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The role of *Qualea multiflora* on Mammary Tumour Cells and Immune System

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ABSTRACT

Species of the genus *Qualea* are used by the Brazilian public as a natural anti-inflammatory. Based on this evidence, we evaluated the effects of terpene fractions (βF and TF) obtained from *Qualea multiflora* on nitric oxide production (Griess assay), cytokines (IL-1, IL-10, IL-12, and TNF-α) and the transcription factor NF-κB by peritoneal macrophages. Since there is a relationship between inflammation and cancer, the cytotoxicity of βF and TF against mammary tumoural cell lineage, and macrophages was evaluated. Inhibition levels close to 90% of the production of NO, IL-1, IL-12 and TNF-α; about 32% of NF-κB; and a large stimulation of IL-10 production (close to the positive control) by peritoneal macrophages were observed in response to βF and TF which are correlated with anti-inflammatory activity. Additionally, the samples showed exceptional cytotoxic activity against tumoural cells but not against macrophages. Since anti-inflammatory activity is important in tumour inhibition, further examination of potential anti-cancerous activity of *Qualea multiflora* is warranted.

INTRODUCTION

Breast cancer is one of the most common cancers worldwide with a high mortality rate. Moreover, the current cancer treatment methods cause severe systemic side effects. For this reason, recent research has focused on the search for alternative treatment extracted from plant-based sources.

Species of the genus *Qualea* (family Vochysiaceae) occur throughout the tropical Americas, ranging from Mexico, passing through Peru, and Guyana, and reaching as far south as Brazil, where it has a marked presence in the Brazilian cerrado (Côrrea, 1974). The powdered bark from *Qualea parviflora* contains tannins and is used as an external antiseptic, while the leaves are used for treating heartburns (Pott and Pott, 1994; Mazzolin *et al*., 2010). The bark, and leaves from *Qualea grandiflora* and *Q. multiflora* are used as astringent, cleansing external ulcers, and inflammations (Almeida *et al*., 1998; Nasser *et al*., 2008). In some tumour types, over 70% of the tumour mass consists of inflammatory cells. These tumour-associated leukocytes, especially macrophages, release angiogenic growth factors, mitogens, proteolytic enzymes and chemotactic factors; thus, recruiting more inflammatory cells and sustaining tumour growth, invasion and angiogenesis (Yan *et al*., 2006). Macrophages constitute one of the main groups of phagocytes in the immune system and part of their effectiveness is due to their nitric oxide (NO) production, and cytokines (Cheng *et al*., 2012). Research in the last few years has shown the involvement of macrophages, and NO in carcinogenesis, through process stimulation such as: initiation, promotion, progression, metastasis, and angiogenesis (Hofseth *et al*., 2003; Hughes *et al*., 2012; Daldrup-Link and Coussens, 2012). There is a strong association between poor cancer patient survival and increased macrophage density in thyroid, lung and liver.

NO production is regulated by the transcription factor NF-κB and cytokines such as the tumour necrosis factor-alpha (TNF-α) and the interferon-γ (IFN-γ) (Kovalovsky *et al*., 2000; Moon *et al*., 2012). Additionally, the interleukins IL-1, IL-12, and IL-10 modulate the macrophages' activity. Among these cytokines, IL-10 is the unique macrophage inhibitor, showing anti-inflammatory properties.

Based on the data collected amongst the rural population of the Brazilian cerra do about empirical use of this plant, for

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instance as an anti-inflammatory, it was possible to direct this study to the probable pharmacological effect. In previous studies, crude chloroform extracts inhibited the production of the pro-inflammatory mediators NO, IL-1 and IL-12 (data unpublished). The aim of this study was to evaluate the cytotoxic activity of TF and βF against tumour cells and anti-inflammatory activity of these fractions on peritoneal macrophages.

MATERIALS AND METHODS

Plant material

Q. multiflora was collected from the Ypê garden (Cerrado region), in the city of Porto Nacional, state of Tocantins by Prof. Dr. Clelia Akiko Hiruma-Lima. The plant was identified by Prof. Dr. Solange Fatima Lolis (UNITINS-Porto Nacional) and deposited in the UNITINS herbarium (Voucher No 4158). The extracts of the plants were prepared at the Instituto de Química of UNESP (São Paulo State University) in Araraquara, Brazil. *Q. multiflora* leaves (200g) were dried at 40 °C, grounded and extracted exhaustively with 2L of chloroform. The solvent was evaporated in a rotatory evaporator and fractioned on a silica gel column using solvents of increasing polarity (hexane → chloroform → methanol), yielding the terpene fraction (TF) and the β-sitosterol rich fraction (βF). The presence of β-sitosterol in βF and of lupeol, lupenone and friedelin in TF, was determined by a thin layer of chromatography, in comparison with authentic samples.

Animals

Swiss mice (6 - 8 weeks old, weighing 18 - 25 g) were maintained in a polycarbonate box at (23 ± 1) ºC, (55 ± 5)% humidity, 10 - 18 circulations/h and a 12-h light/12-h dark cycle, with free access to food (Purina, São Paulo, Brazil) and water. All animals were maintained and handled according to the International Ethical Guidelines for the Care of Laboratory Animals (Faculty Ethics Committee # n 29/2006).

Peritoneal exudate cells

Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested from Swiss mice in 5.0 mL of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed three times by centrifugation at 200 x g for 5 min at 4 °C with 3.0 mL PBS and then resuspended in a 1.0 mL RPMI-1640 culture medium (Sigma, Munich, Germany) containing $2 \cdot 10^{-5}$ M β-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine and 5% fetal bovine serum (Sigma, Munich, Germany). The cell suspension was counted and adjusted in a Neubauer chamber (Boeco, Hamburg, Germany) in accordance with each assay (Moleiro *et al*., 2009).

LM3 cell lineage

The mammary adenocarcinoma (LM3) murine lineage were kindly supplied by the Angel H. Roffo Oncology Institute in Buenos Aires, Argentina. The LM3 lineage originated from a mammary adenocarcinoma obtained from Balb/c mice with lung metastases. Cells were maintained in a minimum essential medium (MEM) (Gibco, São Paulo, Brazil) with 3 mM L-glutamine, 80 μg/mL gentamycin, supplemented with 5% heat-inactivated fetal calf serum (FCS) at 37 °C in a 5% humidified CO_2 air atmosphere. Serial passages were performed by detaching tumour cells with PBS or by trypsinization (0.25% trypsin and 0.02% EDTA in Ca^{2+} and Mg^{2+} free PBS) of the confluent monolayer. The medium was changed every three days.

Assessment of cellular viability

Cell viability was assessed by a MTT assay (Mosmann, 1983). The cells were resuspended in a RPMI-1640 medium containing 5% fetal bovine serum, 100 IU/mL of penicillin, 100 IU/mL of streptomycin and 50 mM β-mercaptoethanol. Then, it was adjusted to $5 \cdot 10^6$ cells/mL. 100 μL of the suspension and 100 μL of TF, μF or Taxol® were added to each microplate well and incubated for 48 h. A MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was added (100 μL) and the plate was incubated for 3 h at 37 °C and 5% CO_2. The absorbance was measured at 540 nm with a Multiskan Ascent ELISA reader (Labsystems, Helsinki, Finland) equipped with a 620 nm reference filter. Only cells and culture medium (RPMI-1640) were used for control which corresponded to 100% of macrophages viability (Mosmann, 1983).

Inhibition of NO production

Adherent PEC were resuspended in RPMI-1640-C medium and adjusted to $5 \cdot 10^6$cells/mL. Suspensions were placed in a 96-well microplate and mixed with 100 μL of TF, βF or Taxol®.

The mixture was completed with 100 μL of LPS (lipopolysaccharide from *Escherichia coli* O111:B4) at 10μg/mL and the plates were incubated for 48 h at 37 °C and 7.5% CO_2. Nitrite concentrations were measured by a quantitative colorimetric assay using the Griess reagent system: 50 μL aliquots of supernatant were added to 50 μL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine and 3% H_3PO_4), incubated at room temperature for 10 min and the absorbance was measured at 540 nm in an ELISA microplate reader (Labsystems, Vantaa, Finland). Samples in triplicate were assayed in four experiments and reported as μmol NO/ $5 \cdot 10^5$ cells (Green *et al*., 1982).

Determination of TNF-α, IL-1, IL-12 and IL-10

For the determination of cytokines TNF-α, IL-1, IL-12 and IL-10 adherent PEC were stimulated with the samples, together with LPS (0.5 μg/mL), except when IL-10 was determined. After 48 h, the supernatants were removed, filter-sterilized and stored at –80 °C until assayed by an immunoenzymatic assay (BD Biosciences, Pharmingen™, San Diego, USA) according to the manufacturer's protocol. Results were expressed in pg/mL.

Determination of the transcription factor NF-κB

The quantification of the transcription factor NF-κB (sub-unit p65) on macrophages stimulated with LPS and exposed to TF, βF and Taxol® for 48 h, was performed with a NF-κB ActivELISA kit (Ingenex, San Diego, United States). The peritoneal exudate cells were aseptically obtained as described above and adjusted to $2 \cdot 10^6$ cells/mL in a RPMI-1640-C medium. The nuclear fraction was obtained by the lysate preparation module contained in the NF-κB ActivELISA (Ingenex®) kit.

Cytotoxicity activity

LM3 cellular growth was quantified by the MTT assay. Cells were detached with trypsin, washed and transferred into 96-well microtiter plates at a concentration of $3 \cdot 10^4$ cells/mL. Tumour cell suspension was plated (190 µL) and pre-incubated for 24 h at 37 °C and 7.5% CO_2. After incubation 100 µL of the tested samples were added to the cells and further incubated for 48 h under the same conditions. After incubation, the medium was replaced with a fresh medium containing 1 mg/mL of MTT. After 3 h, the medium was removed, 100 µL of isopropanol was added to dissolve the formazan crystals formed and the absorbance measured at 540 nm with a Multiskan Ascent ELISA reader (Labsystems, Helsinki, Finland) equipped with a 620 nm reference filter (Mosmann, 1983).

Statistical analysis

Data are expressed as means ± standard deviation (SD) and the Tukey test (Graphpad Instat 3.05) was used to determine the significance of differences between positive control and experimental groups.

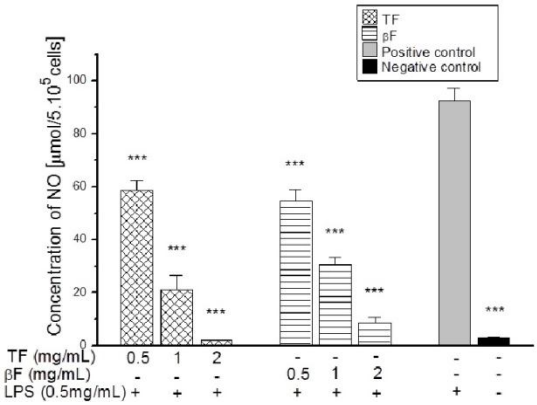

Fig. 1: NO production in peritoneal macrophages stimulated with LPS and exposed to the fractions TF and βF from *Q. multiflora* (***$p < 0.001$, compared to the positive control).

RESULTS

Inhibition of NO, TNF- α, IL-1 and IL-12 production

Production of NO (Fig. 1), TNF-α (Fig. 2), IL-1 (Fig. 3) by the murine peritoneal macrophages was inhibited by all concentrations of TF and βF (0.5, 1 and 2 mg/mL) in a dose-dependent manner. NO (Fig. 1) and TNF-α (Fig. 3) presented inhibition close to 100% in the highest TF and βF concentrations. On the other hand IL-1 production (Fig. 3) was inhibited only partially by TF and βF with the greatest inhibition (51%, in relation to the positive control) observed in TF at 2 mg/mL. The inhibition of IL-12 was very expressive in all concentrations, but it was not in a dose-dependent manner. Even the lowest concentration of TF and βF (0.5 mg/mL) inhibited the production of cytokine IL-12 by nearly 100% (Fig. 4).

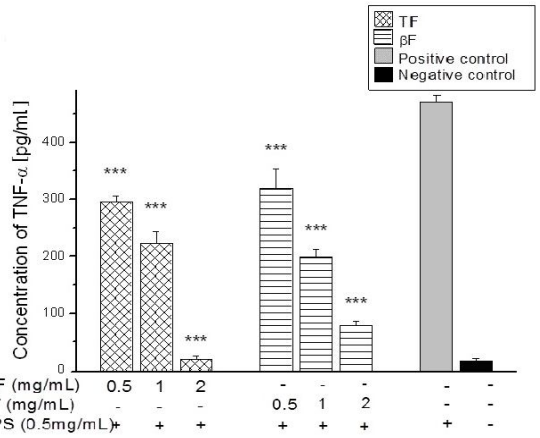

Fig. 2: TNF-α production in peritoneal macrophages stimulated with LPS and exposed to the fractions TF and βF from *Q. multiflora* (***$p < 0.001$, compared to the positive control).

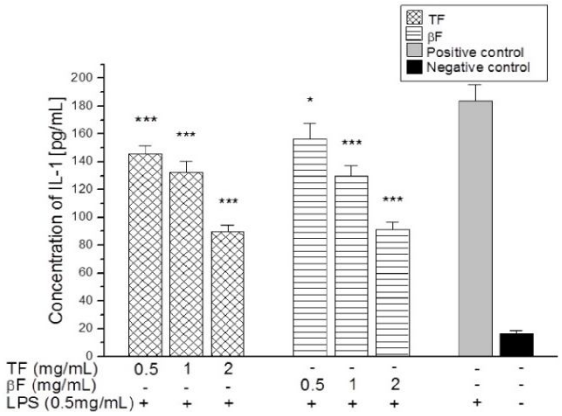

Fig. 3: IL-1 production in peritoneal macrophages stimulated with LPS and exposed to the fractions TF and βF from *Q. multiflora* (***$p < 0.001$, *$p < 0.05$, compared to the positive control).

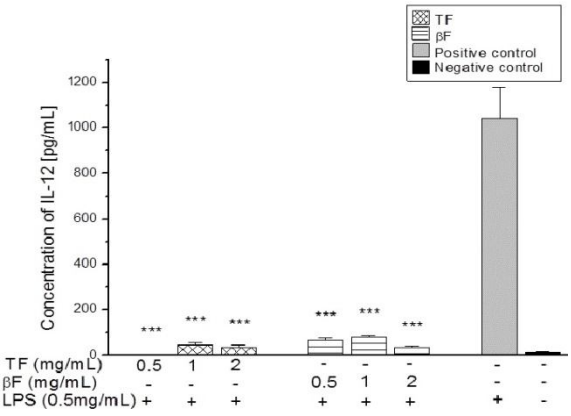

Fig. 4. IL-12 production in peritoneal macrophages stimulated with LPS and exposed to the fractions TF and βF from of *Q. multiflora* (***$p < 0.001$, compared to the positive control).

Production of cytokine IL-10

The production of the interleukin IL-10 was stimulated in a dose-dependent manner in the presence of TF and βF (Fig. 5) and the absence of LPS. This production was greater in higher concentrations of TF and βF. At the highest dose of TF (2 mg/mL), the IL-10 level was even as high as in the positive control.

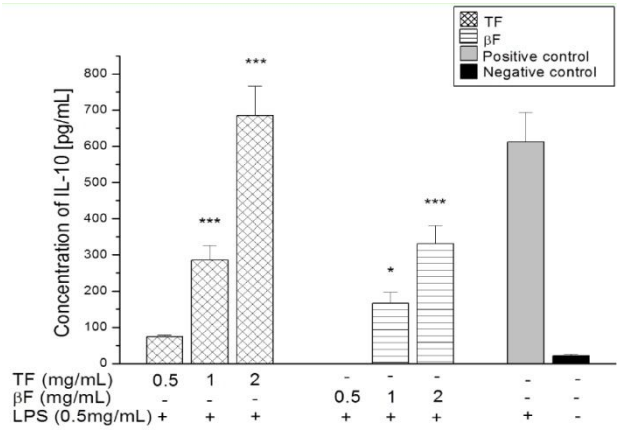

Fig. 5: IL-10 production in peritoneal macrophages and exposed to the fractions TF and βF from *Q. multiflora* (***$p < 0.001$, *$p < 0.05$, compared to the negative control).

Level of the transcription factor NF-κB

Transcription factor NF-κB levels in the nuclear fraction of the macrophages were slightly, but significantly reduced by the higher doses of TF and βF (32% inhibition by 2 mg/mL TF with respect to the positive control, Fig. 6).

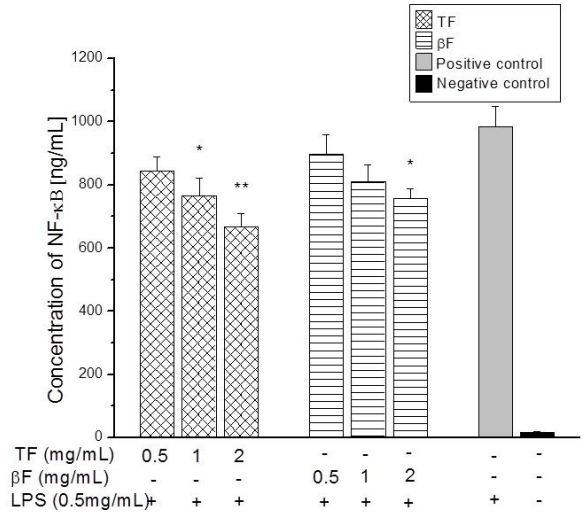

Fig. 6: Concentration of NF-κB in the nuclear fraction of peritoneal macrophages stimulated with LPS and exposed to the fractions TF and to βF from *Q.multiflora* ($p < 0.05$, compared to the positive control).

Cytotoxic activity in mammary tumoural lineage

The IC_{50} values (concentration causing the death of 50% of the cells) obtained for TF, and βF were above that of the standard drug Taxol® in LM3 tumoural lineage (Table I), while even at the highest concentrations TF and βF affected the peritoneal macrophage viability little, if to any extent (Table II). At the concentration of 2 mg/mL of TF, macrophage viability was near 100%, while the viability rates of the LM3 was 0%. As for βF, it was partially toxic to the macrophages at the same concentration (2 mg/mL) with 73.34% viability. Also, the viability of tumour cells was 0%.

Table. 1: IC_{50} (mg/mL) of fractions from *Q. multiflora* for LM3 cells after 48 h of incubation.

Samples	LM3
TF	0.31 ± 0.06
βF	0.27 ± 0.08
Taxol®	0.18 ± 0.06

Table. 2: Cell viability after 48 h in the presence of fractions from *Q. multiflora*.

Samples	Peritoneal macrophages	LM3
TF (2 mg/mL)	99.2%	0%
TF (1 mg/mL)	88.6%	8.3%
TF (0.5 mg/mL)	90.1%	34.3%
βF (2 mg/mL)	73.3%	0.0%
βF (1 mg/mL)	67.9%	1.5%
βF (0.5 mg/mL)	61.8%	28.9%
Negative control	100%	100%

DISCUSSION

In the present study, assays were performed with TF and βF in order to assess the pharmacological properties of the plant *Q. multiflora*. Analysing the results for the production of NO (Fig. 1), TNF-α (Fig. 2) and IL-12 (Fig. 4), we observed that TF and βF exerted immunosuppressive activity on the LPS-stimulated macrophages in a dose-dependent manner, with inhibitory results nearing 100%, in relation to the positive control ($p<0.001$). On the other hand, the inhibition of IL-1 only partial reached 51% at higher concentrations (2mg/mL) of TF and βF (Fig. 3). The modulation of the immune system in cancer control is very important because several cytokine and inflammatory mediators are involved in the tumour process. Several reports have confirmed the role of NO in the initiation, promotion, and progression of tumors. NO can enhance migration, invasion and metastasis of breast and colon cancer cells by activating the mitogen-activated protein kinase pathway (Le Bitoux and Stamenkovic, 2008; Gochman *et al*., 2012). Moreover, NO synthase inhibitors prevent angiogenesis in mammary tumour and cancer metastasis therapy can be accomplished by iNOS inactivation (Kitaura *et al*., 2011). TNF-α acts as a tumour progression factor by modulating tumour motility or augmenting adhesion molecule expression on the target organs (Palladino *et al*., 2003). The interleukin-1 (IL-1) is known to be up-regulated in many tumour types and has been implicated as a factor in tumour progression by stimulating angiogenesis and metastasis. Studies have reported that high levels of IL-1 in the tumour micro-environments are associated with a more virulent tumour phenotype (Hughes *et al*., 2012; Ostrand-Rosenberg *et al*., 2012). On the other hand IL-12 is not directly related to tumour progression but is related to NO production; thus, modulating IL-12 is indirectly involved in the tumour process (Trinchieri and

Scott, 1998; Kitaura *et al*., 2011). Contrary to NO and to all cytokines mentioned above, IL-10 has anti-inflammatory characteristics, by means of macrophage activation inhibition (Liu *et al*., 2012). Bearing this in mind, when testing substances for their anti-inflammatory and antitumour properties, it is desirable that those substances tested be capable of presenting stimulation of the production of this cytokine (Mocellin *et al*., 2003). In this study, TF and βF presented expressive stimulating activity of the production of this cytokine in non LPS-stimulated macrophages ($P<0.001$ when compared with negative control). Results obtained were close to the positive control (Fig. 5) showing grate anti-inflammatory activity of TF and βF on murine macrophages.

In the case of antitumour and anti-inflammatory drugs, it is very important to determine the effect of those substances on the transcription factor NF-*k*B with regard to their action mechanism elucidations, since it is involved in the expression of the genes that codify iNOS and cytokines in macrophages (Wu *et al*., 2012). In Fig. 6, we observe that there was a moderate inhibition (reaching 32% inhibition by 2 mg/mL TF with respect to positive control; $p<0.01$) of this factor's concentration in the nuclear fractions of macrophages exposed to samples tested. This inhibition was not as intense as expected, but this was probably because there are other transcription factors involved in the production of this mediator and cytokines. The samples were also tested for their cytotoxic activities against LM3 tumour cells. All samples tested showed a larger value for IC_{50} than the standard drug Taxol® (Table I). On the other hand, emphasis should be placed on the fact that TF, and βF, both presented an exceptional toxicity against mammary tumour cells but not against macrophages (Table II). At higher concentrations of TF and βF, macrophage viability was near 100% while the viability of the LM3 tumour cells was 0%. This is very important in an antitumour drug, since the immunological system is essential to the contention of cancer.

CONCLUSION

Considering that the samples studied herein presented exceptionally tumoural cytotoxicity and immunomodulatory activity, we can suggest that the fractions obtained from *Q. multiflora* possess a promising therapeutic potential. The results obtained highlight the importance of conducting an in-depth study of the species of the Brazilian biome and show the great potential of its biodiversity in the treatment of chronic inflammatory diseases such as cancer.

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