European Journal of Medicinal Plants
6(3): 167-174, 2015, Article no.EJMP.2015.052
ISSN: 2231-0894

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Immunomodulation of Human Blood Phagocytes by *Orbignya phalerata Mart.* Plus IFN-γ and TGF-β Cytokines

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Authors' contributions

This work was carried out in collaboration between all authors. Author DGC carried out the assay participated in the sequence alignment and drafted the manuscript. Author JCDR participated in the sample collection carried out the assays and help to draft the manuscript. Author LGRV also participated in the sample collection and carried out the assays. Author JPMOS participated in the sample collection carried out the assays and help to draft the manuscript. Author ACHF carried out assay conceived of the study and participated in its design and coordination. She also helped to draft the manuscript. Author ELF carried out assay conceived of the study and participated in its design and coordination. He also helped to draft the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2015/14760
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Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=913&id=13&aid=7726

Original Research Article

Received 19th October 2014
Accepted 30th December 2014
Published 10th January 2015

ABSTRACT

Aims: Babassu is the popular name of the palm *Orbignya phalerata Mart*. That belongs to the family of Arecacae (Palmae). Some studies have attributed biological properties to this plant's fruit; however, there are few studies evaluating the biological properties of its leaves. The aim of this study was to evaluate the potential for the extract from Babassu leaves to be a possible

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immunomodulator of the functional activity of blood mononuclear phagocytes (MN) incubated with the bacteria Enteropathogenic *Escherichia coli* (EPEC) in the presence of IFN-γ and TGF-β cytokines.
Study Design: Experimental study of *in vitro* cell immunomodulation by medicinal plants plus cytokines.
Place and Duration of Study: Institute of Biological and Health Science, Federal University of Mato Grosso, Barra do Garças, MT, Brazil, between June 2012 and July 2014.
Methodology: Mononuclear (MN) phagocytes were separated from blood by density gradient using Ficoll Paque. The cells were incubated with EPEC in the presence or absence of plant extract and IFN-γ and TGF-β cytokines. A control was performed using only cells (spontaneous group). The functional activity was evaluated by superoxide anion release, phagocytosis and bactericidal activity.
Results: There was a significant increase ($p<0.05$) in the superoxide release by phagocytes in all groups that were incubated with plant extract. In the presence of IFN-γ and TGF-β cytokines plus plant extract, was observed lower ($p<0.05$) superoxide release and increased in phagocytosis and bactericidal activity.
Conclusion: Babassu extract is a potent immunostimulator of mononuclear phagocytes and a natural alternative that can be applied for the benefit of human health, specifically in infectious diseases.

Keywords: Orbignya phalerata Mart; EPEC; phagocytes; cytokines.

1. INTRODUCTION

Plants have been used for therapeutic purposes by people for a long time. This practice is observed in different ethnicities and locations around the world [1,2,3].

Currently, medicines produced from plants according to indigenous and popular knowledge are being used by people in urban and rural areas because they are safe, effective and inexpensive [2]. It is observed in the poorest regions that medicinal plants are sold in fares and markets and are popularly found in residential backyards [3].

In Brazilian ethnobotany, babassu, which is a generic name given to an oil palm belonging to the *Arecaceae* [Palmae] family and members of the *Orbignya* and *Attalea* genera, is highlighted among plant species [4]. Studies demonstrate that babassu has analgesic and anti-inflammatory biological properties [5]. Studies performed with a compound of plants, of which the highest concentration was babassu extract, verified that this herb activates the functional mechanisms of human phagocytes [6,7] and can activate the immune system when combined with a modified release system [8,9]. Several studies have also demonstrated that babassu oil has anti-inflammatory activities [10,11] and healing properties [4,10,12].

The main product used in manufacturing phytotherapic drugs in Brazil is Babassu's leaves; however, there are few studies on its therapeutic properties. Recent emerged interest in the identification of the biological properties of its leaves has shown babassu to be an analgesic and anti-inflammatory [5]. T The phytotherapic "Mais vida" is a compound produced by mixing seven different plants, including babassu. Some studies have found that the phytotheraphic action of this compost or of babassu on human phagocytes activates their functional mechanisms [7,8,10,13].

Cytokines are potent modulators involved in immune homeostasis, regulating the inflammatory response and promoting effective control of pathogenic and tolerogenic mechanisms. Therapeutic administration of cytokines has been employed to manipulate the immune response in diseases such as cancer, autoimmune diseases and infectious diseases; the cytokines' adjuvant properties may enhance the effectiveness of the vaccine. Several studies are being conducted on the methods and optimal concentrations for the administration of cytokines [14], but their interaction with medicinal plants such as babassu have not been elucidated yet. Thus, the aim of this study was to evaluate the cell viability of human blood phagocytes in the presence of hydroalcoholic extract produced from the leaves of babassu and to verify the

modulatory activity of plant extracts in phagocytes in the presence of cytokines.

2. MATERIAL AND METHODS

2.1 Blood Sampling and Blood Cell Separation

Blood samples [10 mL] were collected from 43 volunteer donors and were placed in tubes with EDTA. This study was approved by the Federal University of Mato Grosso Research Ethics Committee, and all of the subjects gave written informed consent before beginning the experimental protocol. The samples were centrifuged at 160 x g for 15 min to separate plasma from the cells. Cells were separated over a Ficoll-Paque gradient [Pharmacia, Upsala, Sweden], producing preparations with 95% pure mononuclear (MN) cells as analysed by light microscopy. Purified MN phagocytes were resuspended independently in serum-free 199 medium at a final concentration of 2 x 10^6 cells/mL. The cells were used immediately for superoxide release, phagocytosis and bactericidal activity assays [15].

2.2 Preparation of Hydroalcoholic Extract for the Prospecting of the Materials

The plant used in the preparation of the extract was collected and deposited in the herbarium of the "Centro de Biodiversidade Ambiental, Reserva Ecocerrado Brasil" located in Araxá, MG, Brazil (latitude 19°36'47, 1", longitude 47°08'20,9" and altitude 939 m).

Orbignya sp. leaves were collected, and the preparation involved the mixing process followed by maceration and distillation. For processing of macerates, the plant was macerated by placing 200 g of the plant for one liter of alcohol 70%. The plant was left soaking for thirty days at room temperature. During the first ten days the preparations were shaken once a day. After this period the preparation was filtered. For the distillation process the samples were passed on and still were concentrated until syrupy consistency in temperature up to 60°C Thus, the samples of the plant were passed through the distiller and concentrated to a syrupy consistency, at temperatures up to 60ºC. The extract concentration used was 0.2 g/ml.

2.3 *Escherichia coli* Strain

The enteropathogenic *Escherichia coli* [EPEC] used was isolated from stools of an infant with acute diarrhoea [serotype 0111: H^- AL^-, eae^+, eaf^+, bfp^+]. This material was prepared and adjusted to 10^7 bacteria/ml, as previously described by Honorio-França et al. [15].

2.4 Treatment of Blood MN Phagocytes with Cytokines and Extract of *Orbignya sp*.

To assess the effect of plant extract and/or cytokines (IFN-γ and TGF-β) on superoxide anion release, phagocytic and bactericidal activity, MN phagocytes ($2x10^6$ cells/mL) were incubated with 5 µL of cytokines (Sigma ST Louis, USA, final concentration 100 ng/ml) [16] and/or 50µl of Orbignya sp.'s hydroalcoholic solution (concentration of 0.2 g/ml) for 1 h at 37°C. The phagocytes were then washed once with 199 medium at 4°C and immediately used in the assays. A control was performed with only 199 medium.

2.5 Cell's Viability

To verify cell viability of MN phagocytes in the presence of plant extract, the phagocytes were incubated in 250 µl of cells with 50 µl of extract for 30 minutes at 37ºC under continuous shaking. In sequence, cells were stained for 1 minute with 200 µl of acridine orange (concentration 14.4 mg/ml), resuspended in PBS, and then centrifuged and washed twice more. Next, slides were mounted and a quantity of one hundred cells (live and dead) was counted on a fluorescence microscope (Nikon Eclipse E 2000, Nikon Corporation, Tokyo, Japan) [17].

2.6 Release of Superoxide Anion

Superoxide release was determined by cytochrome C [Sigma, ST Loius, USA] reduction [15]. Briefly, mononuclear phagocytes and bacteria were mixed and incubated for 30 min for phagocytosis. Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160 x g, 10 min). Cells were then resuspended in PBS containing 2.6 mM CaCl2, 2 mM MgCl2, and cytochrome C (Sigma, ST Loius, USA;2 mg/mL). The suspensions (100 µL) were incubated for 60 min at 37°C on culture plates. The reaction rates were measured by

absorbance at 550 nm and the results were expressed as nmol/O_2^-. All the experiments were performed in duplicate.

2.7 Bactericidal Assay

Phagocytosis and Microbicidal activity were evaluated by the acridine orange method [18]. Equal volumes of bacteria and cell suspensions were mixed and incubated at 37°C for 30 min under continuous shaking. Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160 x g, 10 min, 4°C). Cells were resuspended in serum-free 199 medium and centrifuged. The supernatant was discarded and the sediment dyed with 200 µL of acridine orange (Sigma, ST Loius, USA; 14.4 g/L) for 1 min. The sediment was resuspended in cold 199 medium, washed twice and observed under immunofluorescence microscope at 400 x and 1000x magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least 3 bacteria in a pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index is calculated as the ratio between orange-stained (dead) and green- stained (alive) bacteria x 100 [3]. All the experiments were performed in duplicate.

2.8 Statistical Analysis

Data were expressed as the mean ± standard deviation [SD]. Statistically significant differences in superoxide anion release, phagocytosis and bactericidal index in the presence or absence of plant or cytokines were evaluated using analysis of variance [ANOVA]; statistical significance was considered for a p-value less than 0.05.

3. RESULTS AND DISCUSSION

According to ours results, the phagocytes' viability was above 90% in the presence of the plant extract, independent of incubation times; no significant difference was observed when compared to their respective control groups (Table 1).

A previous study showed that the toxicity of ethanol from babassu extract presents low or no acute toxicity in mice [19]. Nanodoses of almond Babassu showed no toxicity in mice [20]. These studies corroborate our results; *in vitro* phagocyte viability of the extract showed rates above 90%.

Table 1. Cell viability of MN phagocytes in the presence of the babassu extract with different incubation times

Time (min)	MN (%)	MN + *Orbignya* (%)
0	91.00±4.54	90.14±5.81
30	93.00±4.00	90.86±6.23
60	92.00±5.14	91.83±2.23
90	94.75±2.90	96.50±1.29
120	91.60±8.59	90.71±5.56

The data represent the mean ± standard deviation (SD) of ten experiments with cells from different individuals. Mononuclears (MN).

However, several authors also relate cell viability with functional activity, demonstrating that stimuli such as medicinal plants [8,21,22] and hormones [23] can increase superoxide anion release from MN phagocytes. In this study, there was a significant increase ($p<0.05$) in the superoxide release by MN phagocytes in all groups that were incubated with the plant extract when compared to the spontaneous release and the cells incubated with only EPEC. In the presence of babassu extract the superoxide release increased, regardless of the presence of EPEC. The phagocytes incubated with EPEC and plant extract showed increased superoxide release. In the presence of IFN-γ and TGF-β cytokines, there was a decreased superoxide release by phagocytes compared with the cells treated with babassu in the presence of EPEC.

There was no significant difference between the cells incubated with cytokines in the presence and absence of plant extract (Fig. 1).

Literature reports that increased superoxide release by phagocytic cells is associated with increased phagocytic and bactericidal activity [17,18]. Phagocytosis and microbicidal activity of phagocytes with production of active oxygen metabolites such as free radicals comprise an important defence mechanism against a number of bacterial [15,17,18]. Fungal [24] and protozoal infections [25].

Here, the phagocytosis index increased significantly in cells incubated with EPEC and babassu extract when compared to those cells incubated only with bacteria. When the cells were treated with cytokines and plant extract a significant increase was observed in phagocytosis and bactericidal activity (Table 2).

The highest increase in phagocytosis index was observed in cells treated with IFN-γ plus plant

extract. The cells of the group containing TGF-β and plant extract also had a significant increase in phagocytosis index compared to the cells of other experimental groups, except the cells incubated only with plant extract. There was no significant difference in the rate of phagocytosis between groups containing IFN-γ plus plant extract and TGF-β plus plant extract (Table 2).

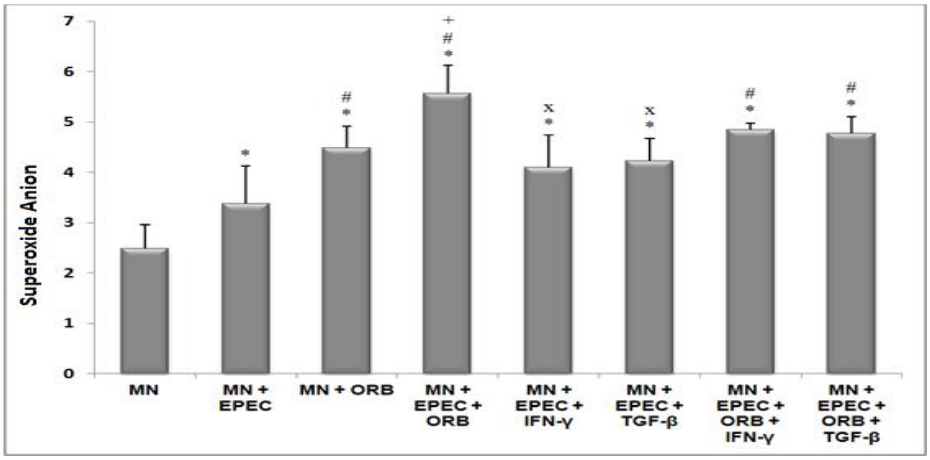

Fig. 1. Superoxide release by MN phagocytes (2x10^6 cells/mL) treated with babassu extract and IFN-γ and TGF-β cytokines in the presence of EPEC

Mononuclear (MN); mononuclear plus enterophatogenic Eschechia coli (MN+EPEC); mononuclear plus enterophatogenic Eschechia coli plus Orbignya (MN+EPEC+ORB); mononuclear plus enterophatogenic Eschechia coli plus Interferon-gamma (MN+EPEC+IFN-γ);mononuclear plus enterophatogenic Eschechia coli plus Transforming growth factor beta (MN+EPEC+TGF-β);mononuclear plus enterophatogenic Eschechia coli plus Orbignyaplus Interferon-gamma (MN+EPEC+ORB+IFN-γ); mononuclear plus enterophatogenic Eschechia coli plus Orbignyaplus Transforming growth factor beta (MN+EPEC+ORB+TGF-β).

*The data represent the mean ± standard deviation (SD)of twenty experiments with cells from different individuals. Mononuclears (MN). *ANOVA (P<0.05) when comparing the experimental groups with the spontaneous group containing only cells. # ANOVA (P<0.05) groups compared with the experimental group containing cells and EPEC (EPEC MN). ANOVA (P <0.05) groups comparing the experimental group containing cells and hydroalcoholic Orbignya leaves (MN* +babassu*).x ANOVA (P<0.05) groups comparing the experimental group containing cells. EPEC and hydroalcoholic Orbignya leaves*

Table 2. Phagocytosis index (%) and bactericidal activity (%) of blood MN phagocytes

Groups	Phagocytosis (%)	Bactericidal activity (%)
MN + EPEC	60.9±2.8	22.6±9.1
MN + EPEC + ORB	66.8±6.7	33.9±8.8
MN + EPEC + IFN-γ	59.8±4.7	51.7±8.0$^{*\#}$
MN + EPEC + TGF-β	54.1±4.9$^{\#}$	34.3±7.8^{+}
MN + EPEC + ORB+ IFN-γ	79.6±4.1$^{*\#+x}$	41.1±2.9*
MN + EPEC + ORB+ TGF-β	71.2±5.2^{*+x}	48.9±3.9$^{*\#x}$

The data represent the mean ± standard deviation (SD) of thirteen experiments with cells from different individuals. Mononuclears (MN).

The data represent the mean ± and the standard deviation (SD). Eschechia coli (MN+EPEC); mononuclear plus enterophatogenic Eschechia coli plus Orbignya (MN+EPEC+ORB); mononuclear plus enterophatogenic Eschechia coli plus Interferon-gamma (MN+EPEC+IFN-γ); mononuclear plus enterophatogenic Eschechia coli plus Transforming growth factor beta (MN+EPEC+TGF-β);mononuclear plus enterophatogenic Eschechia coli plus Orbignyaplus Interferon-gamma (MN+EPEC+ORB+IFN-γ); mononuclear plus enterophatogenic Eschechia coliplus Orbignyaplus Transforming growth factor beta (MN+EPEC+ORB+TGF-β).

**The results represent statistically significant differences between experimental groups compared with the control group (ANOVA. P <0.05). $^{\#}$ANOVA (P<0.05) when comparing the experimental group (MN* +babassu + *EPEC) with the other experimental groups. $^{+}$ANOVA (P<0.05) experimental groups compared with the group containing cells. IFN-γ and EPEC (EPEC + MN + IFN-γ). ^{x}ANOVA (P<0.05) groups compared with the experimental group containing cells. TGF-β and EPEC (EPEC + MN + TGF-β)*

The cells treated with only IFN-γ had the highest rate of bactericidal activity. The cells treated with both cytokines and plant extract had a significantly higher bactericidal index when compared to cells in the control group (Table 2).

There was a significant increase in bactericidal activity in cells treated with both TGF-β and plant extract when compared to the cells treated with only TGF-β. There was no significant difference in bactericidal activity between the cells treated with IFN-γ and plant extract when compared with the cells treated with only IFN-γ. There was also no significant difference in bactericidal activity between cells treated with cytokines and plant extract (Table 2).

In the literature, aqueous Babassu extract was capable of promoting cell migration of MN phagocytes in vivo, releasing proinflammatory mediators such as nitric oxide (NO) by macrophages *in vivo* and *in vitro*. promoting a significant increase in the expression of MHC class II and secreting TNF [26]. In this study, the hydroalcoholic extract of Babassu was able to modulate in vitro MN phagocytes. In addition to the association of the hormone melatonin with the phytotherapic "Mais Vida", prepared from a mixture of seven different plants including the Babassu (75% dry leaf babassu) promoted a potent activation of oxidative mechanisms and MN phagocytes [6]. Other works with the same compound showed similar results [7,8,13]. Immunomodulatory activity of the plant was also reported in a study of blood lymphocytes [27].

When assessing the behaviour of MN phagocytes in the presence of the extract of babassu and the IFN-γ and TGF-β cytokines the release of superoxide anion was slightly decreased when compared to the group containing only plant extract and EPEC. However, in the presence of both cytokines there was no significant increase in the rate of phagocytosis and bacterial killing.

At low concentrations. ROS are capable of mediating cellular functions that are activated by natural stimuli [28]. All activated cells produce a certain amount of ROS, but the natural antioxidant systems maintain low levels. A condition of oxidative stress is generated when a cell accumulates an excessive concentration of ROS [29].

The plant extract was shown to be a potent immunostimulator of MN phagocytes because it induced greater superoxide anion release by these cells. The association between the Babassu extract and cytokines has been shown to be effective in modulating MN phagocytes. The decrease of superoxide release caused by the combination of plant extracts with IFN-γ and TGF-β cytokines may be considered a beneficial response because the functional activity of the cell was not altered, but the cellular oxidative stress was reduced.

4. CONCLUSION

This study demonstrated that the association between the Babassu extract with cytokines was able to control cellular oxidative mechanisms and improved the functional response of MN phagocytes. These data suggest that the Babassu extract associated with cytokines may be an alternative form of therapy against infections.

ACKNOWLEDGMENT

The authors are very grateful to Environmental Biodiversity Center. “Ecocerrado” Reserve. Araxá. MG. Brazil for technical assistance with the plant extractand the authenticity of plant identification. This research received grants FAPEMAT, CAPES and CNPq.

COMPETING INTERESTS

The authors declare no conflict of interest and non-financial competing interests regarding the publication of this article.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=913&id=13&aid=7726