



Appraisal of Immunological Impacts of *Melaleuca leucadendra* Extract Over Macrophage Performance in Vitro

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ABSTRACT

This trial research was performed to discuss the immune-influence of *Melaleuca leucadendra* ‘paper-bark tree’ dried leaves which is an important medical plant known in many regions in the world. The leaves were dissolved in a mixture of (ethanol + water) (3:1) mixture, then filtered, evaporated and dried under reduced pressure to obtain leaves extract. The macrophages of blood derived origin were provided from rats and mixed with three different leaves extracts doses in tissue culture plates and incubated then stained with fluorescent acridine orange and examined under fluorescent microscope to assess the phagocytic and killing potency. The wells contents were aspirated and assayed for nitric oxide and interleukin-2 levels. The results displayed an obvious increase in phagocytic, killing performance as well as nitric oxide and IL-2 level production than control in a dose dependent manner. The obtained results suggested the immune-stimulant impact of the paper-bark tree leaves.

Key words: Interleukin-2, Macrophages, *Melaleuca Leucadendra*, Nitric oxide and Phagocytic activity.

INTRODUCTION

Immuno-regulation is essential for the maintenance of immune system homeostasis and conflict of many diseases and disorders. The recent decades exhibited raising investigations concerned immunomodulatory effects of medical plants and their extracts. These comprise stimulatory impacts on immune cells, molecules, and cytokine production (Cheng *et al.*, 2018; Abdel-Sattar *et al.*, 2019). The widespread usage of herbal extracts and active ingredients of plant origin favor their ability for potentially substitute the chemotherapeutic agents to bypass their known adverse reactions, stress on the beneficial microbiota and the antimicrobial resistance emergence in pathogens (Dkhil *et al.*, 2016).

Melaleuca leucadendra, “commonly known as the paper-bark tree” is a well-known medicinal herb which has many therapeutic properties. *M. leucadendra* have been popularly used for tranquilizing, sedating, ill-removing and pain-soothing agents (Surh and Yun, 2012).

Macrophages are major immune system coordinator and involved in many immunological activities. They pouch and assimilate microbes, cellular debris, malignant

cells, and any non- self-molecules, in a process called phagocytosis. These macro eaters devastate the pathogens via bactericidal molecules output (Shapouri-Moghaddam *et al.*, 2018).

In addition to phagocytosis, macrophages play not only a substantial role in nonspecific defense (innate immunity) but also support the inception of specific acquired immunity (Bonnardel *et al.*, 2015). Macrophages are located in all tissues but with various shapes and names, all forms are acquired from monocyte; the primitive kind of mononuclear phagocyte exist in blood stream which are developed in the bone marrow, and some differentiate then migrate into different lymphoid tissues (Artyomov *et al.*, 2016).

Nitric oxide (NO) is a potent effector molecule that can be induced quantitatively by macrophages and sharing in the innate host immune protection system targeting pathogens (Martinez *et al.* 2000).

Interleukin-2 (IL-2) is a soluble signaling cytokine molecule interposing various chains of immunological effects; the expansion and vivification of macrophages, CD4+ T helper cell polarization, B lymphocyte differentiation, CD8+T cell vastness, memory lineage and natural killer cell activation (Boyman and Sprent 2012).

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Eventually, the novel sight of medical plants is directed towards their extracts and bioactive components bind to the immunological acts. The current study examined the impact of the *M. leucadendra* extract on various activities of macrophages; phagocytic, killing ability, active molecule production.

MATERIALS AND METHODS

Ethical approval: Anesthetic steps and handling with animals followed the ethical guidelines of the Ethical Committee of the National Research Centre in Egypt under number of 17119.

Plant material

Melaleuca lucadendron was obtained from the Giza Zoo, Cairo, Egypt. The plant leaves were shade dried and grounded to a powder. A voucher specimen is deposited at 4°C.

Extraction and isolation

Dried grounded *M. lucadendron* leaves were extracted twice with aqueous- ethanol (3:1) mixture for 24 hours at room temperature. The extract was concentrated, frozen, and lyophilized (Hashim *et al.*, 2018).

Animals

Twenty female Wistar albino rats of average weight (100-130g) were divided randomly into 4 groups; each with 5 rats. They were obtained from the Animal House, National Research Centre, Egypt.

Assessment of macrophage phagocytic and killing activities *in vitro*:

Isolation of monocytes from blood

The rats were anesthetized with diethyl ether and blood samples were collected using heparinized falcon tubes. The heparinized (10 IU/ml) rat blood grouped samples were centrifuged at 3000 rpm for 10 minutes, then the buffy coat was aspirated and carefully layered on histopaque phagocytes isolation medium, pH 7.3 by a ratio 1:3 in siliconized centrifuge tubes and the gradient was centrifuged at 280 xg for 25 min at 4°C. The interphase layer that contained mononuclear cells was aseptically aspirated and centrifuged at 3000 rpm for 5 minutes (Winnicka *et al.*, 2000).

Preparation of bacterial suspension

Staphylococcus aureus ATCC 25923 was obtained from the reference laboratory of the Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain-Shams University. The staphylococci were maintained on nutrient agar slopes (Difco Laboratories, Detroit, MI) and grown overnight in BHI broth (Difco). The overnight cultures served as inoculum; these bacteria were then harvested and washed twice in Hanke's balanced salt solution (HBSS), pH 7.2, and the organism were suspended and adjusted to 52.5% transmission as each 1ml contained 50×10^6 CFU. The bacteria were opsonized with 1ml of 10% inactivated homologous serum (obtained from 5 serum samples collected from 5 different rats) for 30 minutes at 37°C with gentle shaking. After opsonization, the bacteria

suspended in 1ml HBSS (Silva *et al.*, 1988) with some modification (Hakim *et al.*, 2019).

Phagocytic and killing activities

Monocytes were maintained in RPMI- 1640 medium containing 10% fetal bovine serum, and traces of mercaptoethanol at 37 °C in a humidified 5% CO₂ atmosphere (ESPEC CO₂ Incubator) for 48 hours. The assays were done in four 6- wells tissue culture plates, each plate contained one concentration (100, 200 or 300mg /1ml) of the *M. leucadendra* extract in five wells with staphylococcal suspension and the fourth served as control without extract. The plates were incubated for 24 hrs. The wells contents were aspirated and centrifuged at 160 xg 4°C for 7 min and stained with acridine orange.

Acridine orange staining

The pellet was stained with 15 mg/l acridine orange in phosphate buffered saline, pH 7.2 for 1 min, washed twice in ice-cold HBSS, and wet-mounted on microscope slides (Nagl *et al.*, 2002). Intracellular phagocytosed staph cells then killed ones through determining viable (green) and non –viable (red / yellow) fluorescence through examination in a fluorescent microscope using transmitted light was equipped with x 100 oil immersion objective. Photographs were taken using Kodak color-print film with the shutter set for two minutes.

Measurement of nitrite concentration

The nitrite accumulated in plates' wells was measured as an indicator of nitric oxide production, according to (Rajaraman *et al.*, 1998) with some modification (Hakim *et al.*, 2015). Briefly, 100ul of wells' contents was incubated with an equal volume of Griess reagent in triplicate, into flat bottom 96 well plate at 25°C for 10 min in a dark place. The absorbance was measured at 540nm by universal microplate reader ELx 800 UV (Bio-Tek), and the concentration of nitrite was calculated from the sodium nitrite standard curve.

Determination of Interleukin-2

The mouse IL-2 ELISA kit (Koma Biotech, Inc.) was used for the quantitative determination of mouse IL-2 in wells contents of the three plant extract concentrations.

Statistical analysis

Data for immunological parameters were analyzed and main effects were discussed if $P < 0.01$ and were presented as means $\pm$ SE for the indicated number of independently performed experiments. Statistical significance (≤ 0.01) was assessed by t-test.

RESULTS AND DISCUSSION

Fig. 1.; “1 a, b and c” showed a concentration dependent significant increasing in the phagocytic and killing activities of *S. aureus* cells after 12 hours in comparison with control “d”. The Table (1) displayed the dependent significant increasing in nitrite concentration related to nitric oxide production from monocytes. The table (2) showed the dependent significant increasing in IL-2 production from monocytes derived macrophages.

Table 1: displayed the dependent significant increasing in nitrite concentration related to nitric oxide production from monocytes.

	Control	(100mg /1ml) of <i>M. leucadendra</i> extract	(200 /1ml) of <i>M. leucadendra</i> extract	(300mg /1ml) of <i>M. leucadendra</i> extract
OD	0.162± 0.02	0.174±0.05	0.193±0.05	0.212±0.05
Nitrite concentration (µmol/L)	29.5	32.5	35.8	39.1

Each value is a mean ± standard deviation.

Table 2: IL-2 production from monocytes derived macrophages.

	Control	(100mg /1ml) of <i>M. leucadendra</i> extract	(200 /1ml) of <i>M. leucadendra</i> extract	(300mg /1ml) of <i>M. leucadendra</i> extract
IL - 2 production (ng/ml)	14.3± 0.02	21.2± 0.03	28.8± 0.04	33.1± 0.04

Each value is a mean of triplicate analysis ± standard deviation.

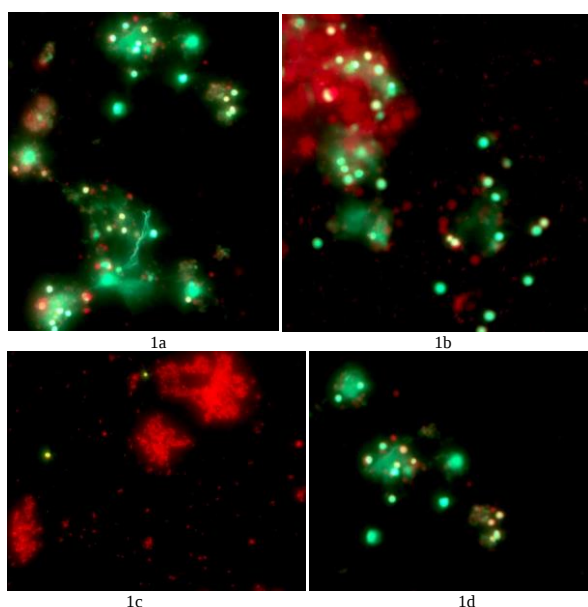


Fig. 1 “a, b and c”: showed a concentration dependent significant increasing in the phagocytic viable (green) and killed non –viable (orange- red) fluorescence *S. aureus* coccal cells after 12 hours in comparison with control “d”.

The achievement of medicinal plants and their extracts as immune-modulators is an effective and valid method to enhance the immune responses against pathogens (Artyomov *et al.*, 2016). *M. leucadendra* represents a member of a popular *Melaleuca* genus, but literature reports about this species still rare (Surh and Yun, 2012).

It is known that macrophages play an important role in host defense as they able to phagocytize microorganisms. Thus, phagocytosis is a fabulous indicator of macrophage effector performance and it constitutes the definitive and most substantial point of the immunological defense system (Guan *et al.*, 2011).

In this study the phagocytic and killing activity of *S. aureus* by three different *M. leucadendra* concentrations was monitored through checking the internalized viable (green) and unviable (red) coccal cells. The data shown in figure 1a, b and c represented a marked increasing in the phagocytosis then killing of *S. aureus* cells as dose-responsive manner in comparison with control 1 d.

Furthermore, the data obtained from tables 1 and 2 exhibited the significant increase in macrophage effector molecules “antimicrobial nitric oxide and immune-promoter IL-2 in a dose dependent manner.

These data coincided with those obtained from previous studies that emphasized the immunomodulatory

activity of genus *Melaleuca*; (Hammer, 2015; Baldissera *et al.*, 2017; Malhi *et al.*, 2017; *et al.*, Casarin *et al.*, 2018). It is illustrated that most of the remedial characteristics of *M. leucadendra* are due to the entity of the flavonoids which are the major bioactive component of the plant essential oil (Baldissera *et al.*, 2014 and Hashim *et al.*, 2018).

Conclusions

The present study gave light on the immune-enhancement potency of paper-bark tree (*M. leucadendra*). Our results asserted that the addition of leaves extract to macrophages elevated their phagocytic and killing abilities in vitro under fluorescent microscopical examination. Also the given data showed that there was a significant increasing in nitric oxide and IL-2 levels produced from macrophages in a dose dependent manner.

Authors contribution

Ashraf Hakim has prepared the experimental design, performed the nitric oxide assay besides the preparation of the initial draft of this manuscript, Mona Elshabrawy has made the statistics. Amany Hashim has prepared the *M. leucadendra* leaves extract, Magdy Bakry has isolated and performed the incubation of macrophage. Sohier Syame has prepared the bacterial suspension besides acridine orange assay and Amany Dapgh performed the interleukin -2 assay. All authors have shared in the manuscript revision.

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