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Immunomodulatory effects of herbal plants plus melatonin on human blood phagocytes

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Abstract

It has been shown a mixture of seven herbal plants was able to trigger cell oxidative mechanism and subsequently inducing cellular activation. Moreover, melatonin hormone has also been shown to perform different actions on the cellular oxidative metabolism. It is possible that the herbal mixture associated with melatonin can activate phagocytes, improving microbicidal activity and further ameliorating resistence to the infections. The aim of this work was to verify in vitro immunomodulatory effects of melatonin and a medicinal plants mixture on blood mononuclear phagocytes (MN). We collected 40 blood samples from normal individuals to obtain the phagocytes. The MN phagocytes were separated by Ficoll-Paque gradient. Preparation of plant extract to obtain the herbal mixture was carried through the process of maceration followed by distillation. Phagocytosis and microbicidal activity of blood phagocytes, treated or not with exogenous superoxide dismutase (SOD), against enterophathogenic *Escherichia coli* (EPEC) were evaluated by acridine orange method. The herbal mixture and/or melatonin were added to the cell suspensions as immunomodulators. We observed increased phagocytosis and microbicidal activities by blood MN phagocytes in the presence of melatonin or the herbal mixture. The association of both potentiated the functional activity of blood MN phagocytes. Phagocytes previously treated by exogenous SOD had decreased microbicidal activity independently of immunomodulators. These data suggest that the herbal mixture is a potent immunostimulatory agent, and that the interaction between plant and hormones may represent an alternative mechanism of defense against infection, especially in immunosuppressed patients.

Keywords: herbal mixture, melatonin, blood phagocytes, phagocytosis, superoxide

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Introduction

Several studies attempt to elucidate the antimicrobial action of phagocytes. On this respect, it was assigned an important role for oxygen-derived radicals and their possible modulators [1-4]. The multiple cascades displayed by free radicals may be associated on phagocytosis and microbicidal activity designed to eliminate potentially pathogenic agents [2].

Phagocytosis represents an important defense mechanism especially against bacterial infections. In phagocytosis occurs massive activation of cellular oxidative metabolism with generation of potent active oxygen metabolites. Oxygen-derived radicals are involved in many important processes such as immune reactions, longevity, and peroxidation of cell lipids, proteins, carbohydrates, and DNA [1,5].

On the order hand, the literature has been demonstrated that many plants can stimulate immune cells constituting a real promise for treatment of infections [6]. The use of medicinal plants has been increased and there is a mojor scientific breakthrough on issues related to both chemical and pharmacological studies and the search for a new therapeutic compounds [7,8] especially of alternative drugs in disease treatment, including infections diseases.

In Brazilian ethnopharmacology a popular mixture of seven plants has been shown to be useful for immune system improvement as well as increased resistance to tumor cells. This herbal mixture includes the extract preparation of the following plants: *Orbignya Marti*, *Tabebuia avellanedae*, *Arctium lappa*, *Rosa centifolia*, *Maytenus ilicifolia*, *Vernonia condensata* and *Thujae occidentalis*. It has been demonstrated that this herbal mixture has ability to massively activate cellular oxidative mechanisms [9], but its implications for phagocytosis and microbicidal activity are still partially understood.

Literature also reports the importance of hormones and neuropeptides as potent immunomodulators, among them the melatonin hormone [10], involved in many different aspects of the regulation of functional activity on the immune system [11].

Melatonin functions are still only partially understood [12]. Research has been pointed out that melatonin, synthesized by pineal gland, is able to modulate the immune response [10,12] in a dose-dependent manner [13,14]. The involvement of melatonin on phagocytes function is controversial. Some authors postulate that melatonin activates immune system [10,15], whereas others had considered it as a immunosuppressant factor [16], while others report that this hormone is a also produced by the activated phagocytes [17].

Previous report in vitro has been pointed out that this herbal mixture and melatonin play an important role in modulating cell activation once it triggers activation of blood phagocytes by increasing the superoxide anion production [10,11]. It is possible that this mixture associated to melatonin can activate the phagocytes and increase the microbicidal activity of these cells which can further improves resistance against infections.

The aim of the present study was to verify in vitro immunomodulatory effects of melatonin and a medicinal plants mixture on blood mononuclear phagocytes.

Materials and Methods

Subjects: After the informed consent, a sample of 15 mL of blood was collected from 40 clinically healthy men ranging from 18 to 35 years of age. All procedures were submitted to ethical evaluation and obtained institutional approve.

Separation of blood cells: Blood samples were collected into heparinized (25U/ml) tubes. The samples were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden), density gradient (density 1.077 g/l), producing preparations with 98% of pure mononuclear (MN) phagocytes, analyzed by light microscopy. Purified MN phagocytes were resuspended independently in serum-free medium 199 at a final concentration of $2x10^6$ cells/mL.

E. coli strain: The Enterophatogenic *Escherichia coli* used was the EPEC O111 – H^- serotype. The stock culture was cultivated in Trypic Soy Broth (TSB, Difco) for 18 hours at 37°C. Bacteria were washed twice in phosphate buffered saline (PBS) and adjusted to an concentration of 1 x 10^7 bacteria/mL as measured by turbidimetry at 540 nm, using a spectrophotometer (Femto). This bacterial concentration was previously determined by colony unit counting on Trypic Soy Agar (TSA, Difco, Detroit) [2].

Preparation of Herbal Mixture: The herbal mixture was composed of 75% leaf dry of *Orbignya martiana*, 2% of the bark of *Tabebuia avellanedae*, 4% of leaf of *Arctium lappa*, 5% of petals of *Rosa centifolia*, 5% of leaf of *Maytenus ilicifolia*, 5% of the leaf of *Vernonia condensata* and 4% of the leaf of *Thujae occidentalis*. All these plants were collected and deposited in the herbarium at the Environmental Biodiversity Center - "EcoCerrado" Reserve, Araxá - MG, Brazil, localized at Lat. 19 ° 36'47, 1" Long. 47 ° 08'20, 9", with a 939m altitude.

The preparation involved the mixing process followed by maceration and distillation according to the Brazilian pharmaceutical code [9]. For processing of macerates, the plant parts were 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 60oC and in the mixture was added preservative NIPAGIN® M.

Phytochemical screening

Phytochemical screening for identification and indication for their main chemical constituents of aqueous extract of herbal mixture was done [18]. Following reagents and chemicals were used alkaloids with dragendroff's reagents, flavonoids with metallic magnesium plus HCl, saponins with the ability to produce foam, reducing sugars with Fehling's reagent, glycosides with Liberman's test, tannins with ferric chloride and polysaccharides with iodine solution.

Treatment of blood phagocytes with melatonin and with herbal mixture

To verify the activity of melatonin and the herbal mixture on phagocytosis, mononuclear phagocytes ($2x10^6$) were treated with 30 min before phagocytosis. To check the phagocytosis and microbicidal activity by mononuclear phagocytes (2 x 10^6), the cells were treated with hormone and/or with the herbal mixture immediately after phagocytosis tests. For each test performed, MN phagocytes (2 x 10^6) were also incubated in culture medium 199 in the absence of the melatonin and the herbal mixture. Concentrations of the hormone melatonin were 10^{-7} molar [19] and the herbal mixture 1mg/ml [9].

Bactericidal assay

Viability test, phagocytosis and microbicidal activity were evaluated using the acridine orange method described by Bellinati-Pires et al. [20]. 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 *g*, 10 min, 4°C), and the cells were resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded and the sediment dyed with 200µL of acridine orange (14.4g/L) for 1 minute. The sediment was resuspended in cold culture 199, washed twice and observed under immunofluorescence microscope at 400x and 1000x magnification. The viability index was calculated by counting 100 cells. For viability, the viable cells were shown to be green and red non-viable. The phagocytosis index was calculated by counting the number of cells ingesting at least three bacteria in a pool of 100 cells. To determine the bactericidal index, cells were stained with acridine orange and the killing of the bacteria were verify in 100 cells counted per slide. The dead EPEC, viewed with orange

stain [20]. The assays of phagocytosis and microbicidal activity were made in the presence or absence of superoxide dismutase (SOD; 140 units) [3,21]. All the experiments were performed in duplicate or triplicate.

Results

Phytochemical screening

Phytochemical screening showed the presence of tannins, especially condensed type or catechist. The mixture also presented in its chemical composition leucoanthocyanidins, flavanones and catechins. To lower degree there was presence of phenols, anthocyanins, proanthocyanidins, flavonoids, flavanones, xanthones, triterpenoids and saponins. The herbal mixture was negative for steroids, resins, alkaloids, quaternary compounds, quinones, flavonoids aglycones and steroid aglycone triterpenoids (Table 1).

Table 1 – Phytochemical screening for identification and indication of main chemical constituents of aqueous extract of herbal mixture (HM).

Analysis	Aqueous Extract (HM)
Tannins	++
Leucoanthocyanidins, catechins and flavanones	++
Phenols	+
Anthocyanins, anthocyanidins and flavonoids	+
Flavanones and xanthones	+
Triterpenoids	+
Saponins	+
Resins	-
Alkaloids	-
Quaternary compounds	-
Quinones	-
flavonoids aglycones	-
Steroid aglycone triterpenoids	-

(++) strong positive reaction (+) positive reaction
(-) negative reaction

Blood MN phagocytes viability in the presence of the herbal mixture

The results of blood MN phagocytes viability in the presence of the herbal mixture are showed in the Table 2. It was observed untreated cells had 99% viability. Similar rates were also observed when these cells were incubated by the herbal mixture and melatonin, demonstrating the lack of toxicity of immunomodulatory agents on used dosage.

Table 2 – Number and index of viability of Blood Mononuclears (MN) phagocytes in the presence of melatonin hormone (MLT) or/and herbal mixture (HM).

Parameter	Experimental Group		
	199 Medium	MLT	HM
Phagocyte count ($x10^6$ cells/ml)	3.9 ± 0.2	3.8 ± 0.6	4.0 ± 0.3
Viability of MN phagocytes (%)	99 ± 1.2	97 ± 1.1	99 ± 1.3

Results represent the mean (± SD) of ten experiments with cells from different individuals. (ANOVA $p>0.05$)

The phagocytic activity of blood MN cells for EPEC in the presence of the herbal mixture and melatonin hormone was significantly increased when the cells were stimulated by herbal mixture and melatonin (Figure 1A).

The herbal mixture plus melatonin increased the bactericidal index when compared to the results presented by cells incubated only with the bacteria or melatonin (Figure 1B).

Treatment of blood phagocytes by exogenous SOD did not interfered on phagocytosis (Figure 2A). In respect of the microbicidal activity of blood MN treated by exogenous SOD, independent of the effect of immunomodulatory agents, it was observed lower bactericidal index (Figure 2B).

Discussion

In this study we evaluated the in vitro immunomodulatory effect of melatonin associated with a mixture of seven herbal plants on phagocytosis and microbicidal activity of MN human blood phagocytes against enterophatogenic *Escherichia coli*, and their interactions with cellular oxidative mechanisms.

Plants are considered an unlimited source of molecules for new treatment of diseases. There is growing interest in the investigation of different plant species to identify their potential for therapeutic application because of the large historical legacy of medicinal plants [22] with fewer side effects, lower cost and toxicity. There is general consensus that the adverse effects of herbal medicines are less frequent when compared to synthetic drugs [23].

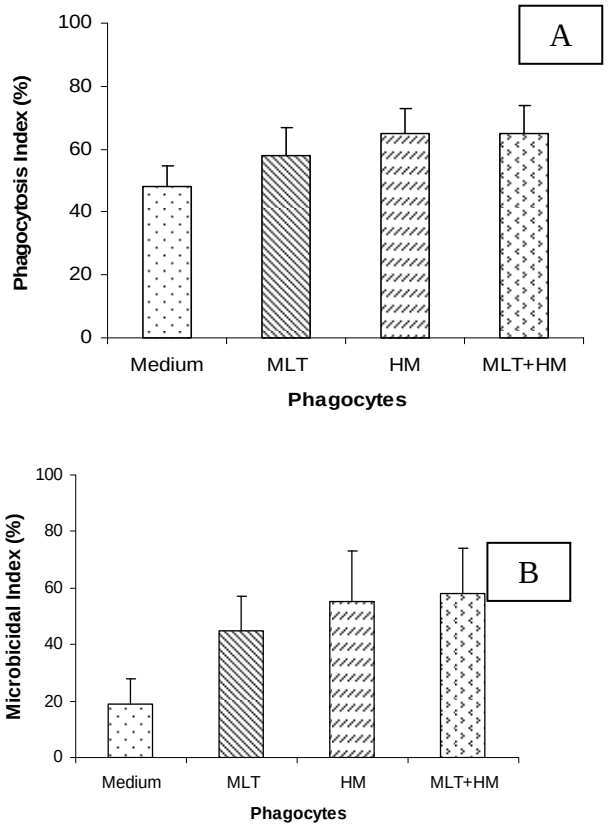

Fig 1 Phagocytosis and microbicidal index of blood MN cells for EPEC in the presence of the herbal mixture and the hormone melatonin.

Literature reports the effectiveness of medicinal plants which have been opened prospect for obtaining new drugs [24] and studies relate that a large number of plants used in traditional healing are employed in often sophisticated mixtures, rather than as individual plants [25]. Several medicinal plants have shown ability to induce immune system activation displaying beneficial effects against disease [25,26].

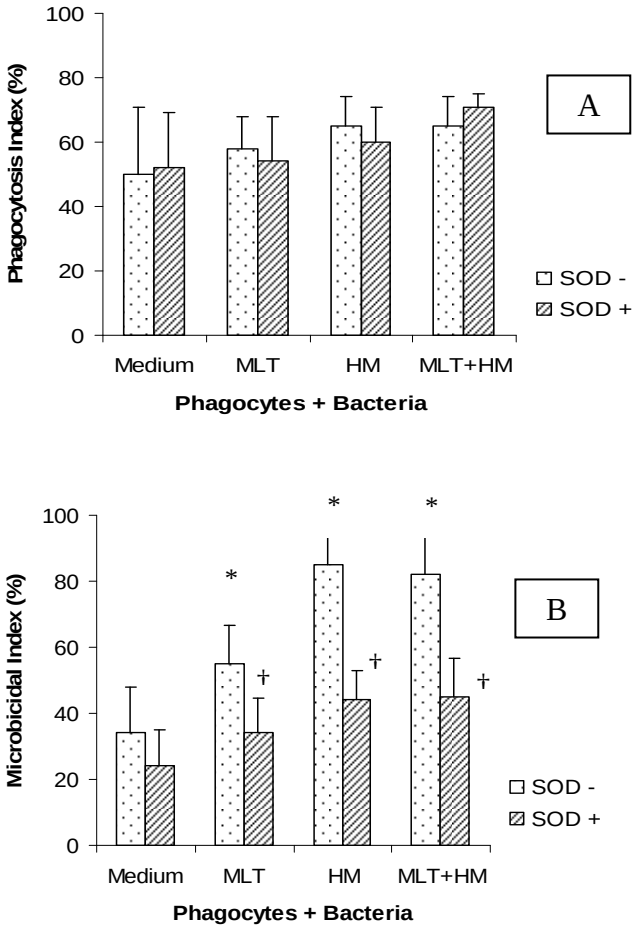

Fig 2 : Phagocytosis index and microbicidal activity of blood MN phagocytes treated with exogenous SOD in the presence of the herbal mixture and the hormone melatonin.

In this study it was found that the herbal mixture presented a potent immunstimulatory effect on the functional activity of blood phagocytes. Association of melatonin with the herbal mixture

potentiated both the phagocytic and microbicidal activities. One of the herbs used in this mixture is *Thujae occidentalis*. One interesting clinical controlled trial reported that an extract containing *Thujae occidentalis* and other two herbs improved common cold symptoms in human patients [27]. Microbicidal activity is an important mechanism for elimination of infection, particularly those caused by bacteria [2,3]. Neurohormonal control is very important to modulate immunobiological effects [28]. Melatonin has beneficial free radical scavenging actions beyond its stimulatory effects on the cytosolic antioxidant enzyme systems [14,29]. Many studies have been reported that melatonin strongly stimulates cells of the immune system [3,19]. In a previous study we demonstrated the functional activity of human blood phagocytes against bacteria and fungi are modulated by hormones. The melatonin hormone has increased microbicidal activity of mononuclear phagocytes as well as rat macrophages [4,10,15]. This study reported that melatonin presented imunostimulatory effects that may be potentiated by the herbal mixture.

Many medicinal plants have shown their ability to stimulate different immunomodulatory pathways [30,31].

This study confirmed an additive effect between endogenous peptides and plants with medicinal activity. The combination of melatonin and the herbal mixture increased the functional activity of phagocytes. In literature, several studies attempted to elucidate the antimicrobial action of phagocytes. Regarding this aspect it was assigned an important role for oxygen-derived radicals and their possible modulators [1-3,32]. Our previous study confirmed that the herbal mixture can modulate oxygen metabolism pathways [10].

Macrophages play an important role on the mechanisms of the body's defense against infections. The functional activity of macrophages in various biological systems has been associated with immunomodulatory activity [33]. The generation of free radicals has been reported as an important mechanism for protecting the body during the infectious processes [2,32].

During the oxidative stress the cellular mitochondrial and peroxisomal metabolism generates large amounts of the superoxide anion [10,15]. The free radical releasing has been reported as an important body's defense mechanism especially in gut infections [2,32].

The action of reactive oxygen metabolites has been considered an important mechanism of bacterial killing since an ineffective phagocytosis is associated with formation of granulomas [21]. The superoxide anion is the first molecule from the oxygen free radical cascade of oxygen metabolism.

Moderate oxidative stress is often accompanied by an increase in enzymatic antioxidant defenses that can neutralize deleterious effects of free radicals [34]. Among these enzymes superoxide dismutase (SOD) act by catalyzing the dismutation of superoxide into H_2O_2 and O_2 resulting as an important antioxidant role [34].

In this study, we evaluated if the functional activity of phagocytes modulated by the association of melatonin and an herbal mixture is oxidative-dependent we used exogenous SOD. Phagocytes treatment by SOD reduced the microbicidal activity of cells stimulated by both the melatonin and the herbal mixture. This result suggests that both melatonin and the herbal mixture are able to activate the cellular oxidative metabolism and exert important immunomodulatory effects on phagocytosis and microbicidal activity.

SOD comprises a very important mechanism on superoxide anion reduction [35] so the results of this study suggested that the microbicidal activity of human blood phagocytes is dependent on superoxide anion release constituting an important defense mechanism against bacterial infections. It is important to note that both the herbal mixture as the melatonin hormone, in the used dosages, did not exert cytotoxic effects in these cells.

Conclusion

These data suggest that the herbal mixture is a potent immunostimulatory agent, and that the

interaction between plant and hormone may represent an alternative mechanism of defense against infection.

List of Abbreviations
DNA- deoxyribonucleic acid
MN phagocytes – mononuclear phagocytes
TSB - Trypic Soy Broth
TSA - Trypic Soy Agar
EPEC – Enteropathogenic *Escherichia coli*
SOD – Superoxide dismutase
MLT – melatonin hormone
HM – Herbal mixture
H_2O_2 - hydrogen peroxide

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