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Original Article

Nitrosative and oxidative anti-stress, and anti-inflammatory potential of *Litopenaeus stylirostris* cytoprotective fraction

Potencial anti-estrés nitrosativo y oxidativo, y anti-inflamatorio de una fracción citoprotectora aislada de *Litopenaeus stylirostris*

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ABSTRACT

Biological activities of a fraction from *Litopenaeus stylirostris* muscle, containing a bioactive novel indolocarbazole alkaloid derivative, eicosapentaenoic acid, and dioctyl phthalate, were determined. Reactive nitrogen species (RNS) scavenging activity was studied by means of the sodium nitroprusside chemical assay, as well as the inhibition of nitric oxide (NO) production and the reduction of reactive oxygen species (ROS) in the RAW 264.7 macrophage cell line, and the regulation of cytokine production in human peripheral blood mononuclear cells, after both cell models were stimulated with *Escherichia coli* lipopolysaccharide. In addition, an *in-silico* analysis was performed to determine the possible interaction of the fraction with proteins related to inflammation. Results showed that the fraction decreases RNS, NO, and the intracellular ROS levels; furthermore, it was found capable of up- and down-regulating anti- and pro-inflammatory cytokines, respectively, suggesting the presence of compounds that could be associated to decreases of nitrosative and oxidative stress, as well as inflammation processes; furthermore, this could be corroborated by predicting the interactions between EPA and selected inflammatory proteins (iNOS, NF-κB and TNF-α).

Keywords: Reactive nitrogen species; reactive oxygen species; nitric oxide; cytokines.

RESUMEN

Se realizaron actividades biológicas de una fracción aislada del músculo de *Litopenaeus stylirostris*, la cual contiene un nuevo compuesto bioactivo derivado del alcaloide indolocarbazol, el ácido eicosapentaenoico y el dioctil ftalato. Se estudió la actividad eliminadora de especies reactivas de nitrógeno (ERN) por medio del ensayo químico de nitroprusiato de sodio, así como la inhibición de la producción de óxido nítrico (ON) y la reducción de especies reactivas de oxígeno (ERO) en la línea celular de macrófago RAW 264.7, y la regulación de la producción de citocinas en células mononucleares de sangre periférica humana, tras ser ambos modelos celulares estimulados con lipopolisacárido de *Escherichia coli*. Además, se realizó un análisis *in-silico* para determinar la posible interacción de la fracción con proteínas relacionadas con la inflamación. Los resultados mostraron que la fracción disminuye los niveles de ERN, ON y ERO; además, se encontró que es capaz de regular positiva y negativamente las citoquinas antiinflamatorias y proinflamatorias, respectivamente; lo que sugiere la presencia de compuestos que podrían estar asociados con disminuciones del estrés nitrosativo y oxidativo, así como con procesos de inflamación; asimismo, esto se pudo corroborar mediante la predicción de las interacciones entre EPA y proteínas inflamatorias seleccionadas (iNOS, NF-κB y TNF-α).

Palabras clave: Especies reactivas de nitrógeno; especies reactivas de oxígeno; óxido nítrico; citocinas.

INTRODUCTION

Accounting for approximately 10 million deceased in recent years, cancer continues as the main cause of deaths in the world (Ferlay *et al*., 2022), with risk factors such as elevated fat consumption, smoking, inflammatory processes, etc., considered forms that contribute to prevent this disease (Alberts *et al*., 2010); this latter factor (inflammation) supports conditions that promotes several cancer-associated cell processes as cellular transformation, survival, proliferation, and metastasis, among others. About 25 % of cancer types are associated to inflammatory developments (Allavena *et al*., 2008; Coussens and Werb, 2002). Inflammation, as a set of factors that involve different physiological radicals that, in general, may raise the nitrosative and oxidative stress of a cell, has been well described (Reuter *et al*., 2010; Valko *et al*., 2006); therefore, reducing either nitrosative or oxidative processes or both, may also contribute to an anti-inflammatory effect (Biswas, 2016; Sies, 2000; Valko *et al*., 2007). At present, the exploration for novel drugs that may control the carcinogenic micro-environment is a continuous task (Albini and Sporn, 2007; Blaylock, 2015; Jezierska-Drutel *et al*., 2013).

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The marine ecosystem covers most of the planet's surface and comprises a vast resource of potentially bioactive compounds, which have been sometimes attributed to extreme environments at which, marine organisms live and, therefore, have to produce metabolites structurally different from those produced by organisms from other environments; therefore, this novel molecules might be of great biotechnological potential (Braekman and Daloze, 1986; Stankevicins *et al*., 2008). Due to the previously mentioned, organisms from the sea have been taken as significant sources of biologically active molecules for both, treatment or prevention of a number of illnesses (Blunt *et al*., 2011; Gerwick and Moore, 2012; Molinski *et al*., 2009). Based on the above, there is an understanding that bioactive compounds from this origin are more expected to end up in obtaining cancer drugs than from those obtained from other environments (Ciavatta *et al*., 2017; Fahmy and Soliman, 2013; Gerwick and Moore, 2012).

Previously, our research group, evaluated the cytoprotective activity (as well as the anti-oxidant) of a fraction (called fM) acquired after a chromatographic separation by open column from an extract (chloroform-soluble mix) isolated from wild shrimp (*Litopenaeus stylirostris*) muscle (García-Romo *et al*., 2020). The fraction fM was structurally studied finding that it mainly consist of dioctyl phthalate (DOP); a compound commonly used as plasticizer (Pivnenko *et al*., 2016; Thomas, 1973; Ventrice *et al*., 2013) and previously reported in marine organisms (Cruz-Ramírez *et al*., 2015; López-Saiz *et al*., 2014), eicosapentaenoic acid (EPA); a polyunsaturated fatty acid that is an FDA-approved drug (Gerwick and Moore, 2012) which was found to make up most of the fraction, and an indolocarbazole alkaloid derivative (IAD); a novel compound first reported by García-Romo *et al*. (2020). However, a full characterization of the bioactivities of this fraction has not been done. The objective of the present research work was to study the nitrosative and oxidative anti-stress, and/or anti-inflammatory potential of fM, earlier described (García-Romo *et al*., 2020), as well as to elucidate the constituents that are accountable for these biological activities.

MATERIAL AND METHODS

Test treatments

Cytoprotective/anti-oxidant fraction (fM)

The isolation of the cytoprotective/anti-oxidant fraction (fM) at different concentrations (25, 50, 100 and 200 µg/mL) and its constituents was carried out as previously described (García-Romo *et al*., 2020).

Controls

In addition to fM, the following compounds were used as constituent controls: eicosapentaenoic acid (EPA) (E-2011, Sigma-Aldrich) and dioctyl phthalate (DOP) (D201154, Sigma-Aldrich). Also, N_{ω}-nitro-L-arginine methyl ester hydrochloride, commonly called L-NAME (N5751, Sigma-Aldrich) and gallic acid (GA) (G7384, Sigma-Aldrich), were employed as positive controls for anti-inflammation and -oxidation potential, respectively.

Sodium nitroprusside assay

To determine the fM potential for scavenging the nitric oxide (NO) radical [related to a reduction in the production of NO-originated reactive nitrogen species (RNS)], the sodium nitroprusside (SNP) test was used (Awad *et al*., 2014). NO is generated from SNP at serum pH, and it associates with O to create nitrite (NO_2^-) (Schroeter *et al*., 2002). Experimentally, using microtitration plates, samples (fM and pure controls: EPA and DOP) (100 to 800 µg/mL in PBS at pH 7.4) and SNP (10 mM), were mixed and subsequently incubated at 25 °C for 2.5 h. Next, the supernatant of each well (100 µL) was combined with Griess reagent [0.1% N-(1-naphthyl)-ethylendiamina, 1 % sulfanilamide in 5 % phosphoric acid] (100 µL) in H_2O for 10 min to determine the NO_2^- generated out of SNP. The absorbance of this mixture was finally measured at 550 nm using a microtitration plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). The equation to calculate the RNS level production (%) is the following:

$$RNS\ level\ production\ (\%) = \frac{(As - Ar)(100)}{Ac - Ar}$$

Ar = Absorbance reagent control (without cells).
Ac = Absorbance of the control (without samples).
As = Absorbance of the samples (with DOP, EPA and fM, and anti-inflammatory and antioxidant controls: L-NAME and GA, respectively).

Test cells

An Abelson murine leukemia virus-transformed commercial cell line titled RAW 264.7 (ATCC® TIB-71™), have being cultivated in Dulbecco's Modified Eagle Medium (DMEM) (D6429, Sigma-Aldrich, St. Louis, MO, USA) with 10 % fetal bovine serum (FBS) (Corning, NY, USA). Peripheral Mononuclear Cells from Human Blood (PBMCs) were isolated by a gradient of density using Ficoll-Paque PLUS (GE Healthcare, Otelfingen, Switzerland) reactive; blood concentration was decreased 1:2 using PBS and layered on top of an equivalent amount of Ficoll-Paque PLUS to be centrifuged at 450 xg for 0.5 h. Next to separation, cells were transferred into test tubes and washed with PBS. Then, they were cultivated in RPMI-1640 medium (R7388, Sigma-Aldrich). All cultures were incubated at 37 °C in 5 % CO_2 (VWR 2325 Water-Jacketed CO_2 Incubator, Pa, USA), and stimulated using lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (L4391, Sigma-Aldrich, St. Louis, MO, USA) at 1 µg/mL for 24 h (this in conjunction with each one of the treatments [fM, and anti-inflammatory and antioxidant controls] at 100 µL final volume).

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

This method was performed corresponding to the producer´s instructions (Roche, cell proliferation kit I, Roche, Cat. No. 11-465-007-001): 5 x 10^4 cells/well of RAW 264.7 cells were suspended in 100 µL-aliquot of medium and placed on flat 96-well microtitration plates for incubation; subsequently, the cells were incubated with various concentrations (25, 50,

100, and 200 µg/mL) of each of the treatments. Controls were cultured in the presence of DMSO (0.5 %) meaning 100 % of cell viability. After a 4-hour of incubation, a 10 µL-aliquot of MTT (5 mg/mL) was added. Sodium dodecyl sulfate (SDS) solution was used to dissolved formazan crystals; cells were let stand overnight, and finally read with a plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA) at 570 nm and 650 nm (test and reference wavelengths, respectively) (Liu *et al*., 2017). This same process was carried out for PBMCs cells, only adjusting cell density to $1x10^5$ (cultured with RPMI 0.5 % FBS and Brefeldin A 1:1000 (Cat. 420601, BioLegend, San Diego, CA, USA)). The equation to calculate the cell viability (%) is the following:

$$Cell\ viability\ (\%) = \frac{(As - Ar)(100)}{Ac - Ar}$$

Ar = Absorbance reagent control (without cells).
Ac = Absorbance of the control (without samples).
As = Absorbance of the samples (with DOP, EPA and fM, and anti-inflammatory and antioxidant controls: L-NAME and GA, respectively).

Nitric oxide measurement assay

The NO measurement assay was carried out according to recommendations from different research works (Gao *et al*., 2012; Lee *et al*., 2013) on macrophage cell line (RAW 264.7). Cells were seeded in 96-well plates (50,000 cells/well) containing each 200 µL DMEM amended with 10 % FBS. Subsequently, they were incubated for a day, discarding the medium after this period. Afterwards, the cells were incubated with LPS (1 µg/mL), either along or without the test treatments for 24 h. Then, a 100 µL-aliquot of the supernatant from each well culture was combined with 100 µL of Griess reagent [0.1% N-(1-naphthyl)-ethylendiamine, 1 % sulfanilamide in 5 % phosphoric acid] and incubated in the the dark for 10 minutes. Finally, nitrite generation was obtained using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA, USA) at an absorbance of 550 nm. The amount of NO related to absorbance was calculated using a $NaNO_3$ standard curve (0–100 µM) in triplicate, each time the reagent was prepared and used in the tests, ensuring that the R^2 was not less than 0.99 and monitoring that it was not less than 20 µM of NO were produced during the tests (Supplementary figure 1). The equation to calculate the NO level production (%) in LPS-stimulated RAW 264.7 cells is the following:

$$NO\ level\ production\ (\%)\ in\ LPS\ stimulated\ RAW\ 264.7\ cells = \frac{(As - Ar)(100)}{Ac - Ar}$$

Ar = Absorbance reagent control (without cells).
Ac = Absorbance of the control (without samples).
As = Absorbance of the samples (with DOP, EPA and fM, and anti-inflammatory and antioxidant controls: L-NAME and GA, respectively).

2, 7-Dichlorofluorescein diacetate assay

The measurement of intracellular oxidative stress in RAW 264.7 cells was carried out by the 2′,7′-dichlorodihydrofluoroscein diacetate (DCFH-DA) assay (Halliwell and Whiteman, 2004). Firstly, to 5 x 10^4 cells suspended on DMEM per well, containing the different concentrations of each test treatment, were seeded on flat 96-well microtitration plates and incubated during 24 h. Subsequently, the cells were exposed to DCFH-DA (10 µM) for 30 min at 37 °C and they were finally washed twice with 100 µL PBS (pH 7.4). Wavelengths of 498 and 530 nm (excitation and emission wavelengths, respectively) were used to measure fluorescence (FLUOstar Omega, BMG Labtech Inc., Ortenberg, Germany). Once these results were obtained, with the aim of observing the impact of the biologically active fraction on reduction of LPS-induced intracellular reactive oxygen species (ROS) generation, cells (stimulated or not with LPS and with a single concentration of the biologically active fraction) and subjected to the same procedure above with DCFH-DA, were visualized with a Leica DMi8 inverted epifluorescence microscope (IEM) (Leica Microsystems GmbH, Wetzlar, Germany) using the following: 480/40 excitation FITC and 527/30 emission filters, a DFC 450C camera (Leica Microsystems, Wetzlar, Germany) and overlay software (LAS AF version 3.1.0, Leica Microsystems CMS GmbH, Mannheim, Germany). The equation to calculate the intracellular ROS level production (%) in LPS-stimulated RAW 264.7 cells is the following:

$$Intracellular\ ROS\ level\ production\ (\%)\ in\ LPS\ stimulated\ RAW\ 264.7\ cells = \frac{(As - Ar)(100)}{Ac - Ar}$$

Ar = Absorbance reagent control (without cells).
Ac = Absorbance of the control (without samples).
As = Absorbance of the samples (with DOP, EPA and fM, and anti-inflammatory and antioxidant controls: L-NAME and GA, respectively).

***In-silico* molecular docking**

Three-dimension (3D) structure of eicosapentaenoic acid (PubChem CID: 446284) for the study of its *in-silico* interaction with selected protein receptors was downloaded from PubChem database. In support of the receptors, 3D structures of NF-κB (3U21), iNOS (4D10), and TNF-α (4TSV) were downloaded from the protein databank. The top 3D structure, established on their best biding energy, was selected (Supplementary Table 1). This assay was performed according to Luna-Vital *et al*. (2017), top binding sites were calculated by MetaPocket 2.0 online utility (Zhang *et al*., 2011), and the estimation of docking events was done using AutoDock tools (Trott and Olson 2010). Finally, top docking binding conformation was graphed using the Discovery Studio Visualizer v. 19.1.0.18287 software (Dassault Systèmes, Vélizy-Villacoublay, France).

Cytokine detection assay

The expression of IL-4, IL-6, IL-8 and IL-10 was assayed using a mouse anti-human antibody (Chávez-Sánchez *et al*., 2010). Human peripheral blood mononuclear cells (PBMCs) were adjusted to $1x10^5$ PBMCs and cultured with RPMI 0.5 % FBS and Brefeldin A 1:1000 (Cat. 420601, BioLegend, San Diego, CA, USA) per assayed treatment. Cells were observed in the

Table 1. Effect of different concentrations (µg/mL) of either fM, DOP, EPA, L-NAME, or GA on Reactive Nitrogen Species (RNS) level production (%) derived from nitric oxide (NO) using sodium nitroprusside (SNP) assay.
Tabla 1. Efecto de fM, DOP, EPA, L-NAME y GA sobre los niveles de producción de especies reactivas de nitrógeno (ERN) (%) derivadas del óxido nítrico (ON) mediante el ensayo de nitroprusiato de sodio (NPS).

	(µg/mL)			
	100	**200**	**400**	**800**
fM	100 ± 1.7 cB	100 ± 8.1 cB	90.3 ± 2.1 cAB	77.8 ± 8.0 cA
DOP	70.9 ± 3.1 bB	66.3 ± 1.5 bAB	64.1 ± 1.7 bA	62.0 ± 0.5 bA
EPA	100 ± 5.7 cA	100 ± 4.5 cA	100 ± 1.1 dA	96.2 ± 6.8 dA
L-NAME	100 ± 0.7 cA	100 ± 6.1 cA	91 ± 4.8 cA	89 ± 3.3 cdA
GA	33.7 ± 1.1 aC	30.2 ± 3.6 aC	24.3 ± 0.6 aB	11.6 ± 1.8 aA

Values represent means ± S.D. from three determinations. Different lowercase letters within the column and capital letters within rows are significantly different ($P \leq 0.05$); Tukey´s least significant difference test. Cytoprotective fraction (fM), dioctyl phthalate (DOP) and eicosapentaenoic acid (EPA) were used as standard controls. Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and gallic acid (GA) were used as anti-inflammatory and antioxidant controls, respectively.

presence of fM, before and after being activated with LPS (during 24 h). Afterward, the cells were treated with inflammatory fluorescent markers (monoclonal antibodies): FITC anti-human IL-4 Clone (MP4 25D2) which reacts with human IL-4 (interlukin-4) (Cat. 500806, BioLegend, San Diego, CA, USA), PE anti-human IL-10 Clone (JES3-9D7) that reacts with human IL-10 (interlukin-10) (Cat. 501404, BioLegend, San Diego, CA, USA), FITC anti-human IL-6 Clone (MQ2-13A5) which reacts with human IL-6 (interlukin-6) (Cat. 501104, BioLegend, San Diego, CA, USA), and PE anti-human IL-8 Clone (E8N1), a marker that reacts with human IL-8 (Cat. 511408, BioLegend, San Diego, CA, USA). At the end, 1 x 10^4 events were analyzed by flow cytometry (BD FACSVerse™ Flow Cytometer - BD Biosciences NY, USA). Data was presented as a means of Relative Fluorescence Intensity (RFI).

Statistical analysis

All assays were performed in triplicate, where each one of them was performed in three trials individually and evaluated using an analysis of variance (ANOVA) test, by means of a Tukey's post hoc multiple comparisons test, defining at 95 % confidence interval and significance level of $P \leq 0.05$ (by a SPSS program). Entire data was described as mean values ± standard deviation.

RESULTS AND DISCUSSION

Inhibition of reactive nitrogen species (RNS) production

The inhibition of RNS production is commonly associated to anti-stress nitrosative activity (Awad *et al*., 2014; Schroeter *et al*., 2002), and different techniques have been implemented to evaluate this parameter. This determination was performed employing the Griess reagent. The determination of NO radical scavenging potential was performed to 1) assess the antioxidant potential on biological reactive species (as RNS), and 2) to assure that any decrease in NO level production (%) was due to an actual effect on anti-inflammatory regulation processes and not just to the scavenging of the NO radical present in the supernatant. There are anti-oxidant systems that affords cell protection against damage related to ROS; at specific environments, these systems could be deficient or incorrect and toxicity might occur due to the rise of ROS (Muravchick, 2006). Therefore, when the NO is mixed with natural radicals, as superoxide (O_2^-), subsequently generates peroxynitrite ($ONOO^-$) and it may induce apoptosis as ROS does (Brüne *et al*., 2003; Weigert and Brüne, 2008). The peroxynitrite radical induces irreversible enzymatic inactivation, protein nitration, genetic insult, damage in mitochondria and in other cell components; all of these negative impacts are either by direct interaction or are subsequent to peroxidation of cell lipids (Muravchick, 2006; Peng *et al*., 2019); for all this, NO radical is considered an important toxic intermediary (Moncada, 1991).

The effect of the fM and controls on the production of RNS and its decrease (%) as anti-nitrosative potential is shown on Table 1. About the controls, GA at 800 µg/mL (highest concentration tested), showed the highest anti-nitrosative potential, allowing the production of no more than 11.6 % RNS. The GA belongs to hydrolysable tannins (which has numerous hydroxyl functional group substitutions within its phenolic ring structure), which confers it a great anti-oxidant potential (Badhani *et al*., 2015).

Instead, DOP also showed anti-nitrosative potential at all concentrations tested. The anti-radical activity of compounds often relates to their ability to donate electrons or hydrogen atoms to free radicals, resulting in radical neutralization. DOP, like other phthalates, has a chemical structure that may allow interaction with reactive species and potentially scavenge these harmful molecules. Some studies have indicated that derivatives of phthalates, including DOP, can exhibit biological activities that may be included through antioxidant effects. For instance, a study highlighted that certain phthalate derivatives extracted from marine organisms showed antimicrobial properties, which could be linked to their ability to combat oxidative stress in microbial cells (Shafeian *et al*., 2022).

The fM, showed anti-nitrosative potential just at 400 and 800 µg/mL (the highest concentrations tested); this might be attributed to the presence of DOP, however, according to Garcia-Romo *et al*. (2020), the chemical characterization of fM showed that DOP is a minoritarian compound, therefore, the inhibition of the generation of RNS might be associated to the presence of IAD since EPA standard failed to show any inhibitory potential. These results suggest that fM has the capability to decrease RNS levels, which is directly related to decreases of cellular stress. Cellular stress is directly related to interferences with an inflammatory process. This assay showed that fM at 100 - 200 µg/mL did not bind NO, which suggests that in the subsequent anti-inflammation tests, decreases in NO production are not attributable to fM.

Inhibition of cell viability

In order to discard that any decrease of NO might be attributable to cell mortality during the evaluation of the anti-inflammation potential, the MTT assay was performed to establish the possible cytotoxicity resulting in RAW 264.7 cells (Chun *et al*., 2007; Khajuria *et al*., 2017; Lee *et al*., 2013). This assay is used to estimate the metabolic status of cells, this due to NADPH-dependent cellular oxidoreductase enzymes action, which may be used to estimate the proportion of metabolically active cells, which reflect viability (Stockert *et al*., 2018); consequently, the reduction of cell viability indicates a cytotoxic effect. The results showed that, at 25 and 50 µg/mL (the lowest concentrations tested), none of the samples decreased cell viability (Table 2). With respect to fM, a significant ($P \leq 0.05$) decrease was observed only at greater concentrations used (100 µg/mL); this observation suggests that fM is not cytotoxic for RAW 264.7 cells at lowest fM concentrations, also possibly indicating that cell toxicity would not be an influence through the following anti-inflammation assays performed at fM concentrations lower than 50 µg/mL.

The RAW 264.7 cell line has high efficiency for DNA transfection, easy propagation, sensitivity to RNA interference, and supports replication of murine noroviruses (ATCC, 2018), which provides this cell line a good model to evaluate inflammation processes. Nevertheless, the inhibition of its proliferation is associated with beneficial effects, as anti-carcinogenesis as an example (Luque-Alcaraz *et al*., 2012). Therefore, these results could suggest that fM has the potential to stop the growth of cancer cells.

Inhibition of NO production

Aiming to determine the anti-inflammatory potential based on the decrease of NO production (%), the measuring of NO was performed through the quantification of the nitrite concentration by the Griess reagent assay. Nitric oxide synthases (NOS) family synthetizes NO, which is a signaling molecule that has several functions such as participation in the defense system, viral replication, and as regulator in inflammatory environments responses (Kim *et al*., 2011). About the latter, the reduction of NO generation suggests anti-inflammatory potential (Korhonen *et al*., 2005; Moncada, 1991; Vane *et al*., 1994). Results showed that every one of the samples decreased NO (%), actually, DOP achieved the lowest ratio of inhibition of NO production, significantly ($P \leq 0.05$) different from the rest of the compounds tested (Table 3), these suggests that DOP lacks anti-inflammatory activity (at least, by means of this pathway). On the other hand, L-NAME and GA had an effect as positive controls, decreasing NO production in a dose-dependent way.

The above mentioned is in agreement with L-NAME be an inhibitor of NO synthesis (Wang *et al*., 2000), and GA is recognized as an anti-oxidant (Locatelli *et al*., 2013). Also, EPA had the capability of inhibiting the NO (%) as its concentration increased, this is consistent with that previously reported for EPA as an inhibitor of the inflammatory environment, which has been correlated to a supplantation of arachidonic acid in the cell membranes (Bagga *et al*., 2003). Also, the activity is related to the potential to decrease nuclear factor kappa B (NF-κB) expression (Novak *et al*., 2003), tumor necrosis factor alpha (TNF-α) (Babcock *et al*., 2002a; Babcock *et al*., 2002b) and NO (%) in LPS-stimulated macrophages due to changed iNOS protein expression (Aldridge *et al*., 2008).

As well as GA, L-NAME and EPA, fM was also capable of inhibiting NO production at 25 and 50 µg/mL, fM concentrations found not to cause cell toxicity; nevertheless, this potential was not different ($P \leq 0.05$) from EPA; hence, fM behaved in a similar way to EPA, suggesting that the other

Table 2. Effect of different concentrations (µg/mL) of either fM, DOP, EPA, L-NAME, or GA on RAW 264.7 cell viability (%) at 24 h of incubation.

Tabla 2. Efecto de fM, DOP, EPA, L-NAME y GA sobre la viabilidad de las células RAW 264,7 (%) a las 24 h de incubación.

	Testing compound concentration (µg/mL)			
	25	**50**	**100**	**200**
fM	100 ± 6.1 aC	100 ± 12.4 aC	80.6 ± 1.6 bB	59.9 ± 1.7 cA
DOP	100 ± 4.3 aA	100 ± 3.7 aA	100 ± 11.9 bA	100 ± 2.0 dA
EPA	100 ± 10.7 aB	100 ± 3.3 aB	49.3 ± 1.0 aA	38.0 ± 1.6 aA
L-NAME	100 ± 6.1 aA	100 ± 4.9 aA	100 ± 15.0 bA	100 ± 1.9 dA
GA	100 ± 6.1 aB	89.6 ± 15.7 aB	71.3 ± 16.5 abAB	49.1 ± 2.2 bA

Values represent means ± S.D. from three determinations. Different lowercase letters within the column and capital letters within rows are significantly different ($P \leq 0.05$); Tukey´s least significant difference test. Control cell cultures were incubated with DMSO (0.5 %) and represent 100 % viability. Cytoprotective fraction (fM), dioctyl phthalate (DOP) and eicosapentaenoic acid (EPA) were used as standard controls. N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) and gallic acid (GA) were used as anti-inflammatory and antioxidant controls, respectively. LPS control (1 µg/mL) had viability of 99.7 ± 8.0 %.

Table 3. Effect of different concentrations (µg/mL) of either fM, DOP, EPA, L-NAME, or GA on nitric oxide (NO) level production (%) in LPS-stimulated RAW 264.7 cells at 24 h of incubation.
Tabla 3. Efecto de fM, DOP, EPA, L-NAME y GA sobre los niveles de producción de óxido nítrico (ON) (%) en células RAW 264.7 estimuladas con LPS a las 24 h de incubación.

	Testing compound concentration (µg/mL) + LPS (1 µg/mL)			
	25	50	100	200
fM	63.0 ± 9.7 aC	46.4 ± 4.1 abB	38.7 ± 5.5 abAB	28.2 ± 1.7 abA
DOP	100 ± 10.6 bA	100 ± 7.2 dA	90.8 ± 4.9 dA	86.7 ± 13.9 dA
EPA	70.7 ± 5.7 aC	43.2 ± 9.8 aB	27.3 ± 3.5 aAB	27.0 ± 3.1 aA
L-NAME	79.8 ± 2.8 aC	75.3 ± 7.3 cBC	66.0 ± 5.8 cB	45.4 ± 1.7 bcA
GA	74.7 ± 3.6 aC	65.1 ± 6.6 bcBC	52.6 ± 8.9 bcAB	46.3 ± 3.7 cA

Values represent means ± S.D. from three determinations. Different lowercase letters within the column and capital letters within rows are significantly different ($P \leq 0.05$); Tukey´s least significant difference test. All the treatments were compared with control (cells + LPS at 1 µg/mL), which represent 100 % of NO production. Absorbance values were contrasted to a $NaNO_3$ standard curve (R^2= 0.9986), where the maximum production of NO was 0.5 µg/mL. Untreated negative control cell cultures were incubated only with DMSO (0.5 %) and represents the natural NO production of the cells (26.6 ± 4.7) due to spontaneous stress. Cytoprotective fraction (fM), dioctyl phthalate (DOP) and eicosapentaenoic acid (EPA) were used as standard controls N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and gallic acid (GA) were used as anti-inflammatory and antioxidant controls, respectively.

components of fM (IAD and EPA) might be the compounds responsible for the inhibition of NO production by fM (with the exception of DOP). However, to confirm the inhibitory potential of IAD, further investigation is needed using IAD in a more highly purified form.

Inhibition of intracellular reactive oxygen species (ROS) production

The inhibition of ROS production is commonly associated to anti-stress oxidative activity (Brüne *et al*., 2003; Feng *et al*., 2001), and different techniques have been implemented for the evaluation of this parameter.

With the purpose of measuring the inhibition of intracellular ROS production, the DCFH-DA assay was performed. As mentioned, the elevated ROS induce oxidative stress, hence, successive inflammation responses, which might start several physiological alterations (Conforti *et al*., 2009). Also, when an inflammatory environment is produced, an extreme amount of reactive oxygen species is produced, causing cell injuries such as damage in DNA, that later causes alterations (Lonkar and Dedon, 2011). As a result, pro-inflammation and ROS production consists of a loop of biochemical reactions with reciprocal promotion; ROS are critical inflammatory mediators, and for this reason, anti-inflammatory potential involves a decrease in ROS production.

All the samples in the present study diminished intracellular ROS (%) (Table 4). Most reduction in ROS production was visualized with GA at its maximum concentration; this agrees with previously published reports, since GA is recognized as a powerful molecule against oxidative stress (Erol-Dayi *et al*., 2012). Besides, this study showed that L-NAME does not have great potential to reduce ROS, compared to other treatments. On the other hand, once all the treatments, at their lowest concentration (25 µg/mL), were compared, fM, as well as DOP and EPA, showed statistically ($P \leq 0.05$) similar activity as GA. This means that fM (composed of IAD,

Table 4. Effect of different concentrations (µg/mL) of either fM, DOP, EPA, L-NAME, and GA on intracellular ROS level production (%) in LPS-stimulated RAW 264.7 cells at 24 h of incubation.
Tabla 4. Efecto de fM, DOP, EPA, L-NAME y GA sobre los niveles de producción de ROS intracelular (%) en células RAW 264.7 estimuladas con LPS a las 24 h de incubación.

	Testing compound concentration (µg/mL) + LPS (1 µg/mL)			
	25	50	100	200
fM	59.5 ± 7.9 aB	55.0 ± 7.6 abB	48.5 ± 5.2 abAB	37.1 ± 5.5 abA
DOP	68.6 ± 1.4 abB	62.0 ± 11.5 abB	58.6 ± 4.2 bcAB	42.2 ± 8.8 abA
EPA	75.8 ± 9.0 abB	50.2 ± 12.7 abAB	45.4 ± 9.9 abA	38.5 ± 10.6 abA
L-NAME	81.0 ± 2.1 bB	74.7 ± 10.6 bAB	68.9 ± 8.7 cAB	54.2 ± 8.5 bA
GA	60.7 ± 9.3 aB	41.8 ± 3.9 aA	31.7 ± 5.2 aA	30.6 ± 4.5 aA

Values represent means ± S.D. from three determinations. Different lowercase letters within the column and capital letters within rows are significantly different ($P \leq 0.05$); Tukey´s least significant difference test. All the treatments were compared to controls (cells + LPS at 1 µg/mL), which represent 100 % of ROS production. Untreated negative control cell cultures were incubated only with DMSO (0.5 %), and they represent cell's natural ROS production level (23.2 ± 11.6) by spontaneous stress. Cytoprotective fraction (fM), dioctyl phthalate (DOP) and eicosapentaenoic acid (EPA) were used as standard controls. N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) and gallic acid (GA) were used as anti-inflammatory and antioxidant controls, respectively.

DOP and EPA), acted in the same way DOP and EPA standards did; thus, the potential of fM to inhibit ROS production might be associated to these two compounds, nevertheless the influence of IAD to this bioactivity remains undetermined. Evidence exists that PUFAs (ω-3) are capable to decrease RNS and ROS levels in LPS-activated macrophages, this capacity has been associated to its potential to regulate enzymes responsible for the production of reactive species (Ambrozova *et al*., 2010); rather the opposite was found in the literature, since there is evidence that phthalic acid can increase ROS levels on murine macrophages (Naarala and Korpi, 2009); then, continuing studying DOP and its potential to decrease ROS is important. Once the potential of fM of reducing cellular ROS levels (quantitatively) was established, this capacity was also qualitatively verified by microscopical examination using a dying technique.

The results showed that fM, at 25 µg/mL, caused a significant reduction in the fluorescence intensity, this when compared to LPS-stimulated cells and in the absence of fM. This observation confirms that, when cells are exposed to LPS, fM contents are capable of decreasing ROS levels; consequently, this would decrease its levels of stress, which is also associated with a reduction in inflammatory processes.

Figure 1 shows that fM, at 25 µg/mL, decreased the fluorescence intensity observed under a microscope (compared to cells stimulated with LPS and without fM). This observation contributes to confirm that, when a cell is exposed to LPS, the incorporation of fM can decrease ROS levels, consequently it will decrease its level of stress, which is also associated with a reduction in inflammatory processes.

Nitric oxide synthase (iNOS) that generates NO (which has important roles in several pathological environments) does not exert regulation over the immune system (Pando and Verma, 2000); however, at high concentrations, NO takes the lead to the rise of reactive oxygen species (Tegeder *et al*., 2001). Activation of inflammatory environments requires a signaling regulated by NF-κB; consequently, this has the capacity of manipulating the expression of genes related to inflammation and immunity, as PGE2, NO, IL-1b, IL-6, and MCP-1 do (Davis *et al*., 2001; Ghosh *et al*., 1998). Later activation, in answer to lipopolysaccharide, is translocated into the nucleus to control transcription (Funk, 2001; Hwisa *et al*., 2013); accordingly, the quest for molecules with the capacity of exerting regulation on these responses is a chief effort of interest in search for means of reducing inflammatory environments.

In summary, fM at low concentration decreases around 50 % of the production of NO and intracellular ROS; however, pure EPA standard also decreased it [there were no statistical differences between them ($P \leq 0.05$)]; this suggests that these biological activities might be attributed only to EPA. Therefore, molecular docking studies (*in silico*) were neces-

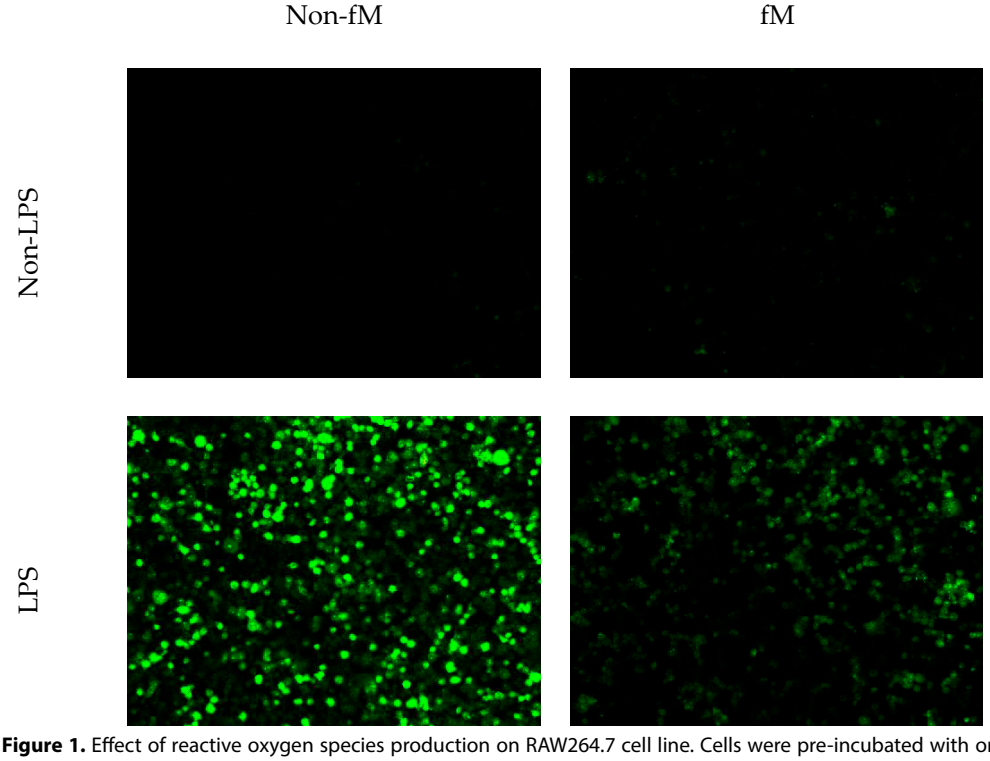

Figure 1. Effect of reactive oxygen species production on RAW264.7 cell line. Cells were pre-incubated with or without fM (25 µg/mL) and activated or not with lipopolysaccharide (LPS, 1 µg/mL) for 24 h. Cells were stained with H_2DCFDA (10 µM) and incubated for 30 min (37 °C). 2, 7-Dichlorofluorescein fluorescence was observed using epifluorescence microscopy (20x).

Figura 1. Efecto de la producción de especies reactivas de oxígeno en la línea celular RAW264.7. Las células se preincubaron con o sin fM (25 µg/mL) y se activaron o no con lipopolisacárido (LPS, 1 µg/mL) por 24 h. Las células se tiñeron con H_2DCFDA (10 µM) e incubaron durante 30 min (37 °C). Se observó fluorescencia de 2, 7-diclorofluoresceína mediante microscopía de epifluorescencia (20x).

sary to know the possible interaction of EPA with some of the proteins involved in the previously reported biochemical reaction cascade (iNOS, NF-κB and TNF-α).

In-silico interactions between EPA and selected inflammatory proteins

Based on the above, studies on the possible interactions of the EPA with different proteins involved in inflammation processes such as iNOS, NF-kB and TNF-α were performed using computer programs. These interactions predict the type of bond that can be formed based on an affinity between a receptor (protein) and a ligand (molecule of interest); these interactions (Van der Waals, alkyl, conventional hydrogen, carbon hydrogen bonds, etc.) occur through amino acid residues and can be compared using a parameter called binding energy, which the greater their negativity (kcal / mol), the greater the affinity among receptor-ligand is.

This indicates that, if there is a coupling with proteins, there may be a change in their conformation, causing an activation or deactivation of these proteins; this is directly related to possible signaling in a specific metabolic pathway, which in this case can be for or against the inflammatory cascade. The results of *in-silico* suggested interactions between EPA and each of the inflammatory proteins iNOS, NF-κB or TNF-α, are shown in figure 2.

EPA showed the highest binding energy (-5.80 kcal/mol) when interacted with the amino acid residues PHE468 (by alkyl bond), ASN466 (by conventional hydrogen bond) and PRO93 (by carbon hydrogen bond) of iNOS protein (Figure 2A). iNOS catalyzes the transition of L-arginine to L-citrulline generating NO from the terminal nitrogen atom of the guanidino group of arginine (Aktan, 2004); this explains why the deactivation of this protein is related to the decrease of NO, and later the decrease of an inflammatory process (Korhonen *et al*., 2005).

A binding energy of – 4.70 kcal/mol was obtained for the interaction of EPA with the amino acids HIS142 (by alkyl bond), ASN139 (by carbon hydrogen bond) and PRO256 (by conventional hydrogen bond) of the NF-κB protein (Figure 2B); NF-κB is a protein complex that modulates DNA transcription and it is associated to the cellular response exerted to stimuli from cytokines, stress, oxidized LDL, ultraviolet radiation, and bacterial or viral antigens. In addition, its regulation (activation or inhibition) is associated to pro- or anti-inflammatory processes; since NF-κB is a molecule acting as a shift button to change inflammation on/off within the organism in response to stress, NF-κB "turn on" genes that cause inflammation (Brasier, 2006; Gilmore, 2006; Schottelius and Baldwin, 1999).

Finally, a binding energy of – 4.20 kcal/mol was obtained for the interaction of EPA with the amino acids SER133, GLU135, ALA134, ILE136, PRO139, GLN25, GLN27, ASN46 and LEU26 (all by Van der Waals bonds) of the TNF- α protein (Figure 2C). TNF- α is a part of cytokines proteins group released by immune system cells that intervene in inflammation process (as well as other pathologies) (González-Flores *et al*., 2014). Also, TNF-α is a protein that has an inflammatory capacity, in addition to being able to modulate the generation of other pro-inflammatory molecules (such as the release of prostaglandins), therefore, its negative regulation is directly related to a decrease in inflammatory activity.

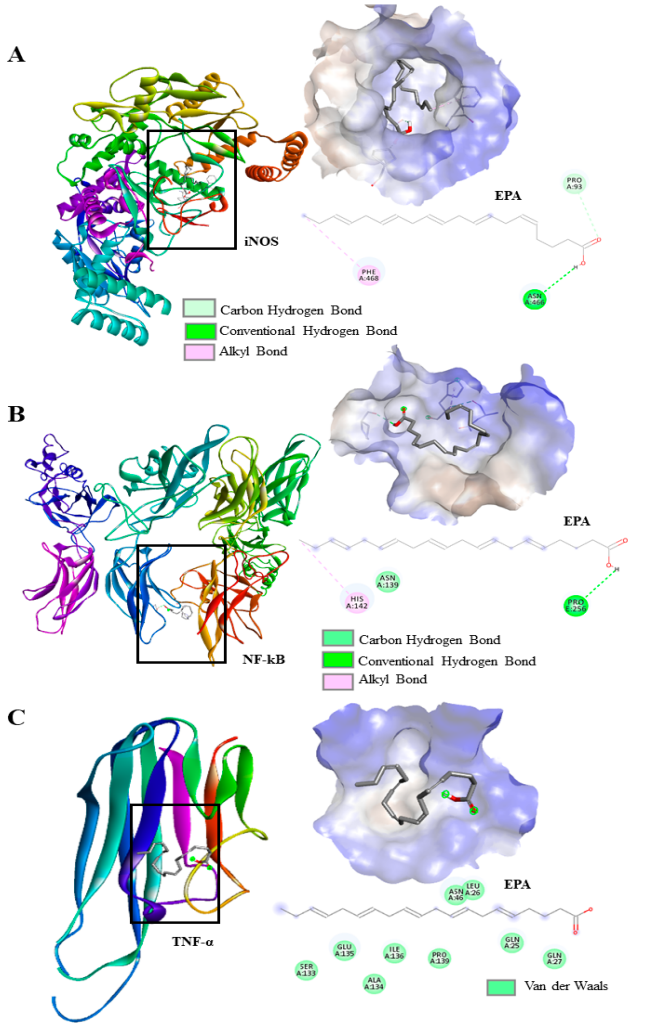

Figure 2. Interactions predicted between EPA and selected inflammatory proteins studied by *in-silico* molecular docking. A) EPA interaction with iNOS, B) EPA interaction with NF-kB, C) EPA interaction with TNF-α.
Figura 2. Predicción de las interacciones entre EPA y proteínas inflamatorias estudiadas mediante acoplamiento molecular *in silico*. A) Interacción de EPA con iNOS, B) Interacción de EPA con NF-kB, C) Interacción de EPA con TNF-α.

All binding energies are described in Supplementary Table 1 and the amino acid residues interacting with EPA are shown in Supplementary Table 2. According to this information, EPA is known to have an affinity for iNOS, NF-κB and TNF-α (all these signals being involved in intermediate metabolic pathways); but, since the expression or suppression of these proteins represents a final reaction of an inflammation process, the study of possible effects of fM on cytokines becomes important.

The inflammation process is a wide cascade of associated biochemical reactions; an example of these reactions is regulation by cytokines; therefore, investigating the possible

capabilities of fM to up- and down-regulate the anti- and/or pro-inflammatory cytokines, respectively, becomes important. For that, it is necessary to have a better understanding whether the above activities (Inhibition of RNS, NO and ROS) are related to direct biochemical reactions of intra cellular inflammatory developments.

Determination of up- and down-regulation in cytokine production

To measure the regulation of peripheral blood mononuclear cells (PBMCs) cytokine production after *in vitro* stimulation with fM and LPS, cytokine production was assayed using a cytokine detection assay (Hernández-Zazueta *et al*., 2023). PBMCs are cells from the innate and adaptive immune systems, mainly composed of lymphocytes and monocytes, including dendritic cells, natural killer cells, T and B cells (Autissier *et al*., 2010). In the innate immune system, monocytes have an important role defending the organism from exogenous and endogenous "dangerous" signals, via pattern recognition receptors (PRR) (Sandor and Buc, 2005). After activation, monocytes can phagocyte, process and present antigens, generate chemokines, and reproduce as a consequence of infection and/or damage (Qureshi *et al*., 2005; Qureshi *et al*., 2012). Through PRR, including Toll-like receptors (TLR), monocytes can recognize constant patterns in pathogens including LPS, teichoic acid, unmethylated CpG DNA sequences, mannose, and double stranded RNA, among others (Sandor and Buc, 2005). The family of TLRs share a common architecture which can lead to the activation of transcription factors, including the NF-κB, which play a modulating role in the induction of survival and pro-inflammatory genes expression (Akira and Kiyoshi, 2004; Akira *et al*., 2001; Arancibia *et al*., 2007), therefore regulating the inflammatory monocytes reaction by pro-inflammatory regulators (MCP-1, TNF-α, IL-1β, IL-6, NO, PGE2 and LTB4) (Qureshi *et al*., 2012). One of the most effective activators of monocytes is LPS (Geng *et al*., 1993; Guha and Mackman, 2001), which immediately stimulates NF-kB, consequently promoting the expression of pro-inflammatory cytokines, IL-6 included; hence, down-regulating it might reduce inflammation events (Furuya *et al*., 2015; Wan and Lenardo, 2010), suggesting anti-inflammatory activity.

Table 5 shows that cell viability on PBMCs does not decrease once they are exposed to fM, but only at its highest concentration (200 µg/mL), where the decrement was below 90 % viability. Also**,** table 5 shows the potential of fM to down-regulate IL-6 and IL-8 following exposure to LPS, evidencing differences ($P \leq 0.05$) when contrasted against control cells, suggesting that fM might have the potential of interfering with the inflammatory events. IL-6 and IL-8 are cytokines released by monocytes that can not only autoactivate them following a retro-alimentary cycle, but also activate B cells and promote specific differentiation of $CD4^+$ T cells, stimulating innate and adaptive responses (Tanaka *et al*., 2014). Therefore, in a practical scenario, fM could in turn be potentially implicated in the modulation of inflammatory responses like those implicated in cancer.

Regulation events of cell cycles, related to inflammation associated developments, not only include regulation of signals that encourage pro-inflammation; additionally, they include signals that promote anti-inflammation and, since LPS can activate PBMCs, they may induce the release of pro-inflammatory molecules including IL-6 and IL-8; they can also promote a decrease in the expression of anti-inflammatory molecules such as IL-4 and IL-10 (Qureshi *et al*., 2012).

Based on the above, the expression of IL-4 and IL-10 in PBMCs was assayed. The fM potential of up-regulating anti-inflammatory molecules (IL-4 and IL-10) is shown in table 5. fM significantly ($P \leq 0.05$) promotes the production of IL-4 and IL-10 showing a dose-depending on response, suggesting that fM might not solely have a regulatory role by intervening in pro-inflammation developments (decreasing IL-6), but also might promote anti-inflammation events (inducing the production of IL-4 and IL-10). As we already mentioned, fM is not only composed of EPA, therefore the activity of

Table 5. Effect of the cytoprotective fraction (fM) on cell viability (%) at 24 h of incubation, and the up-regulation of anti-inflammatory cytokines (IL-4 and IL-10) and down-regulation of pro-inflammatory cytokines (IL-6 and IL-8) on interleukins production, in the activation by LPS-activated human monocytes cells.
Tabla 5. Efecto de la fracción citoprotectora (fM) sobre la viabilidad celular (%) a las 24 h de incubación y la regulación positiva de las citocinas antiinflamatorias (IL-4 e IL-10) y negativa de las citocinas proinflamatorias (IL-6 y IL-8) en la activación de la producción de interleucinas por células de monocitos humanos activados por LPS.

	Cell viability (%)	Relative Fluorescence Intensity (RFI)			
		IL-4	IL-10	IL-6	IL-8
Control	100 ± 2.74 a	421.7 ± 6.7 b	192.3 ± 12.9 ab	181.0 ± 4.0 a	355.66 ± 8.14 a
LPS	99.00 ± 0.37 a	287.0 ± 3.5 a	114.3 ± 8.5 a	811.3 ± 2.1 d	2234.00 ± 96.19 a
fM (µg/mL)					
25	97.39 ± 1.27 a	509.7 ± 12.7 c	226.3 ± 12.0 bc	243.3 ± 4.0 c	2626.40 ± 360.85 a
50	95.90 ± 0.67 a	532.0 ± 8.9 c	315.0 ± 6.0 c	194.0 ± 1.0 b	1705.00 ± 244.41 a
100	94.60 ± 1.01 a	626.7 ± 17.9 d	588.0 ± 72.1 d	188.0 ± 3.6 ab	1109.00 ± 95.84 a
200	86.33 ± 5.03 a	978.3 ± 28.2 e	591.0 ± 31.2 d	185.3 ± 6.4 ab	814.66 ± 185.39 a

Values represent means ± S.D. of RFI from three determinations. Different letters within the column are significantly different ($P \leq 0.05$); Tukey´s least significant difference test. LPS control cells means cells + LPS at 1 µg/mL. Control untreated cell cultures were incubated only with DMSO (0.5 %); also, control cell cultures were incubated with DMSO (0.5 %) and represent 100 % viability. Treated cells were preincubated with fM (µg/mL) and activated with lipopolysaccharide (LPS, 1 µg/mL) for 24 hr.

regulating cytokines may be due to a possible cooperative action of all its components (including IAD); however, to confirm this, testing these components in their isolated form is necessary.

However, EPA is involved in different biochemical reactions within the inflammatory process (such as NF-κB on the cytosol), which precisely are directly related to the regulation of cytokines on the nucleus (as a final product of the chain of reactions mediated by pathway's related to induction by LPS), possibly explaining why the regulatory action showed fM could be because of intervening the EPA with NF-κB.

CONCLUSIONS

The fraction fM, at high concentrations (>400 µg/mL) has the capability of inhibiting the creation of reactive nitrogen species; furthermore, intracellularly, this fraction does not exert cytotoxicity below < 50 µg/mL, consequently, it may border the generation of NO to about 55 % at 50 µg/mL. Likewise, fM can decrease the generation of reactive oxygen species by about 50 %. In addition, fM was found capable of up- and down-regulating anti- and pro-inflammatory cytokines in a dose-response manner. All these findings suggest that fM has anti-inflammatory activity, however, further research is needed for a full assessment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest

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