified by densitometric analysis, and the results are expressed as a percentage of LPS-induced degradation. Densitometric analysis of protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin. °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

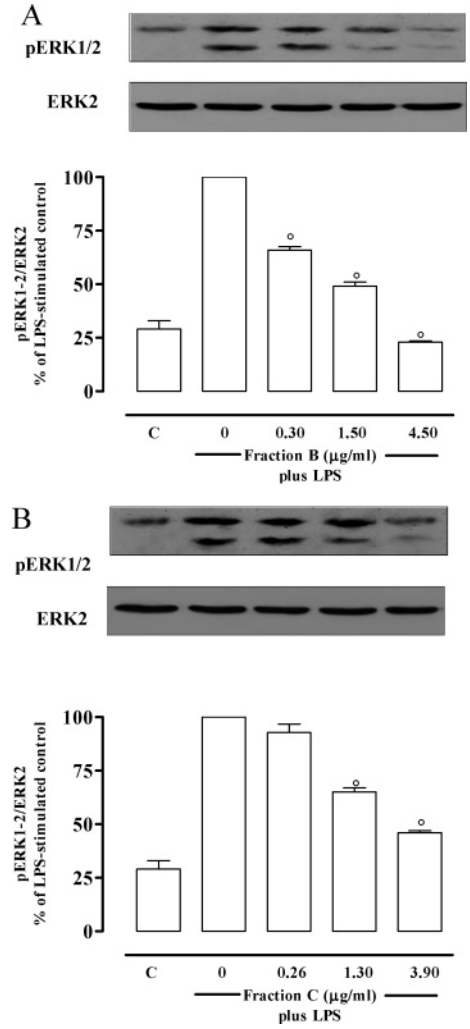

Figure 7. Effect of fractions B (**A**) and C (**B**) of the *F. sellowiana* acetonic extract on LPS-induced ERK-1/2 activation. J774 cells, pretreated (for 2 h) with 0, 0.30, 1.50, and 4.50 μg/mL of fraction B (**A**) and 0, 0.26, 1.30, and 3.90 μg/mL of fraction C (**B**), amounts corresponding to the concentrations present in the acetonic extract, were stimulated with LPS (10 μg/mL) for 15 min. Cell lysates and western blot analysis were performed as described (see Materials and Methods). The bands were quantified by densitometric analysis, and the results are expressed as a percentage of LPS-stimulated cells. Densitometric analysis of protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of ERK-2 levels. °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

the concentration of 1.30 μg/mL (29 and 35%, respectively). At the highest concentration utilized the inhibition of IκBα degradation was 50% (**Figure 6B**, $P < 0.001$, $n = 3$), whereas the inhibition of ERK-1/2 activation was 54% (**Figure 7**B, $P < 0.001$, $n = 3$).

Effect of Fraction B and C Constituents on LPS-Induced Nitrite Production. To characterize the chemical composition of active fractions, fractions B and C were subjected to spectroscopic analysis. The results indicated that fraction B consisted entirely of pure flavone, as resulting by comparison of its spectroscopic data (UV, NMR, and MS) with literature values (*19*). Fraction C, which was analyzed by GC and GC-MS, consisted mainly of stearic acid (28%) and linoleic acid (13%). The other components of fraction C were impurities or compounds devoid of any biological interest, such as phthalates and hydrocarbons. An aliquot of standard flavone, stearic acid, and linoleic acid was subsequently assayed for anti-inflammatory activity at the same concentrations present in each fraction.

Flavone was tested at the concentrations of 0.30, 1.50, and 4.50 μg/mL, stearic acid at the concentrations of 0.073, 0.365, and 1.095 μg/mL, and linoleic acid at the concentrations of 0.034, 0.170, and 0.510 μg/mL.

Similarly to what observed with fraction B, pretreatment of the LPS-stimulated cells with increasing concentrations of flavone (0.30, 1.50, and 4.50 μg/mL) inhibited nitrite production in a concentration-dependent manner (**Figure 8A**, $P < 0.001$, $n = 3$) with IC_{50} of 1.24 μg/mL (95% IC = 0.501−3.064 μg/mL; $r^2 = 0.9969$). Moreover, the effect of fraction C seems to be due to stearic acid (**Figure 8B**), because the LPS-induced nitrite increase in J774 macrophages was not inhibited by linoleic acid (**Figure 8C**), but only by stearic acid. In fact, similarly to what was observed with fraction C, stearic acid (0.073, 0.365, and 1.095 μg/mL) inhibited nitrite production in a concentration-dependent manner, but only from the concentration of 0.365 μg/mL, with $IC_{50} = 0.98$ μg/mL (95% IC = 0.51−1.88 μg/mL; $r^2 = 0.9979$).

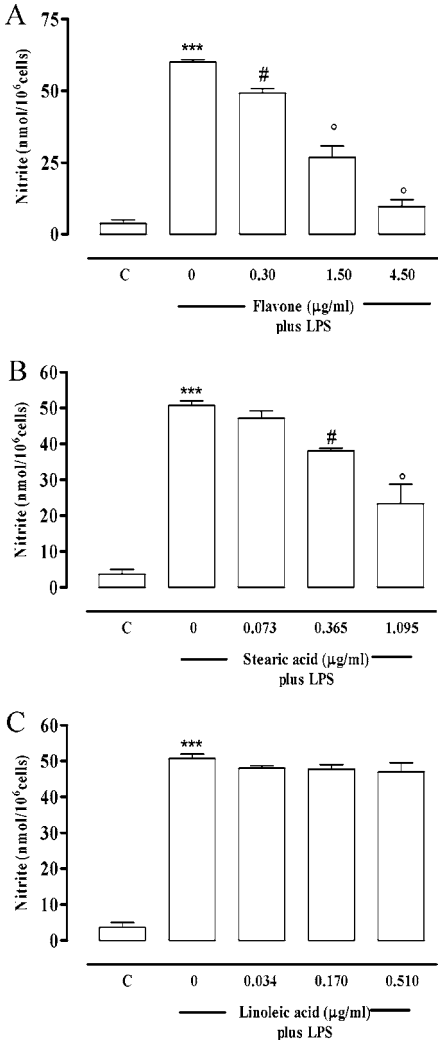

Figure 8. Effect of flavone (**A**), stearic acid (**B**), and linoleic acid (**C**) on nitrite production. J774 cells were pretreated for 2 h with flavone (0, 0.30, 1.50, and 4.50 μg/mL, amounts corresponding to the concentrations present in fraction B) (**A**), stearic acid (0, 0.073, 0.365, and 1.095 μg/mL, amounts corresponding to the concentrations present in fraction C) (**B**), and linoleic acid (0, 0.034, 0.170, and 0.510 μg/mL, amounts corresponding to the concentrations present in fraction C) (**C**) prior to LPS stimulation (10 μg/mL) for 24 h. Nitrites, stable end-products of NO, were measured by the Griess reaction. Data are expressed as mean ± SEM from three separate experiments performed in triplicate. ***, $P < 0.001$ versus unstimulated cells (C); #, $P < 0.05$; °, $P < 0.001$ versus LPS alone (0 μg/mL acetonic extract from *F. sellowiana* fruit).

DISCUSSION

Free radicals have aroused significant interest among scientists in the past decade because of their broad range of effects in biological systems. Moreover, it has been proved that they may be important in the pathogenesis of certain diseases and aging. In fact, there are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (*20*). Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention toward the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidant activity. For example, it has been demonstrated that *F. sellowiana* fruits possess antioxidant activity (*7*).

F. sellowiana Berg., also called the pineapple guava, belongs to the Myrtaceae family and has been cultured mainly in the tropics and subtropics such as southern Brazil, Uruguay, Paraguay, and northern Argentina. This plant has a pleasant flavor, and it is eaten raw, stewed, in jams and jelly, or as juice. It has different biological activities (*6*−*8*) that seem particularly ascribed to the fruit. The fruit contains many compounds including terpenes, tannins, quinines, steroidal saponins, and flavonoids. Like all exotic fruits, *F. sellowiana* contains large amounts of ascorbic acid, hydrocarbons, and minerals. In particular, it has been suggested that the antioxidant activity of *F. sellowiana* could depend on the content of flavonoids as well as on ascorbic acid (*7*).

In addition to radical oxygen species, overproduction of NO, due to the expression of iNOS, also plays an important role in inflammation. Moreover, during inflammatory responses, increased NO levels derived from iNOS activity result in the formation of peroxynitrite after reaction with oxygen free radicals. In particular, in a number of pathophysiological conditions associated with inflammation or oxidant stress, peroxynitrite has been proposed to mediate cell damage (*21*). Moreover, recently, it has been suggested that iNOS overexpression may be intimately involved in the pathogenesis of many diseases, such as colon cancer, multiple sclerosis, neurodegenerative diseases, and heart infarction. The iNOS derived from macrophages is predominantly responsible for the overproduction of NO in injured tissues and inflammation processes. Thus, to study the anti-inflammatory activity of *F. sellowiana* we have utilized the murine macrophage cell line J774, which expresses iNOS, following stimulation with LPS. Because the literature reports (*6, 7*) that biological activity of *F. sellowiana* seems to be ascribed mainly to the acetonic extract of fruits, which appears to be the most effective among all of the extracts studied, we have decided to deepen the study of anti-inflammatory activity of this extract.

Our results demonstrate that *F. sellowiana* acetonic extract exhibits anti-inflammatory activity because it inhibits in J774 cells the LPS-induced NO production and iNOS expression probably at the transcriptional level as demonstrated by the decrease in LPS-induced IκBα degradation and ERK-1/2 phosphorylation. The effect on NO production does not seem to be related to a direct action on iNOS activity, because when added to the cells 24 h after LPS, the extract failed to inhibit nitrite production.

Analysis of the biological activity of the fractions obtained through a chromatographic purification of the extract has shown that only 2 of the 11 fractions, fractions B and C, are responsible for the inhibitory activity. The finding that the chemical constituent responsible for the action of fraction B was flavone was not surprising because an inhibitory action on NO production by flavone and its derivatives has been reported in the literature in several types of cells including LPS-stimulated murine peritoneal macrophages (*22*, *23*). With regard to the component of fraction C that mimicked the anti-inflammatory action of the extract, our results partially agree with the data reported in the literature. In our experimental conditions stearic, but not linoleic, acid inhibited the NO pathway. In the literature contrasting results are present with regard to linoleic acid, which seem to be related to the different cell types utilized. Indeed, it has been demonstrated that linoleic acid inhibits NO production in bovine aortic endothelial cells (*24*) and NO metabolism in humans (*25*), whereas in combination with IFN-γ it induces NO synthesis in hepatocytes (*26*). Our data on linoleic acid effect are in agreement with those shown by Ohata in LPS-stimulated

macrophage cell RAW.264 (*27*), but not with what was demonstrated by the same author concerning stearic acid, which was ineffective in his cellular system, supporting the view of a certain type of specificity with respect to the experimental conditions (i.e., cell type) utilized. Moreover, it must be emphasized that, actually, few results concerning the effect of stearic acid on inflammation model, both in vitro and in vivo, and particularly on the NO pathway, are present in the literature.

It is well-known that NO produced by iNOS in J774 macrophages is regulated at the transcriptional level, predominantly via a NF-κB-mediated pathway, and it is interesting to note that NF-κB is involved in the induction of iNOS also in humans (*28*). Our results demonstrate that the acetonic extract from *F. sellowiana* fruit as well as fractions B and C decrease iNOS protein by inhibition of LPS-induced IκBα degradation and thus NF-κB activation. Accumulating evidence indicates that NF-κB activation, which is essential for gene transcription regulation, is modulated by MAPK/ERK kinase kinase-1 (MEKK-1). In fact, the activation of ERK-1 and -2 has been shown to be involved, in murine macrophages (*29*), in the stimulation of NF-κB activity and iNOS expression through phosphorylation and subsequent degradation of the inhibitory subunit IκBα (*15*). Moreover, it has been demonstrated, in mouse macrophages and in human colon epithelial cells (*30*), that the inhibition of ERK-1/2 directly suppresses endotoxin-induced NO synthesis. Our results demonstrate that the acetonic extract from *F. sellowiana* fruit, and particularly fractions B and C, inhibited in a concentration-dependent manner both IκBα degradation and ERK-1/2 activation. Thus, the acetonic extract seems to inhibit iNOS expression by attenuating IκBα degradation via ERK-1/2 or by inhibiting directly ERK-1/2 activation or by both mechanisms.

In conclusion, our study demonstrates for the first time that the acetonic extract from *F. sellowiana* fruit is able to inhibit NO production because of the presence of flavone and stearic acid. The mechanism of this inhibition seems to be due to an action on the expression of the enzyme, and not on its activity, through the attenuation of NF-κB and/or MAPK activation.

ABBREVIATIONS USED

NO, nitric oxide; NOS, nitric oxide synthases; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκB, inhibitor κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinases; ERK-1/2, extracellular signal-regulated kinase 1/2; NAP, Herbarium Neapolitanum; GC, gas chromatography; MS, mass spectrometry; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); MEKK-1, MAPK/ERK kinase kinase-1.

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LITERATURE CITED

(1) Binder, R. G.; Flath, R. A. Volatile components of pineapple guava. *J. Agric. Food Chem.* **1989**, *37*, 734−736.

(2) Salvo, F.; Toscano, M. A.; Dugo, G. Chemical composition of *Feijoa sellowiana* fruit. *Riv. Soc. Ital. Sci. Aliment.* **1987**, *16*, 471−474.

(3) Ruberto, G.; Tringali, C. Secondary metabolites from the leaves of *Feijoa sellowiana* Berg. *Phytochemistry* **2004**, *65*, 2947−2951.

(4) Lintas, C.; Cappelloni. Dietary fiber content of Italian fruit and nuts. *J. Food Compos. Anal.* **1992**, *5*, 146−151.

(5) Homnava, A.; Rogers, W.; Eitenmiller, R. R. Provitamin A activity of specialty fruit marketed in the United States. *J. Food Compos. Anal.* **1990**, *3*, 119−133.

(6) Basile, A.; Vuotto, M. L.; Violante, U.; Sorbo, S.; Martone, G.; Castaldo-Cobianchi, R. Antibacterial activity in *Actinidia chinensis*, *Feijoa sellowiana* and *Aberia caffra*. *Int. J. Antimicrob. Agents* **1997**, *8*, 199−203.

(7) Vuotto, M. L.; Basile, A.; Moscatiello, V.; De Sole, P.; Castaldo-Cobianchi, R.; Laghi, E.; Ielpo, M. T. L. Antimicrobial and antioxidant activities of *Feijoa sellowiana* fruit. *Int. J. Antimicrob. Agents* **2000**, *13*, 197−201.

(8) Motohashi, N.; Kawase, M.; Shirataki, Y.; Tani, S.; Saito, S.; Sakagami, H.; Kurihara, T.; Nakashima, H.; Wolfard, K.; Mucsi, I.; Varga, A.; Molnar, J. Biological activity of feijoa peel extract. *Anticancer Res.* **2000**, *20*, 4323−4329.

(9) Szabo, C. Alterations in nitric oxide production in various forms of circulatory shock. *New Horiz.* **1995**, *3*, 2−32.

(10) Ohshima, H.; Bartsch, H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **1994**, *305*, 253−264.

(11) Alderton, W. K.; Cooper, C. E.; Knowles, R. G. Nitric oxide synthases: structure, function, and inhibition. *Biochem. J.* **2001**, *357*, 593−615.

(12) Wang, Y.; Marsden, P. A. Nitric oxide synthases: gene structure and regulation. *Adv. Pharmacol.* **1995**, *34*, 71−90.

(13) Lowenstein, C. J.; Alley, E. W.; Raval, P.; Snowman, A. M.; Snyder, S. H.; Russel, S. W.; Murphy, W. J. Macrophages nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9730−9734.

(14) Griseavage, J. M.; Wilk, S.; Ignarro, L. J. Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-kappa B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3308−3312.

(15) Surh, Y. J.; Chun, K. S.; Cha, H. H.; Han, S. S.; Keum, Y. S.; Park, K. K.; Lee, S. S. Molecular mechanism underlying chemopreventive activities of anti-inflammatory ohytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mutat. Res.* **2001**, *480*, 243−268.

(16) Hirano, M.; Osada, S.; Aoki, T.; Hirai, S.; Hosaka, M.; Inoue, J.; Ohno, S. MEK kinase is involved in tumor necrosis factor alpha-induced NF-kappaB activation and degradation of IkBα. *J. Biol. Chem.* **1996**, *271*, 13234−13238.

(17) Jennings, W.; Shibamoto, T. *Qualitative Analysis of Flavour and Fragrance Volatiles by Glass Capillary Gas Chromatography*; Academic Press: New York, 1980.

(18) Adams, R. P. *Identification of Essential Oils Components by Gas Chromatography/Mass Spectroscopy*; Allured Publishing: Carol Stream, IL, 1995.

(19) Agrawal, P. K. *Carbon-13 NMR of Flavonoids*; Elsevier: London, U,K., 1989.

(20) Rose, W. M.; Creighton, M. O.; Stewart, D. H. P. J.; Sanwal, M.; Trevithick, G. R. In vivo effects of vitamin E on cataractogenesis in diabetic rats. *Can. J. Ophtalmol.* **1982**, *17*, 61−66.

(21) Crow, J. P.; Beckman, J. S. The role of peroxynitrite in nitric oxide-mediated toxicity. *Curr. Top. Microbiol. Immunol.* **1995**, *196*, 57−73.

(22) Krol, W.; Czuba, Z. P.; Threadgill, M. D.; Cunningham, B. D.; Pietsz, G. Inhibition of nitric oxide (NO) production in murine macrophages by flavones. *Biochem. Pharmacol.* **1995**, *50*, 1031−1035.

(23) Garcia-Mediavilla, V.; Crespo, I.; Collado, P. S.; Esteller, A.; Sanchez-Campos, S.; Tunon, M. J.; Gonzalez-Gallego, J. The anti-inflammatory flavones quercetin and kaempferol cause

inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappa B pathway in Chang liver cells. *Eur. J. Pharmacol.* **2007**, *28*, 221−229.

(24) Kuroda, R.; Hirata, K.; Kawashima, S.; Yokoyama, M. Unsaturated free fatty acids inhibit Ca^{2+} mobilization and NO release in endothelial cells. *Kobe J. Med Sci.* **2001**, *47*, 211−219.

(25) Turpeinen, A. M.; Basu, S.; Mutanen, M. A high linoleic acid diet increases oxidative stress in vivo and affects nitric oxide metabolism in humans. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1998**, *59*, 229−233.

(26) Liang, J. F.; Akaike, T. Protective effect of linoleic acid on IFN gamma-induced cellular injury in primary culture hepatocytes. *J. Biochem.* **1998**, *123*, 213−218.

(27) Ohata, T.; Fukuda, K.; Takahashi, M.; Sugimura, T.; Wakabayashi, K. Suppression of nitric oxide production in lipopolysaccharide-stimulated macrophage cells by omega 3 polyunsaturated fatty acids. *Jpn. J. Cancer Res.* **1997**, *88*, 234−237.

(28) Zhang, X.; Laubach, V. E.; Alley, E. W.; Edwards, K. A.; Sherman, P. A.; Russell, S. W.; Murphy, W. J. Transcriptional basis for hyporesponsiveness of the human inducible nitric oxide synthase gene to lipopolysaccharide/interferon-gamma. *J. Leukocyte Biol.* **1996**, *59*, 575−585.

(29) Ajizian, S. J.; English, B. K.; Meals, E. A. SpeciWc inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J. Infect. Dis.* **1999**, *179*, 939−944.

(30) Lahti, A.; Lahde, M.; Kankaanranta, H.; Moilanen, E. Inhibition extracellular signal-regulated kinase suppresses endotoxin-induced nitric oxide synthesis in mouse macrophages and in human colon epithelial cells. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 1188−1194.

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