Immunostimulatory and cytotoxic activities of *Indigofera suffruticosa* (Fabaceae)

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One of the major disadvantages of the current cancer therapy is the suppression of the immune system. Brazilian flora is considered one of the most diverse in the world and many plants were found to contain active constituents that can be valuable sources of new drugs. The plant *Indigofera suffruticosa* was studied to determine its potential to stimulate the immune system and also to be effective against tumor cells. We investigated the effects of the alkaloidal fraction and the pure alkaloid indigo obtained from *I. suffruticosa* on macrophage activation by measuring nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production. Cytotoxic activity was also evaluated against two tumor murine cells lines, LM2 (breast adenocarcinoma) and LP07 (lung adenocarcinoma). The alkaloidal fraction induced a high NO production and a moderated TNF-α release. The pure indigo demonstrated an elevated NO and TNF-α production. The fraction and the pure compound also exhibited cytotoxic activity against both adenocarcinoma cell lines and indigo showed the strongest cytotoxic activity with IC_{50} value of 0.89 μg/ml against LM2 and 1.44 μg/ml against LP07. Our results presented the immunostimulatory and cytotoxic activity of *I. suffruticosa*, enhancing macrophage function and therefore contributing to the host defense against tumors.

Keywords: alkaloidal fraction; indigo; nitric oxide; tumor necrosis factor-α; tumor cell lines

1. Introduction

One of the major disadvantages of the current cancer therapy is the suppression of the immune system (Devasagayam & Sainis, 2002). The main function of the immune system is the defense of the host organism against infectious agents and malignant tumors (Bogdan, Rollinghoff, & Diefenbach, 2000). This system has three primary roles in the prevention of tumors. First, it can protect the host from virus-induced tumors by eliminating or suppressing viral infections. Second, the timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment conducive to tumorigenesis. Third, it can specifically identify and eliminate tumor cells on the basis of their expression of tumor-specific antigens or molecules induced by cellular stress (Swann & Smyth, 2007).

Macrophages are key components of the antimicrobial and tumoricidal immune responses (Bogdan, Rollinghoff, & Diefenbach, 2000). These cells are part of the innate immune system and can respond to chemotactic signals, phagocytize antigens and other particulates, destroy pathogens and tumor cells, process and present antigens to T cells, a critical activity for specific immunological responses and for antibody production. Thus, through their abundance, distribution, motility and responsiveness, macrophages can influence almost every aspect of immune and inflammatory processes (Nathan, 1987; Laskin & Laskin, 2001). The activities of the macrophages may be mediated by the large amount of molecules that these cells can generate such as nitric oxide (NO) (Hajri, Metzger, Vallat, Coffy, Flatter, & Evrard, 1998; Hibbs Jr, 2002) and tumor necrosis factor-α (TNF-α) (Beutler & Cerami, 1989; Vilcek & Lee, 1991).

Nitric oxide is involved in a wide range of physiological processes in humans and in animals. It controls vascular tone, acts as a neurotransmitter and neuromodulator in the central and peripheral nervous systems and influences the activity of the immune system. Substances that selectively enhance or inhibit its synthesis or removal and modify its effects are likely to yield interesting therapeutic agents (Vallance & Moncada, 1994).

TNF-α was first identified as product of macrophages that caused the lysis of certain types of cells, especially tumor cells. It is a multifunctional cytokine involved in immunity, inflammation, apoptosis, autoimmunity and organogenesis (Locksley, Killeen, & Lenardo, 2001). Studies employing neutralizing anti-TNF antibodies demonstrated that the host defense against pathogens is severely impaired in the absence of TNF-α (Pfeffer, 2003). Currently, this cytokine is used in cancer treatment in the isolated limb perfusion setting for soft tissue sarcoma, irresectable tumors of various histological types, and melanoma in-transit metastases confined to the limb (Eggermont, de Wilt, & ten Hagen, 2003).

Each year, 5 million people in low- and middle-income countries die from cancer, about 10 percent of the 50 million deaths in those countries. This proportion, and the total burden of cancer, will continue to grow as the tobacco-induced cancer epidemic accelerates, and as the world population ages (Sloan & Gelband, 2007). Lung cancer kills more people than any other cancer - a trend that is expected to continue until 2030, unless efforts for global tobacco control are greatly intensified (Krech, Davis, Walsh, & Curtis, 1992). It is also important to mention that one in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast, and it is the principal cause of death from cancer among women globally (Bray, McCarron, & Parkin, 2004).

The Brazilian flora is considered one of the most diverse in the world and many plants were found to contain active constituents that can be valuable sources of new drugs (Suffredini, Varella, & Younes, 2006). The Brazilian plant *Indigofera suffruticosa* (Fabaceae) was studied to determine its potential to stimulate the immune system and also to be effective against tumor cells. In this research, we investigated the effects of the alkaloid fraction and pure indigo obtained from this specie on macrophage activation by measuring NO and TNF-α production. The cytotoxic activity was also evaluated against two tumor murine cells lines, LM2 (breast adenocarcinoma) and LP07 (lung adenocarcinoma).

2. Material and methods

2.1. Plant material and samples

Aerial parts of *I. suffruticosa* were collected in June 2003, at Rubião Junior, Botucatu, São Paulo State, Brazil. The plant was identified by Dr. Jorge Tamashiro of the Institute of Biology at UNICAMP. A voucher specimen (UEC: 131.827) documenting the collection is deposited in the Herbarium at UNICAMP (Campinas State University, Brazil).

The air-dried material (1.5kg) was finely pulverized and sequentially extracted at room temperature with methanol (5l) by maceration (3 days each). Removal of solvents in vacuo produced a methanolic extract (30.3g, 2.7%).

The methanol extract (3.0g) was dissolved in 15ml methanol, centrifuged and the supernatant was subjected to column chromatography over Sephadex LH-20 (100 x 5cm) and eluted with an isocratic system. The fractions (50ml) were collected and analyzed by thin layer chromatography over Silica gel TLC plates on glass (20cm x 20cm) developed with a solvent mixture composed of butanol:acetic acid:water (4:1:2, v:v), and visualized using UV light (254 and 365nm) and further sprayed with Draggendorff, iodoplatinate, anisaldehyde/sulfuric acid and NP/PEG (Natural Products/Polyethyleneglycol) reagents (Wagner, Bladt, & Zgainski, 1986). The collected fractions were combined into fractions A (1.26g), B (0.56g) and C (0,98 g). Chromatographic and spectrometric analyses demonstrated that these fractions contained glycerolipids, flavonol derivatives of quercetin and bis-indole alkaloids (indigoid pigments), respectively (Calvo et al., 2009).

The fraction C containing alkaloids was cromatographed on Sephadex LH-20 colunm (30 x 2cm) to give pure indigo (360mg) and a mixture of alkaloids, which was purified by high-speed counter-current chromatography (HSCCC, solvent system: ethyl acetate–*n*-butanolic alcohol–water [3.8:1.2:5.0 – 3.5:1.5:5.0 v/v/v], mobile phase: organic phase, flow-rate: 1.0 ml/min, revolution speed: 850 rpm).

The alkaloidal fraction and the pure indigo obtained from *I. suffruticosa* were dissolved in dimethyl sulfoxide (DMSO) and then diluted in RPMI-1640 culture medium. None of the samples had more than 0.5% of DMSO. The cytotoxic effects of the samples on macrophage viability were determined by MTT assay. The high viability levels observed allowed the development of NO and TNF-α assays (data not shown).

2.2. Animals

Swiss mice (6-8 weeks old, weighing 18 to 25g), supplied by animal house of the Faculty of Pharmaceutical Science of Araraquara were maintained in a polycarbonate box (at 23±1°C, 55±5% humidity, 10-18 circulations/h and a 12-h light/dark cycle), with water and food available *ad libitum*. Animal studies were performed according to the regulations of Research Ethics Committee (# 01/2005), Faculty of Pharmaceutical Sciences – UNESP, Brazil.

2.3. Peritoneal macrophages

Thioglycollate-elicited peritoneal exsudate cells (PEC) were harvested from Swiss mice using 5.0ml of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200*g* for 5 min at 4°C and resuspended in RPMI-1640 medium. Next, PEC were incubated in microplates for 60 min at 37°C for macrophage adherence. Non adherent cell were removed by washing the culture with RPMI-1640 medium.

2.4. Measurement of NO production

NO production was determined by assaying culture supernatants for nitrite using Griess reagent. PEC (adherent cells) at $5x10^6$cells/ml was incubated with the alkaloidal fraction and the pure indigo for 24h at 37°C in a 7.5% CO_2 atmosphere. Cell-free supernatant (100µl) was mixed with 100µl of Griess reagent (sulfanilamide 0.1%, phosphoric acid 3%, naphthylethylenediamine 0.1%) and incubated at room temperature for 10min. Cells incubated with lipopolissacharide (LPS) were used as a positive control and cell in culture medium (RPMI-1640) as a negative control. After incubation, the absorbance of the wells was determined by using a microplate reader (Multiskan, Labsystem) equipped with a 540nm

filter. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards (Green, Wagner, Glogowski, Skipper, Wishnok, & Tannenbaum, 1982).

2.5. Measurement of TNF-α production

For the cytokine immunoassay, PEC (adherent cells) at $5x10^6$/ml was incubated for 24h with alkaloidal fraction and the pure indigo at 37°C in a 7.5% CO_2 atmosphere. Supernatants were removed and TNF-α production was quantified by a sandwich immunoassay kit (BD Biosciences Pharmingen) following the manufacturer's instructions.

2.6. Tumor cells lines

The murine tumor cell lines LM2 (breast adenocarcinoma) and LP07 (lung adenocarcinoma) were obtained from Angel H. Roffo Oncology Institute, Buenos Aires, Argentina by Prof. Lucas Colombo.

2.7. Cell culture

The tumor cell lines were maintained in MEM (Gibco, USA), supplemented with 10% fetal calf serum and garamicin at 37°C with 5% CO2. The medium was changed every two days until the cells reached confluence, at which point they were subcultured.

2.8. Measurement of cytotoxic activity against LM2 and LP07 murine tumor cell lines

Cytotoxicity was quantified using MTT assay (Mosmann, 1983). Cells were detached with trypsin, washed and transferred into 96-well microtitre plates at a concentration of 3×10^4 cell/ml. 190µl of the tumor cell suspension were plated in the wells and incubated at 37°C under 5% CO_2. The microtiter plates containing cells were pre-incubated for 24h to allow stabilization prior to the addition of the samples. After this period, alkaloidal fraction and the

pure indigo were added and incubated for 48 hours in the same conditions. Doxorubicin was used as positive control. After cultivation, the medium was changed for fresh medium containing 1mg/ml of MTT. After three hours, the medium was removed and 100μL of isopropilic alcohol (Mallinckrodt) was added to solubilize the formazana crystals formed. The absorbance was measured at 570nm with reference filter of 620 nm on a spectrophotometer (Multiskan Ascent, Labsystems).The 50% inhibitory concentration for cell growth (IC_{50}) values were expressed as the dose resulting in 50% reduction of tumor cell growth and were calculated using Microcal Origin 7.5.

2.9. Statistical analysis

The results are expressed as means ± S.D. Each experiment was performed at least five times. One-way ANOVA with Dunnett's post test was performed using GraphPad InStat version 3.00 for Windows XP, Graph-Pad Software, San Diego, California, U.S.A. Values of $p< 0.05$ were considered statistically significant.

3. Results

3.1. Phytochemical Studies

Extraction and chromatographic analyses were performed as described in Material and methods. The further purification of alkaloidal fraction by HSCCC gave more indigo (7 mg), indirubin (5 mg), indican (35 mg), dioxindole-β-D-glucoside (5 mg) and alantoin (30 mg).

The compounds in alkaloidal fraction were identified by their UV, NMR and MS spectra, confirmed by comparing spectroscopic/spectrometric data with those in the literature (Guengerich et al., 2004; Chanayath, et al., 2002; Maugard et al., 2001; Kalinowski et al., 1988).

Indigo, the majority compound of alkaloidal fraction, and indirubin were identified by comparison with synthetic materials (standard Fluka) and cochromatography, the developing solvent system was chloroform-hexane-methanol (7:4:1 v/v/v). The colors of the products suggest that they are indigo (blue) or indirubin (red), and the MS and UV confirmed this. The UV/visible spectra of indigo and indirubin (UV 603 and 552 nm λ_{max} respectively) match the literature for indigo (indigotin) and indirubin (isoindigotin) (Maugard et al., 2001). Mass spectrum yielded an apparent MH^+ ion at *m/z* 263 for both indigoids, indicating a molecular mass of 262. The data of NMR spectroscopy of the indigo and indirubin were confirmed by comparison with data of Guengerich et al., 2004.

3.2. Effect of alkaloidal fraction and pure indigo on NO and TNF-α production

Figure 1 show the effect of the alkaloidal fraction and pure indigo on NO production by peritoneal macrophages. The alkaloidal fraction induced a high NO production (from 16.66 to 34.31μmols) that decreased in a dose-dependent manner (Figure 1a). The NO production by the pure indigo was also very elevated, ranging from 22.36 to 48.56μmols and increased in a dose-dependent manner (Figure 1b).

Both alkaloidal fraction and pure indigo also stimulated TNF-α synthesis. The alkaloidal fraction induced a moderate to strong TNF-α release (from 140.06 to 785.12 pg/ml) that decreased in a dose-dependent manner (Figure 2a). The TNF-α release by pure indigo was very strong ranging from 484.63 to 843.85pg/ml and also increased in a dose-dependent manner (Figure 2b).

3.3. Cytotoxic activity of alkaloidal fraction and pure indigo against LM2 and LP07 murine tumor cell lines

As shown in Table 1, alkaloidal fraction and pure indigo have cytotoxic activity *in vitro*. The alkaloidal fraction showed a moderated activity, with IC_{50} value of 37.39 and 24.13

for LM2 and LP07 cell lines, respectively. The results also revealed that pure indigo has a potent cytotoxic activity as potent as the doxorubicin control with IC_{50} value of 0.89 for LM2 and 1.44 for LP07.

4. Discussion

Plants represent a rich source of potential immunomodulating substances and useful anti-cancer agents (Williams, 2001; Cragg & Newman, 2005). Several immunomodulatory and cytotoxic effects induced by medicinal plants and their isolated compounds have been described (Lopes, Benzatti, Jordão Junior, Moreira, & Carlos, 2005; Lopes, Calvo, Vilegas, & Carlos, 2005; Réthy, Hohmann, Minorics, Varga, Ocsovszki, & Molnár, 2008). However, there are no previous studies describing macrophage stimulation and cytotoxic activity in cells treated with *Indigofera suffruticosa*.

Nitric oxide, nitric oxide derivatives and reactive oxygen intermediates are molecules of the immune system which contribute to the control of microbial pathogens and tumors. There is recent evidence for additional functions of these oxygen metabolites in innate and adaptive immunity; these functions include the modulation of the cytokine response of lymphocytes and the regulation of immune cell apoptosis, as well as immunodeviating effects (Bogdan, Rollinghoff, & Diefenbach, 2000). NO is synthesized from a guanidino nitrogen atom of L-arginine by the action of NO synthases (NOS) which form citrulline as a co-product (Leone, Palmer, Knowles, Francis, Ashton, & Moncada, 1991). The macrophage NOS (iNOS) is an inducible isoform that is absent in strictly resting cells, is strongly induced by cytokines and other immunological stimuli and is regulated on transcriptional and post-transcriptional levels involving a number of signal transduction pathways and molecules. The most characteristic feature of iNOS is its prominent regulation by activating cytokines such as TNF-α. Besides that, it is also known that NO can affect the production of more than twenty

cytokines, including Interleukin-1, Interleukin-6, Interleukin-8, Interleukin-10, Interleukin-12, Interferon-γ, TNF-α and Transforming Growth Factor-β (Bogdan, Rollinghoff, & Diefenbach, 2000).

TNF-α plays a beneficial role as immunostimulant cytokine and it is an important mediator of host resistance to many infectious agents and, probably, malignant tumors (Old, 1985). It elicits two fundamentally distinct types of cellular responses: it can cause apoptotic cell death (cytotoxic activity) or modulate cell proliferation, activation and differentiation (proinflammatory/immunomodulatory activity) (Baker & Reddy, 1998; Wallach, Varfolomeev, Malinin, Goltsev, Kovalenko, & Boldin, 1999).

Alkaloidal fraction obtained of aerial parts from *I. suffruticosa* yielded indigo and indirubin (bis-indoles derivatives), indican and dioxindole-β-D-glucoside (bis-indoles precursors), and alantoin (alkaloid found in plants in general). Bis indoles derivatives (indigoids) have been just obtained from some plant sources such as *Indigofera* (tropical species, Africa, Asia, East Indies, South America), *Polygonum tinctorium* (Far East, China, Korea) and *Isatis tinctoria* (Europe) (Maugard et al., 2001).

Our data indicated that the alkaloidal fraction and pure indigo obtained from *I. suffruticosa* strongly induce NO and TNF-α synthesis by unstimulated murine macrophages. Both NO and TNF-α production induced by the alkaloidal fraction decreased in a dose-dependent manner (Figure 1 and Figure 2). It is important to comment that plant fractions may contain novel bioactive compounds and the presence of these different compounds can explain the reduction of NO and TNF-α production in a dose-dependent manner observed in this research with the alkaloidal fraction. Synergism or antagonism due to the complex nature of the fraction can be postulated as an explanation. It is possible that, at some concentrations, certain compounds of the fraction could act in synergism and cause a significant effect. At

lower or higher concentrations, these compounds could be antagonized by different ones (Phillipson, 2001; Liu, 2004).

In addition, it is considerable that the release of both mediators by the pure indigo isolated from *I. suffruticosa* (Figure 1 and Figure 2) was higher than the production of the same mediators by the alkaloidal fraction. The pure indigo showed the best results, and the NO and TNF-α production was increased in a dose-dependent manner.

Both the fraction and the pure alkaloid showed cytotoxic activity in mammary (LM2) and lung (LP07) tumor cell lines, but indigo had a very strong activity as potent as the doxorubicin control. The cytotoxic activity of indigo demonstrated in this study (Table 1) indicates the potential use of this plant for cancer treatment. Different kinds of alkaloids have already been reported as anticancer agents (Kam & Sim, 2003; Jagetia & Baliga, 2006). Indirubin is a 3, 2' bisindole isomer of indigo that has originally been identified as the active principle of a traditional Chinese preparation and has been proven to exhibit antileukemic effectiveness in chronic myelocytic leukemia (Eisenbrand, Hippe, Jakobs, & Muehlbeyer, 2004).

Also, several antitumor drugs have demonstrated the ability to stimulate NO production (Lind, Kontaridis, Edwards, Josephs, Moldawer, & Copeland, 1997). Besides, Stuehr & Nathan (1989) showed that activated macrophages produce nitrite and it can kill tumor cells. Nascimento *et al.* (2006) justified the popular use of babaçu mesocarp (*Orbignya phalerata* - Arecaceae) in the treatment of tumor diseases by observation of macrophages activation and the production of hydrogen peroxide (H_2O_2), NO and TNF-α.

The innate immune system governs the interconnecting pathways of microbial recognition, inflammation, microbial clearance, and cell death. It thereby offers diverse targets for compounds that modify innate immune responses and these compounds could act as potential therapeutics for viral infections and various tumors (Schiller, Metze, Luger,

Grabbe, & Gunzer, 2006). The innate immunity also can be modulated by medicinal plants that offer an alternative to conventional chemotherapy for different types of diseases, especially when the host defense needs to be enhanced under certain health conditions (Ganju, Karan, Chanda, Srivastava, Sawhney, & Selvamurthy, 2003). *I. suffruticosa* may be one of these plants.

In conclusion, the results of the present study indicate that *I. suffruticosa* has immunostimulatory and cytotoxic properties. Consequently, it can enhance macrophages function and contribute to the host defense against tumors. Our findings can promote a better comprehension of the beneficial properties of this plant.

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Figures and table captions

Figure 1. Effect of the alkaloidal fraction (a) and indigo (b) on NO production in peritoneal macrophages.

Adherent cells were incubated for 24 h with the alkaloidal fraction or the alkaloid indigo. Cell free supernatant was mixed with Griess reagent. Cells incubated just with LPS were used as a positive control and cells in culture medium (RPMI-1640) as a negative control (C-). One-way ANOVA with Dunnett's post test was performed.

* $p<0.01$ *vs*. C- control.

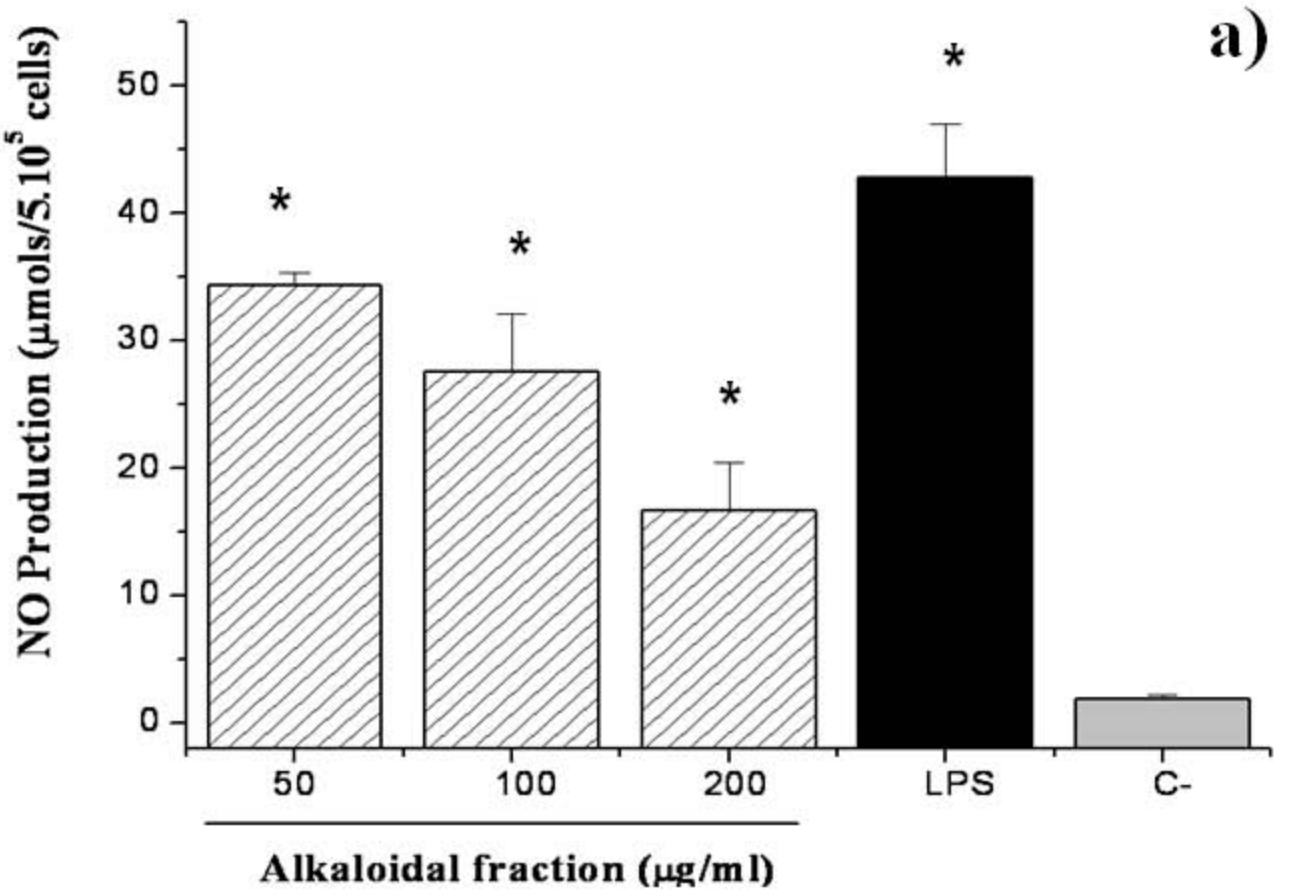
Figure 2. Effect of the alkaloidal fraction (a) and indigo (b) on TNF-α productin in peritoneal macrophages.

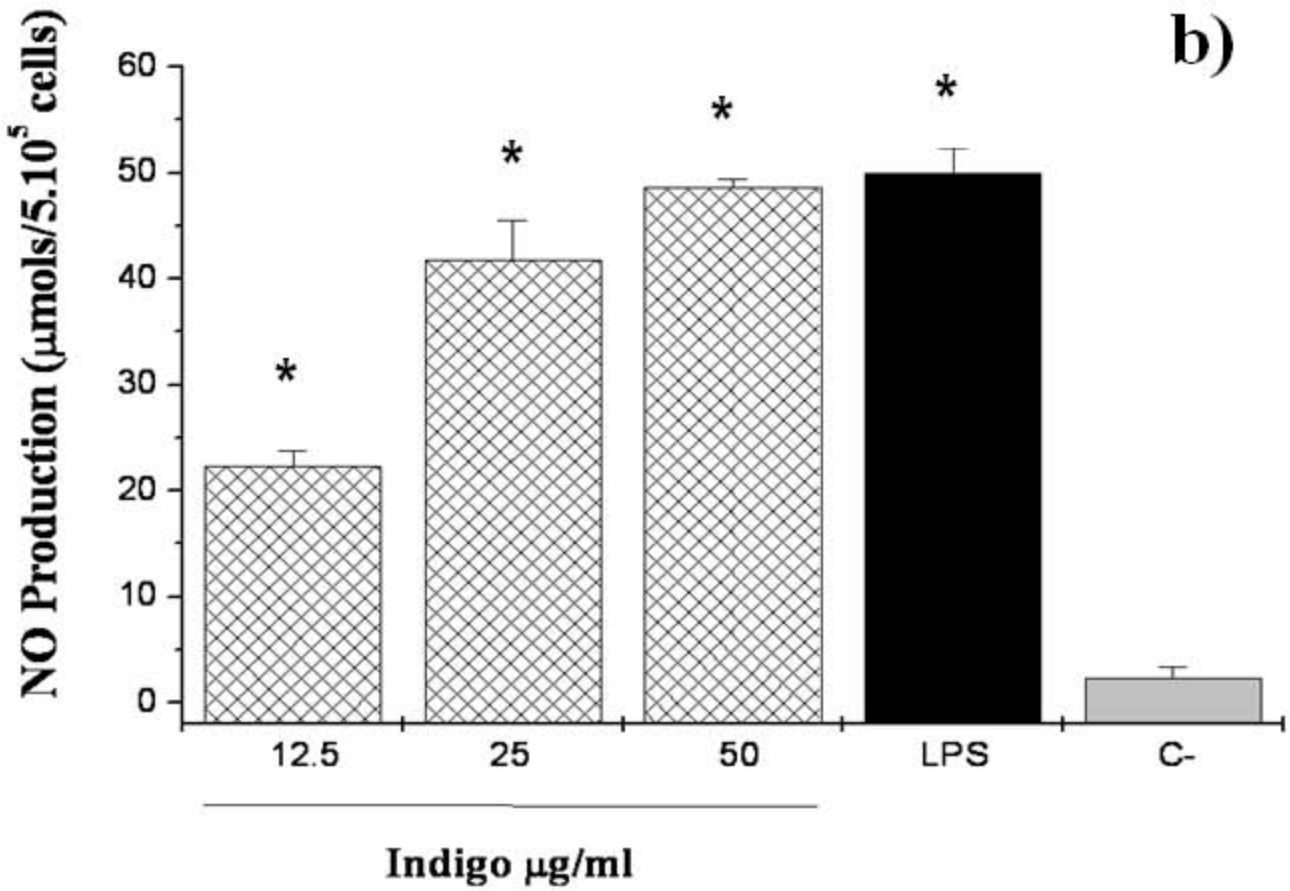
For the cytokine immunoassay, adherent cells were incubated for 24 h with the alkaloidal fraction or the alkaloid indigo. Cells incubated just with LPS were used as a positive control and cell in culture medium (RPMI-1640) as a negative control (C-). Data are reported as the mean ± SD for at least four independent experiments carried out in triplicate. One-way ANOVA with Dunnett's post test was performed.

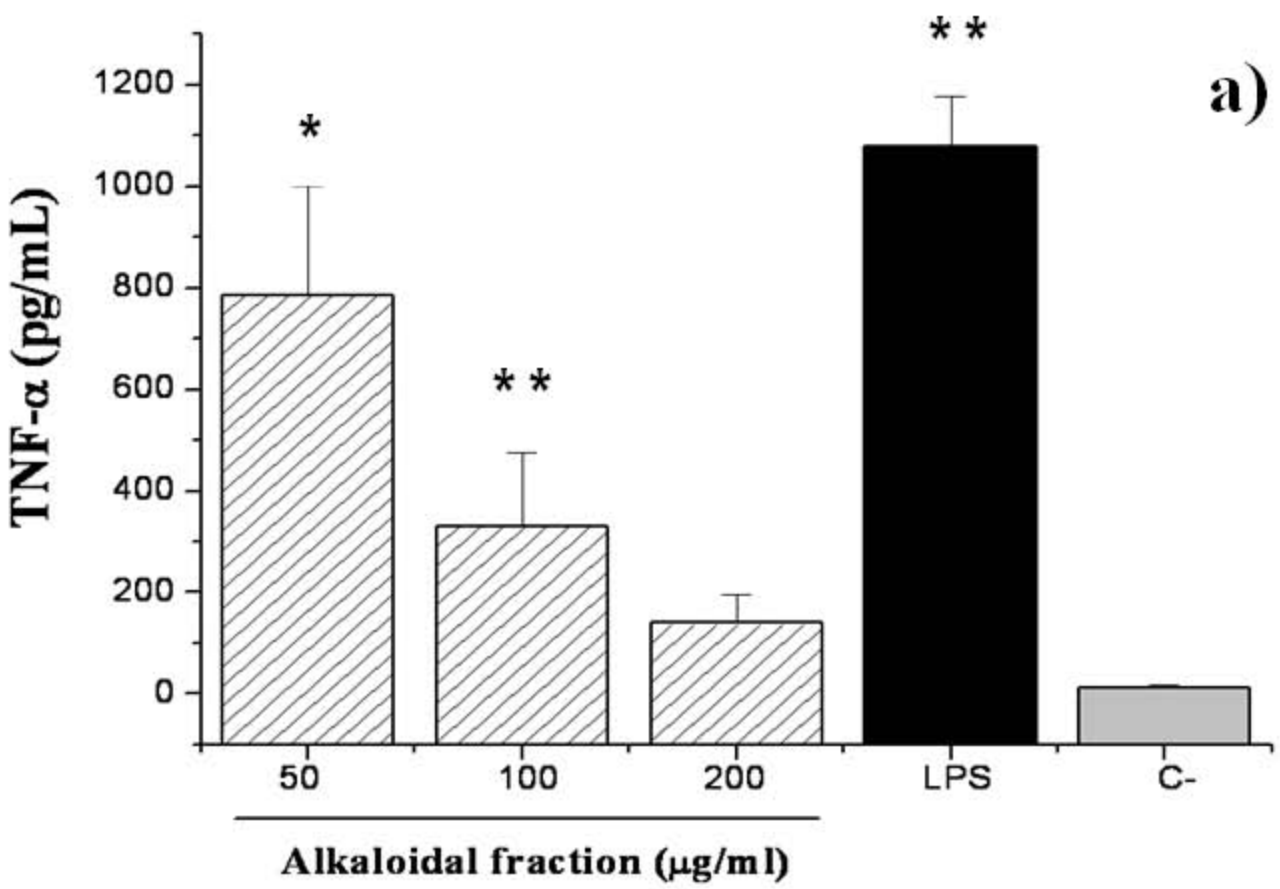
* $p<0.01$ *vs*. C- control.

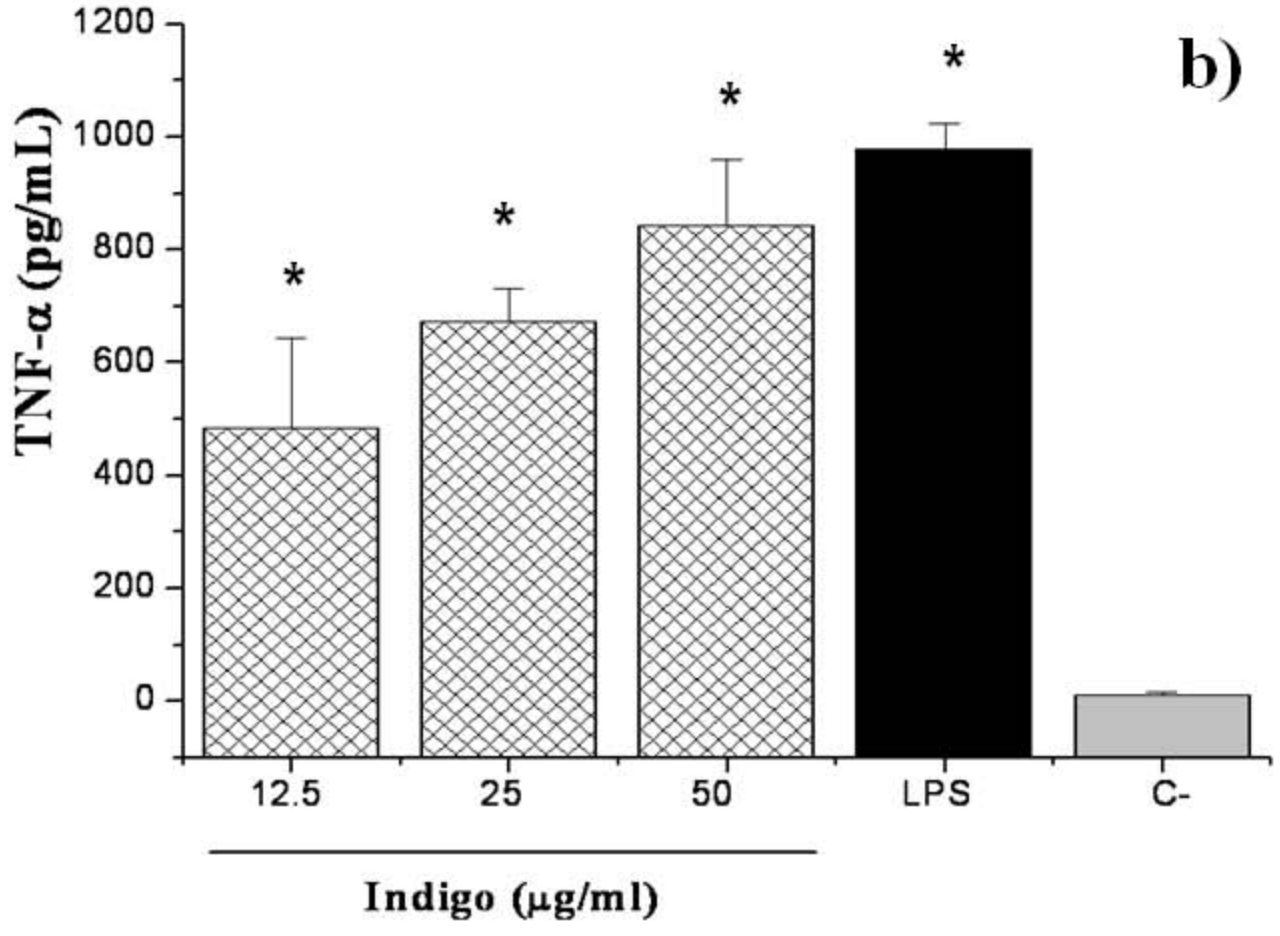
** $p<0.05$ *vs*. C- control.

Table 1. Cytotoxic effects of the alkaloidal fraction and indigo obtained from *I. suffruticosa* on LM2 and LP07 tumor cell lines.


a)
NO Production (μmols/5.10^5 cells)
50
40
30
20
10
0
*
*
*
*
50
100
200
LPS
C-
Alkaloidal fraction (μg/ml)


b)
NO Production (μmols/5.10^5 cells)
60
50
40
30
20
10
0
*
*
*
*
12.5
25
50
LPS
C-
Indigo μg/ml


a)
TNF-α (pg/mL)
1200
1000
800
600
400
200
0
*
**
**
50
100
200
LPS
C-
Alkaloidal fraction (μg/ml)


b)
TNF-α (pg/mL)
1200
1000
800
600
400
200
0
*
*
*
*
12.5
25
50
LPS
C-
Indigo (μg/ml)

Tumor Cell Line	Fraction IC_{50} µg/ml	Alkaloid IC_{50} µg/ml	Doxorubicin IC_{50} µg/ml
LM2	37.39± 1.05	0.89±0.056	0.83±0.01
LP07	24.13± 2.41	1.44±0.16	0.52±0.007

Data are presented as IC_{50} values obtained for breast (LM2) and lung (LP07) adenocarcinoma cell lines from five independent experiments. Doxorubicin was used as positive control.