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ORIGINAL ARTICLE

Thymus vulgaris L. and thymol assist murine macrophages (RAW 264.7) in the control of in vitro infections by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*

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Abstract
Microorganisms are capable to combat defense cells by means of strategies that contribute to their stabilization and proliferation in invaded tissues. Frequently antimicrobial-resistant strains appear; therefore, alternative methods to control them must be investigated, for example, the use of plant products. The capacity of the thyme extract (*Thymus vulgaris* L.) and phytocompound thymol in the control of in vitro infections by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* in murine macrophages (RAW 264.7) was evaluated. Minimal inhibitory concentrations (MIC) of the plant products were used. The effect of these MIC were analyzed in the assays of phagocytosis and immunoregulation by analysis of the production of cytokines (IL-1β, TNF-α, and IL-10) and nitric oxide (NO). The plant products effectively assisted the macrophages in the phagocytosis of microorganisms, presenting significant reductions of *S. aureus* and *P. aeruginosa*. The macrophages also regulated the production of inflammatory mediators in the infections by *S. aureus*, *P. aeruginosa*, and *C. albicans.* In addition, thyme provided a satisfactory effect in response to the bacterial infections, regarding generation of NO. Thus, the effectiveness of the thyme and thymol to control in vitro infections by *S. aureus*, *P. aeruginosa*, and *C. albicans* was observed.

Highlights
- Phagocytosis of *S. aureus* by RAW 264.7 was enhanced with thymol
- Thyme enhanced the phagocytosis of *P. aeruginosa* by RAW 264.7
- Plant products provided immunoregulation of inflammatory cytokines
- Production of nitric oxide was improved with the treatments in bacterial infections

Keywords *Thymus vulgaris* · Thymol · In vitro infection · *Staphylococcus aureus* · *Pseudomonas aeruginosa* · *Candida albicans*

Introduction

Microorganisms present strategies that facilitate their proliferation in invaded tissues, promoting infection and relevant inflammatory processes. During an infection, microorganisms can use their own resources to inactivate some functions of the host's immune system [1], such as the synthesis of some proteins capable of generating cytotoxic effect to immune cells, as well as the alteration of some of their surface molecules, which it prevents the recognition of microorganisms by phagocytic cells and consequently it promotes inhibition of their phagocytosis [2]. However, if they were phagocytosed, the microorganisms can use some proteins capable of modifying the acidity of phagosomes, giving them survival within these structures [2]. They can also be freed from the phagosomes and live freely in the cytoplasm [3]. In addition,

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there are also certain microbial proteins capable of blocking the chemotaxis of neutrophils [4].

The microorganisms evaluated in this study are clinically relevant due they are capable of causing infections in several systems, which can start as an infection located in the oral cavity. *Staphylococcus aureus* presents high rates of systemic infections and mortality mainly related to the accumulation of its biofilm in medical devices [5]. In hospitalized patients with pulmonary infection, both dental and periodontal biofilms were considered as the source of this infection [6]. In the oral cavity, this microorganism was isolated from supra- and subgingival biofilms in cases of periodontitis [7].

Pseudomonas aeruginosa can be considered an opportunistic pathogen with the capacity to cause severe infections in immunocompromised patients [8]. In the oral cavity, this microorganism can cause a more aggressive form of periodontitis by its presence in the supragingival biofilm [9] and, from these biofilms, can be disseminated systematically and cause infections in other organs, being considered a microorganism responsible for respiratory infection, mainly in hospitalized and immunocompromised patients [10].

Candida albicans is considered as an opportunistic pathogen capable of causing infections mainly in cases of immunological weaknesses caused by the use of medicines, by the presence of other diseases such as cancer or other infectious agents such as HIV [11]. In the oral cavity, the yeast adheres easily to the mucosa and can contribute for the manifestation of pseudomembranous or erythematous candidiasis and also for the appearance of angular cheilitis [12].

Thyme was the medicinal plant evaluated in this study. It was chosen due its recognized antimicrobial activity [13] and its regulator effect in immunological processes [14]. However, the analysis of its effect on in vitro infection has not yet been performed. Thyme is native to the Mediterranean region; nevertheless, it is distributed all over the globe, and it can be used in culinary and also as a medicinal plant [15]. It presents a variety of phytocompounds with emphasis for thymol, a phenolic monoterpene with a biocidal character, besides presenting antiinflammatory, antioxidant, antinociceptive, antiseptic, healing, and local anesthetic effects [16].

Studies have demonstrated the role of phagocytic cells in the in vitro control of microbial infections [17]; nonetheless, reports of the action of plant products such as extracts and phytocompounds on this process are scarce. The action of various medicinal plants has been greatly demonstrated on biofilms and planktonic cultures [18–21]; on the other hand, there is a lack of studies on the interaction of microorganisms with cells of the immune system that have been treated with plant products. The effect of medicinal plants has been proven regarding production of inflammatory molecules; however, in in vitro model where cell culture is used, only microbial constituents have been used to stimulate macrophages to production of cytokines, for example, lipopolysaccharide [22, 23]. Nevertheless, in this study, viable microorganisms were used to provide stimulus and verify the potential of plant products in the process of elimination of infectious agent, as well as in the mediation of synthesis of cytokines and NO.

Thereby, this study aimed to verify the effect of both the thyme extract and the phytocompound thymol in the control of in vitro infections by *S. aureus*, *P. aeruginosa*, and *C. albicans* in murine macrophages (RAW 264.7) by analysis of phagocytosis and immunoregulation.

Material and methods

Plant products

Thyme glycolic extract was commercially purchased at a concentration of 200 mg/mL (Seiva Brazilis, São Paulo, Brazil), chemically composed of thymol, carvacrol, linalool, geranoil, citral, tannins, organic acids, flavonoids, minerals, small amount of saponins, carotene, vitamin C, and other components in less expressive amounts, according to manufacturer. Thymol (2-isopropyl-5-methylphenol—$C_{10}H_{14}O$) was also commercially purchased at ≥99.5% (Sigma-Aldrich, St Louis, USA). Stock solution of thymol was prepared in dimethyl sulfoxide (DMSO—Sigma-Aldrich), and it was diluted in distilled water. The solution was filtered using 0.22 μm membrane (TPP, Trasadingen, Switzerland), and the final concentration was of 800 μg/mL.

Microbial strains

Reference strains (American Type Culture Collection (ATCC)) of *S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 15442), and *C. albicans* (ATCC 18804) from the Institute of Science and Technology (ICT/UNESP) were used in this study. Strains were kept frozen (−80 °C) in Brain Heart Infusion Broth (BHI—HiMedia, Mumbai, India) with 20% glycerol, for bacteria, and Yeast Extract Peptone Dextrose broth (YPD – HiMedia) with 16% glycerol, for *C. albicans*.

Broth microdilution test

This test was used to determine the minimal inhibitory concentrations (MIC) of the thyme and thymol, according to Clinical and Laboratory Standards Institute (CLSI) [24–26]. Briefly, from a 24-h culture, a microbial suspension was prepared in sterile saline solution (NaCl 0.9%), whose turbidity was adjusted to 10^6 CFU/mL (colony-forming units per milliliter) in a spectrophotometer (Micronal, São Paulo, Brazil). This suspension (100 μL/well) was added in microtiter plate wells (TPP) containing each plant products serially diluted in 100 μL of broth, being Mueller Hinton (HiMedia) for bacteria and RPMI 1640 with glutamine, without bicarbonate, and phenol red indicator (HiMedia) buffered with MOPS [3-(*N*-

Morpholino)propanesulfonic acid] (Sigma-Aldrich) at pH 7.0 ± 0.1 for *C. albicans*. The final concentration of the bacterial inoculum was 5×10^5 CFU/mL, and the fungal inoculum was between 5×10^2 and 2.5×10^3 CFU/mL. Ten concentrations of each plant product were verified, i.e., from 50 to 0.09 mg/mL (thyme) and from 200 to 0.39 μg/mL (thymol).

Cultivation of murine macrophages

The murine macrophages (Rio de Janeiro Cell Bank, APABCAM, Rio de Janeiro, Brazil) were cultured in Dulbecco's modified Eagle's medium (DMEM—LGC Biotechnology, Cotia, Brazil) supplemented with 10% fetal bovine serum (FBS—Invitrogen, New York, USA) and 1% penicillin-streptomycin (5000 U/mL-5000 μg/mL—LGC) at 37 °C and 5% CO_2 with atmospheric humidity. After subconfluency, viable cells were quantified by trypan blue (0.4%—Sigma-Aldrich).

In vitro infection of murine macrophages

In a 24-well plate were added 300 μL/well of DMEM containing 10^5 viable cells. After 24 h of incubation, infections by *S. aureus*, *P. aeruginosa*, and *C. albicans* were separately induced in the proportion of 1:5 (cell/microorganism) [17, 27]. Stock solutions of microbial suspensions adjusted to 10^7 CFU/mL were prepared in a spectrophotometer, from a culture in solid medium for 24 h. The inoculum was diluted to reach the appropriate concentration of microorganisms per cell. The solutions of the plant products were prepared in DMEM free of antimicrobials and FBS with final concentration corresponding to the MIC of each product (Table 1). Six replicates were performed per experimental group. In Fig. 1, all groups can be visualized.

For infection, a part of microbial suspension was added in a part of solution containing each plant product or controls. Then, 1000 μL of these new solutions were added to the cells in each well corresponding to the type of treatment. The infection was performed under incubation (37 °C) for 30 min [17]. After, the supernatants were collected and maintained at −20 °C for further immunological analysis. The phagocytosis was stopped adding 1000 μL of ice-cold phosphate-buffered saline (PBS) to the wells [27]. These washes were performed twice and also aided in the elimination of non-phagocytized microorganisms.

The action of the products on the phagocytosis was analyzed in lysed macrophages by addition of 1000 μL/well of sterile distilled water at room temperature. After 10 min of stirring, 100 μL of suspension from each well were seeded on BHI or SD agar. The CFU count was performed after 24 h of incubation, and the concentration was given in colony-forming units per milliliter.

Table 1 Final concentrations used for the control of in vitro infections by *S. aureus*, *P. aeruginosa*, and *C. albicans*

Experimental group	*S. aureus*	*P. aeruginosa*	*C. albicans*
Thyme	100 mg/mL	100 mg/mL	50 mg/mL
Thymol	200 μg/mL	200 μg/mL	10 μg/mL
Antimicrobial[a]	1%	1%	1%
DMEM[b]	Pure	Pure	Pure

[a] One percent of penicillin-streptomycin (5000 U/mL-5000 μg/mL) for bacteria and nystatin (100.000 IU/mL) for yeast

[b] Medium free of antimicrobials and FBS

Cell viability of murine macrophages during in vitro infections

The neutral red (NR) test was applied to check the viability of infected RAW 264.7 in the experimental groups. The NR dye presents affinity for lysosomes of viable cell; thus, the hypothesis of the results corresponds to the action of the microorganisms rather than the macrophages was excluded, since this organelle is absent in microorganisms. For this end, 200 μL/well of DMEM containing 4×10^4 viable cells were added to the microtiter plate (n = 10/group). After 24 h of incubation, the cell culture was infected for 30 min as previously described. Phosphate-saline buffer was used to wash the wells and also discarded affected cells. Solution of NR (20 μg/mL PBS) was added (100 μL/well), and after 2 h of incubation, under protection of light, the supernatant was withdrawn and 100% ethanol was added (100 μL/well). After 15 min of stirring, the absorbance of the wells was read in a microplate spectrophotometer (570 nm—BioTek, Vermont, USA) and values of optical density (OD) were converted to percent of viability in relation to the group treated with DMEM free of antimicrobials and FBS. In addition, non-infected macrophages were also analyzed for comparative purposes.

Quantification of cytokine during in vitro infections

Supernatants of macrophages infected by *S. aureus*, *P. aeruginosa*, and *C. albicans* were collected for quantification of proinflammatory (IL-1β and TNF-α) and antiinflammatory (IL-10) cytokines. Their levels were quantified by ELISA, sandwich method. Therefore, commercial kits (R & D Systems, Minneapolis, USA) for IL-1β (DY401 catalog), TNF-α (DY410 catalog), and IL-10 (DY417 catalog) were used, according to the manufacturer's guidance. The absorbance of the wells was read in a microplate spectrophotometer (450 nm—BioTek), and the values were converted to picograms per milliliter (pg/mL), considering the cytokine standard curve. For this, GraphPad Prism 5.0 was used. Cellular supernatants from non-infected groups were also collected and analyzed for comparative purposes.

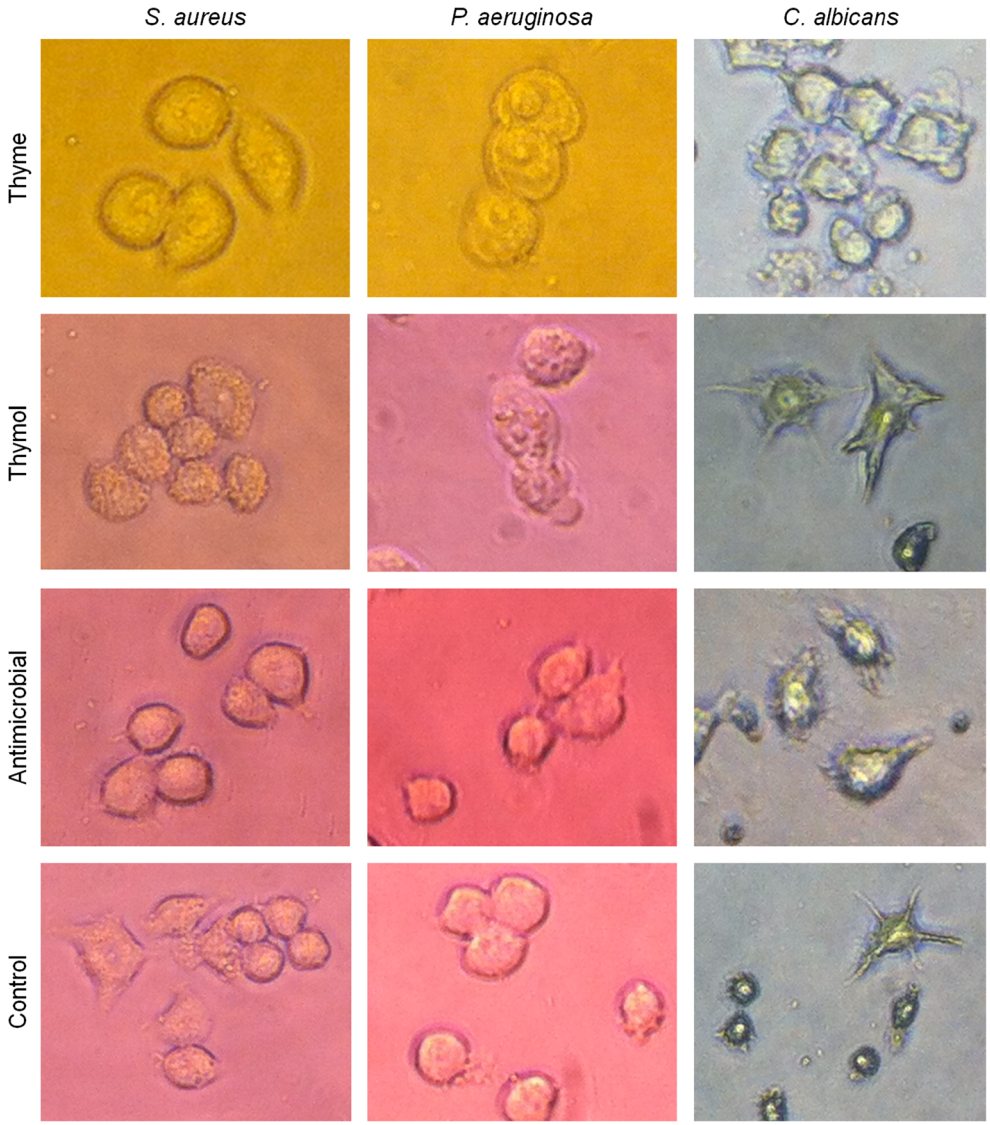


Fig. 1 In vitro infection. Murine macrophages (RAW 264.7) infected by *S. aureus*, *P. aeruginosa*, and *C. albicans* and treated with thyme extract, thymol, antimicrobials (penicillin-streptomycin for bacteria and nystatin for yeast), or DMEM free of antimicrobials and FBS

Quantification of NO during in vitro infections

In a microtiter plate, 100 μL of cell supernatant and 100 μL of Griess reagent were added. After 10 min of stirring, the absorbance of the wells was read in a microplate spectrophotometer (570 nm—BioTek) and the values were converted to micromolar (μM), according to the standard nitrite curve from 100 to 0 μM (0.1 M NO_2^- —Sigma-Aldrich). The GraphPad Prism 5.0 was used for this conversion. The concentration of nitrite in non-infected groups was also quantified for comparative purposes.

Giemsa method

Murine macrophages (10^6 cells) were cultured on sterile glass slides accommodated in a sterile polystyrene chamber with four compartments (Nunc, Roskilde, Denmark) in 3000 μL of DMEM at 37 °C/24 h. Infections were induced as previously described. The cells were washed with ice-cold PBS and fixed with 100% methanol for 10 min. Alcohol was removed, and Giemsa dye (3000 μL/slide) was added. Giemsa dye was prepared adding 500 μL of 100% methanol (pH 6.8) and 500 μL of glycerol in 0,008 g of dye (Merck, Darmstadt, Germany); then, this solution was diluted 2× in distilled water. After 10 min of contact, the glass slides were washed in running water, dried at room temperature, and analyzed in light microscope using the oil immersion objective lens. Giemsa staining can be seen in Fig. 2a.

Scanning electron microscopy

Polystyrene test specimens (PTS, 5 mm^2) were sterilized overnight by 2% glutaraldehyde and then were added in wells of 24-well plate. On the PTS were added 800 μL of DMEM containing 10^5 cells. After 24 h of incubation, the cells were infected as previously described. After infection, the cells were washed with ice-cold PBS and the macrophages were fixed by solution containing 0.25% glutaraldehyde, 4% paraformaldehyde, and PBS

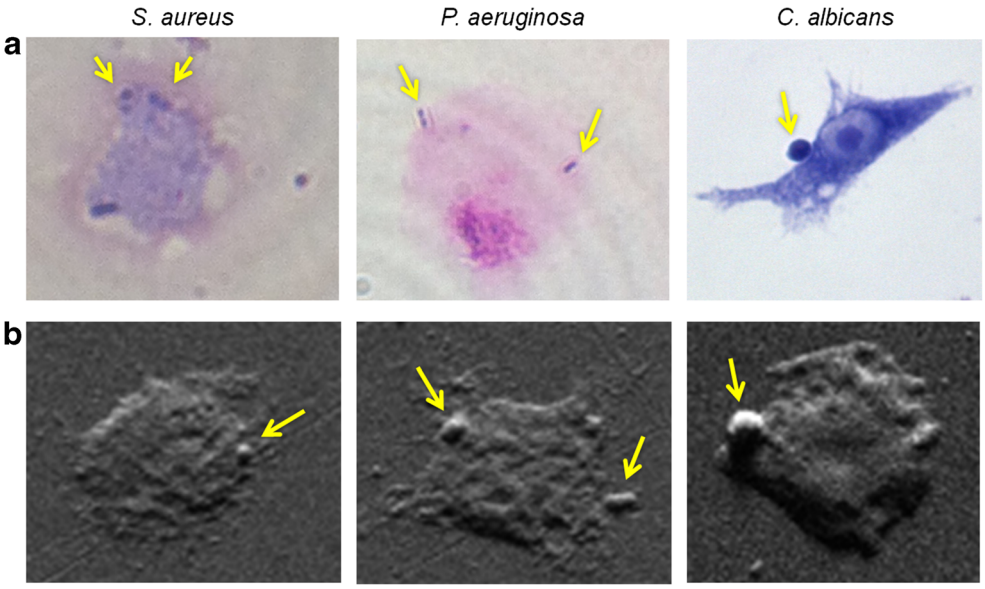


Fig. 2 Microorganism-macrophage interaction. Interaction of *S. aureus*, *P. aeruginosa*, and *C. albicans* with murine macrophages (RAW 264.7). **a** Giemsa, visualization by immersion optical microscopy (×1000). **b** Scanning electron microscopy (×3000). Scale 50 μm. *Arrows* indicate microorganisms interacting with the macrophage

(ratio per milliliter: 125:400:475 μL), for 20 min. Subsequently, the specimens were washed with PBS and dehydrated with 70% alcohol (10 min), 90% alcohol (10 min), 100% alcohol (10 min), and 100% alcohol (20 min). The samples were dried at room temperature overnight and were metalized with gold particles (Emitech SC7620 Sputter Coater, UK). Posteriorly, PTS were analyzed in SEM (Inspect S50, Oregon, USA). Interactions of microorganisms with macrophages can be visualized in Fig. 2b.

Statistical analysis

The data were presented in mean values (±standard deviation) and were analyzed statistically by ANOVA and Tukey's test, considering statistically significant difference when $P \leq 0.05$. GraphPad Prism 5.0 was used for this purpose.

Results

Inhibitory effect of the plant products on microorganisms

Thyme presented MIC of 100 mg/mL for *S. aureus* and *P. aeruginosa* and MIC of 50 mg/mL for *C. albicans*. For thymol, MIC of 200 μg/mL for bacteria and MIC of 10 μg/mL for *C. albicans* were observed.

Phagocytosis

In the infection by *S. aureus*, significant reductions of colony-forming units per milliliter were observed in the treated groups, in comparison to the control group. Thymol presented the highest percentage of reduction than other treated groups (Fig. 3a, b). The plant products also significantly reduced the concentration of colony-forming units per milliliter in the infection by *P. aeruginosa*, when compared to the control group. However, the thyme presented the highest index of reduction (Fig. 3c, d). Regarding infection by *C. albicans*, it was noted that the reductions demonstrated by the plant products were not significant in comparison to the control group; nevertheless, nystatin presented the highest percentage of reduction (Fig. 3e, f).

Cell viability of the macrophages

In the infection by *S. aureus*, the viability of RAW 264.7 was similar to the control group, in the treatment with thyme extract; however, in the treated groups with thymol or antimicrobials, there were significant reductions of viability (Fig. 4a). Groups with no microorganism presented significant reduction after exposition to thyme extract, thymol, and antimicrobials, when compared to the control group (Fig. 4b). During the infection, the lysosomal activity was more pronounced in infected groups and treated with plant products than in non-infected groups (see asterisks).

In the infection by *P. aeruginosa*, there was also a similarity between the group treated with thyme and the control group, besides reductions in groups exposed to the thymol and antimicrobials (Fig. 4c). The infected groups and treated with plant products presented similar reductions to the groups free of this bacterium (Fig. 4d). In addition, it was found that the cell viability was higher in infected groups and treated with the plant products (see asterisks).

In the infection by *C. albicans*, the viability of RAW 264.7 in the treatments with plant products was similar to the control group (Fig. 4e). In the absence of yeast, the cell viability of the treated groups with thyme and antimicrobials was similar to

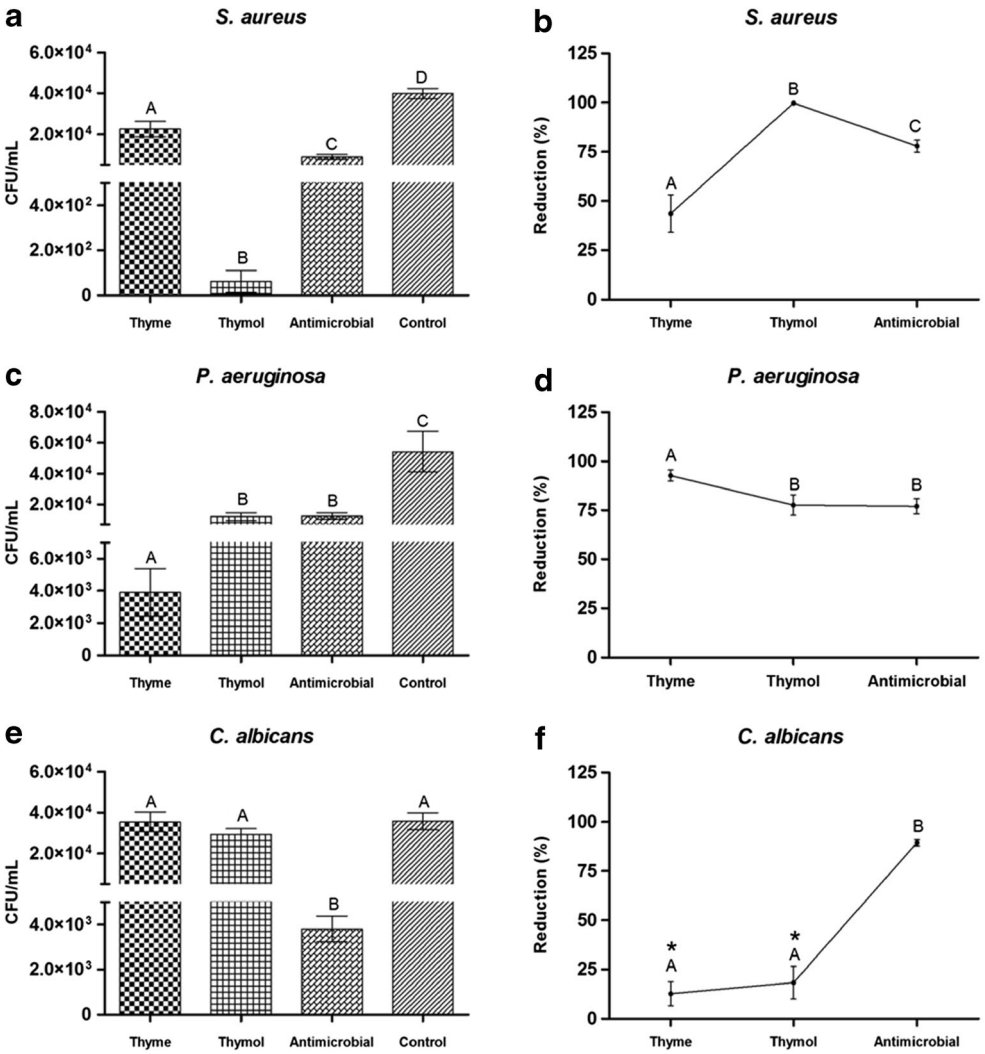


Fig. 3 Phagocytosis. **a**, **c**, **e** Mean (±standard deviation) of colony-forming units per milliliter obtained from the lysis of murine macrophages (RAW 264.7) infected by *S. aureus*, *P. aeruginosa*, and *C. albicans* and treated with thyme extract, thymol, antimicrobials (penicillin-streptomycin for bacteria and nystatin for yeast), or DMEM free of antimicrobials and FBS. **b**, **d**, **f** Mean (±standard deviation) of the percentage of reduction of colony-forming units per milliliter. *Different letters* indicate significant statistical difference ($n = 6$; $P \leq 0.05$; ANOVA, Tukey's test). *Asterisk* indicates that reduction was not significant in relation to the control group

the control group (Fig. 4f). Similarity between infected groups and free of *C. albicans* was observed in all groups.

Quantification of cytokine

In the infection by *S. aureus*, the level of IL-1β in the groups treated with plant products was similar to the control group (Fig. 5a (I)), TNF-α was lower or similar (Fig. 5a (II)), and IL-10 was superior or similar (Fig. 5a (III)), in relation to the control group. In the absence of infection, the level of IL-1β was similar to the control group (Fig. 5b (I)), in the same way, were the levels of TNF-α (Fig. 5b (II)) and IL-10 (Fig. 5b (III)). It was verified that in the groups with infection and treated with thyme, there was a higher production of IL-1β and IL-10 than in the non-infected groups (see asterisks).

In the infection by *P. aeruginosa*, the level of IL-1β in groups treated with the plant products was higher or similar to the control group (Fig. 5c (I)), TNF-α was lower or similar (Fig. 5c (II)), and IL-10 was similar or lower (Fig. 5c (III)), when compared to the control group. In groups free of microorganism, the level of IL-1β was higher or similar to the control group (Fig. 5d (I)), TNF-α was lower (Fig. 5d (II)), and IL-10 was higher or similar (Fig. 5d (III)) to the control group. Additionally, the level of IL-1β was lower during the infection and treatment with thyme, in comparison to the group with no microorganism (see asterisks). Levels of TNF-α were higher in the infection and treatments with thymol and antimicrobial groups, as well as in control group (see asterisks). The level of IL-10 was higher in the group with microorganism and treated with antimicrobials and also in the control group (see asterisks).

In the infection by *C. albicans*, the levels of IL-1β (Fig. 5e (I)) and IL-10 (Fig. 5e (III)) in the groups treated with the plant products were higher or similar to the control group and TNF-α (Fig. 5e (II)) was similar or higher to the control group. In groups free of yeast, the levels of IL-1β (Fig. 5f (I)) and IL-10 (Fig. 5f (III)) in groups treated with the plant products were higher or similar to the control group. The level TNF-α was lower or similar (Fig. 5f (II)) to the control group. During the

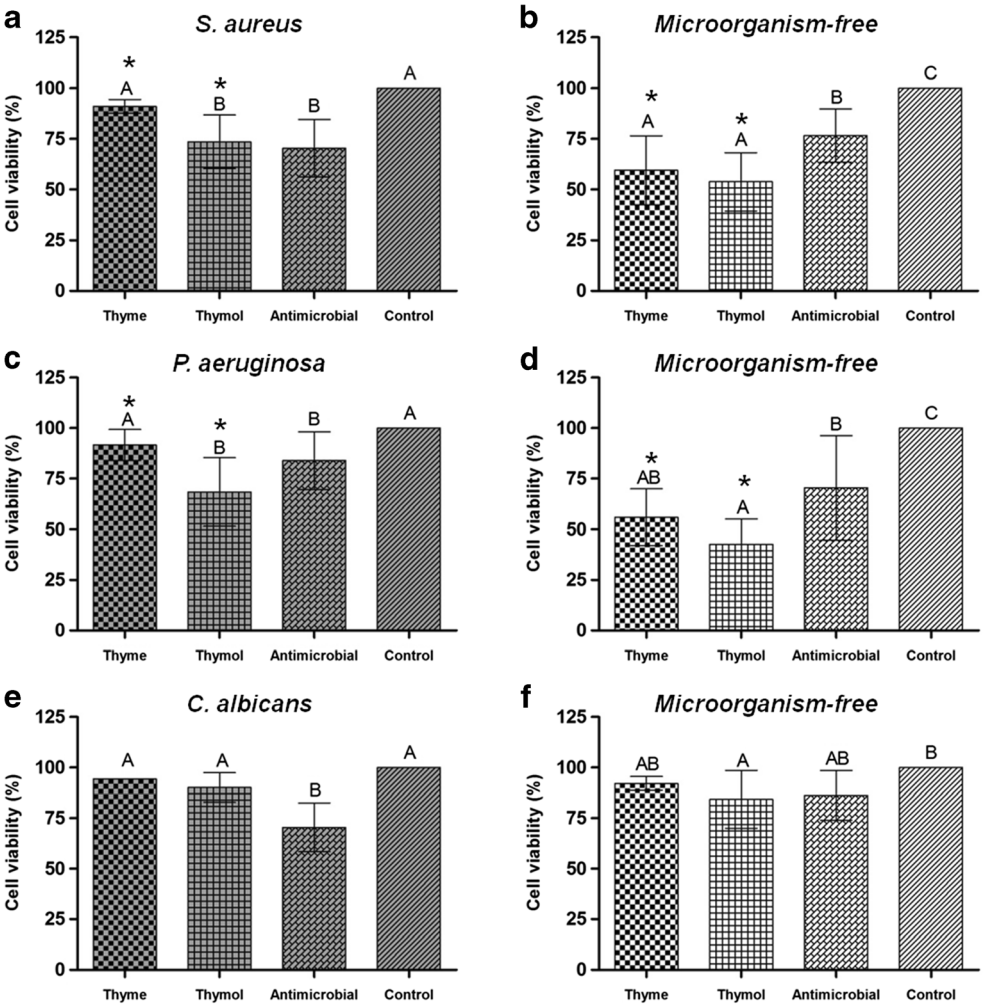


Fig. 4 Cell viability. Mean (±standard deviation) of the percentage of viability of murine macrophages (RAW 264.7) infected by *S. aureus*, *P. aeruginosa*, and *C. albicans* (**a**, **c**, **e**) or free of microorganisms (**b**, **d**, **f**) and treated with thyme, thymol, antimicrobials (penicillin-streptomycin for bacteria and nystatin for yeast), or DEMEM free of FBS and antimicrobials. *Different letters* indicate significant statistical difference ($n = 10$; $P \leq 0.05$; ANOVA, Tukey's test). *Asterisk* indicates significant statistical difference in the comparison between groups with infection and free of microorganisms in each treatment

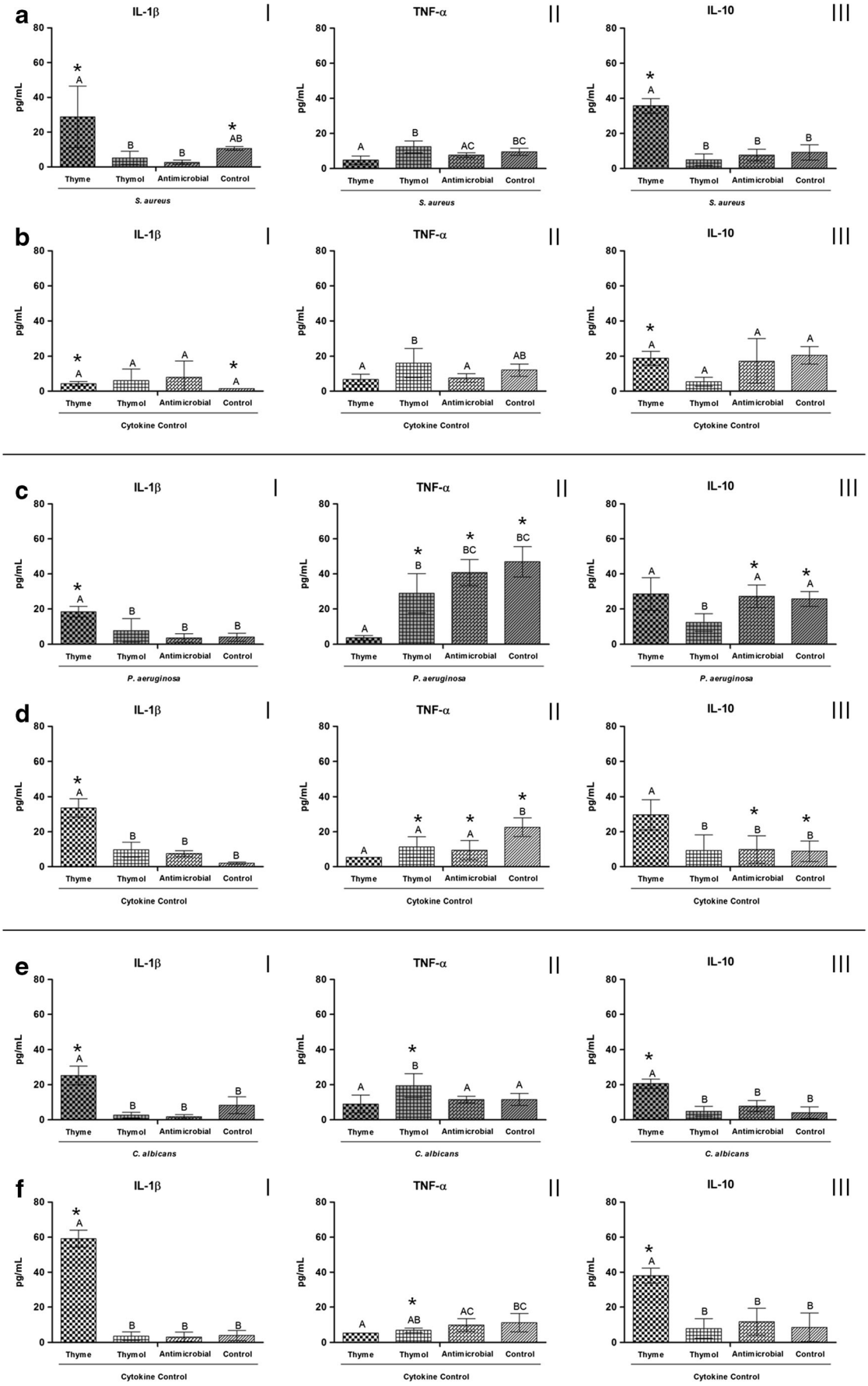
infection, only the level of TNF-α was higher, in treatment with thymol. The levels of IL-1β and IL-10 were higher in non-infected groups and treated with thyme (see asterisks).

Quantification of NO

In the infection by *S. aureus* (Fig. 6a (I)) and *P. aeruginosa* (Fig. 6a (II)), the generation of NO in the group treated with thyme was superior to the control group. In the absence of microorganism, this same scenario was observed (Fig. 6b (I, II)). However, there was higher production of NO during the infection by *S. aureus* and treatment with thyme. In the infection by *P. aeruginosa*, higher production of NO in the group free of microorganism (see asterisks) was observed. In the infection by *C. albicans*, the synthesis of NO in groups treated with the plant products was similar to the control group, both in the presence (Fig. 6a (III)) and in the absence (Fig. 6b (III)) of yeast. In addition, no significant statistical difference was found between the groups infected and the non-infected by *C. albicans*.

Discussion

In this study, it was verified that both the MIC of the thyme and thymol aided the macrophages to control infections by *S. aureus* and *P. aeruginosa*, as proven by effective reductions of colony-forming units per milliliter) in Fig. 3a–d. These results proved the ability of plant products in assisting macrophages in the elimination of microorganisms in an infection, since it has been reported that *S. aureus* is able to defend itself against phagocytosis by fighting leukocytes, eliminating them, or inhibiting their recruitment; their biological functions; and also the production of antimicrobial agents by them [28]. This microorganism can also affect phagocytes (macrophages) producing specific toxins [29], besides proteins capable of neutralizing opsonins, such as immunoglobulin G, and complement C3b protein [30]. After phagocytosed, some strains of *S. aureus* are able to survive for days in primary human macrophages [3], since they can produce modulins that affect the integrity of phagolysosome, allowing its access to the cytoplasm where its proliferation will occur


a
IL-1β
TNF-α
IL-10
pg/mL
Thyme
Thymol
Antimicrobial
Control
S. aureus
b
Cytokine Control
c
P. aeruginosa
d
e
C. albicans
f

◀ **Fig. 5** Inflammatory cytokines. Mean (±standard deviation) of the concentration of IL-1β, TNF-α (proinflammatory) and IL-10 (antiinflammatory) produced by murine macrophages (RAW 264.7) infected by *S. aureus* (**a**), *P. aeruginosa* (**c**), and *C. albicans* (**e**) or free of microorganisms (**b**, **d**, **f**) and treated with thyme extract, thymol, antimicrobials (penicillin-streptomycin for bacteria and nystatin for yeast), or DMEM free of antimicrobials and FBS. *Different letters* indicate significant statistical difference ($n = 6$; $P \leq 0.05$; ANOVA, Tukey's test). *Asterisk* indicates significant statistical difference in the comparison between groups with infection and free of microorganisms in each treatment

followed by the lysis of this infected macrophage [31]. The survival of this bacterium within the cell is also mediated by production of apoptosis inhibitory agents [27].

We verified that both thyme and thymol were effective in helping macrophages to combat infection by *P. aeruginosa*. In addition, it has also been reported that *Wnt3a*, a multifunctional molecule, was capable of modulating the inflammatory response and also effectively contributed for the elimination of *P. aeruginosa* phagocytosed by RAW 264.7, by the induction of antimicrobial peptides. Additionally, this molecule can promote decrease of the expression of proinflammatory cytokines and act as an antiinflammatory molecule, generating apoptosis in macrophages, a phenomenon indispensable for the control of inflammation [32].

In the infection by *C. albicans*, no significant reduction of this yeast was observed with application of thyme or thymol. Reduction was verified only with nystatin (Fig. 3e, f). In this sense, the plant products were not effective in aiding macrophages to control fungal infection. In another study, macrophages RAW 264.7 were pretreated with adequate concentrations of quercetin, a product obtained from plants, and after were infected by *C. albicans*. It was found that the quercetin reduced the efficiency of the phagocytosis and also the production of TNF-α; however, the viability of macrophages was not affected. Thus, it has been suggested that the inhibition of phagocytosis may have occurred due to morphological alterations in the macrophages, especially in the structure of cytoskeleton, being related to the loss of actin [33].

Macrophages infected by *S. aureus* or *P. aeruginosa* showed higher lysosomal activity in groups treated with thyme or thymol in comparison to groups with no microorganisms (Fig. 4a–d). The viability of macrophages was around 50% in some cases, as analyzed in non-infected groups and treated by the plant products. However, they could effectively assist macrophages to eliminate microorganisms, as can be seen in the infected groups that presented significantly higher percentages. Therefore, we could suggest that the lysosomal activity of the cells was not affected, an important cellular biological activity for the control of microorganisms, since in these structures, the pathogens can be inactivated or destroyed by enzymatic action, resulting in the control of infection [34]. In the case of the infection by *C. albicans*, it was found that the

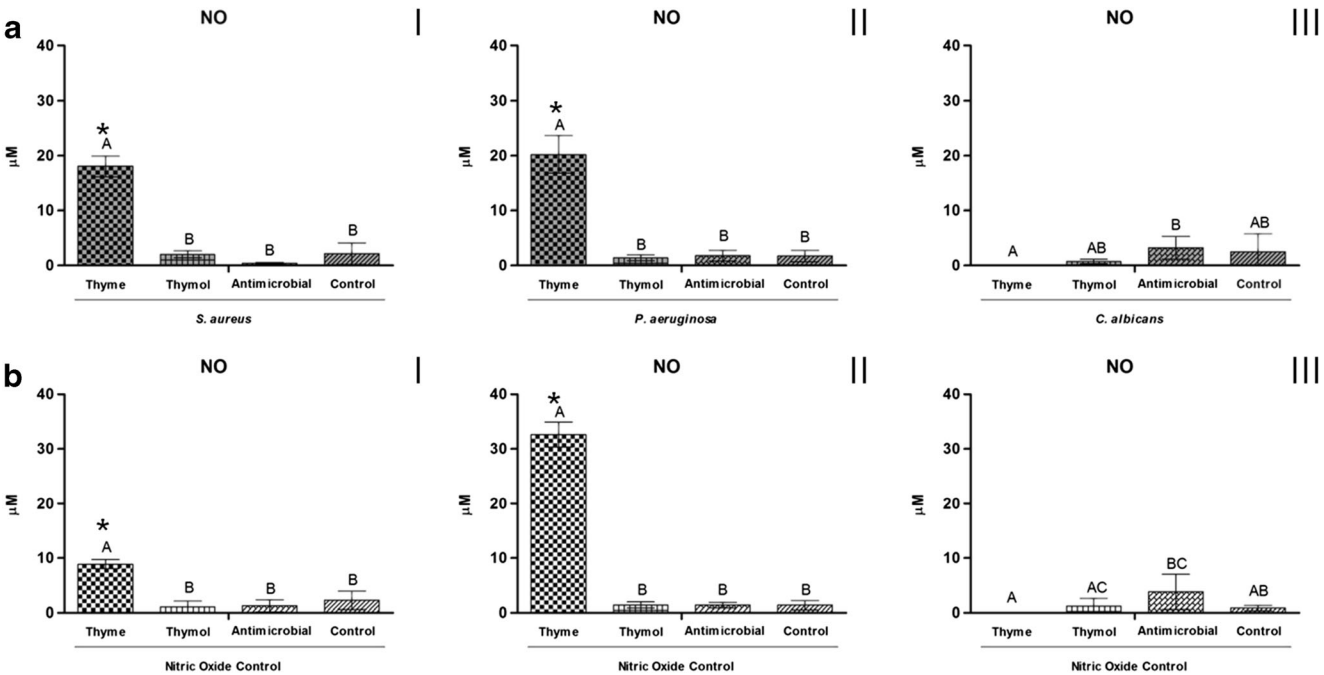


Fig. 6 Nitric oxide (NO). Mean (±standard deviation) of the concentration of NO (μM) generated by murine macrophages (RAW 264.7) infected by *S. aureus*, *P. aeruginosa*, and *C. albicans* or free of microorganisms and treated with thyme extract, thymol, antimicrobials (penicillin-streptomycin for bacteria and nystatin for yeast), or DMEM free of antimicrobials and FBS. *Different letters* indicate significant statistical difference ($n = 6$; $P \leq 0.05$; ANOVA, Tukey's test). *Asterisk* indicates significant statistical difference in the comparison between groups with infection and free of microorganisms in each treatment

outcomes were similar between the non-infected and the infected groups (Fig. 4e, f).

Activated macrophages can secrete a variety of molecules in response to the agent that triggered this activation, the production, and release of inflammatory cytokines by these cells is an example of this [35]. In this study, proinflammatory (IL-1β and TNF-α) and antiinflammatory (IL-10) cytokines were quantified from macrophages infected by *S. aureus*, *P. aeruginosa*, and *C. albicans*.

During infection by *S. aureus*, the synthesis of IL-1β was significantly higher in the group treated with thyme extract (Fig. 5a (I)), compared to the non-infected group (Fig. 5b (I)). Increased levels of this cytokine might indicate that there was a higher stimulus to the cell; however, IL-1β is essential for the control of infection, by recruiting inflammatory cells to the site of the invasion, as well as activating other protective mechanisms [35], besides initiating and increasing the inflammatory response due to microbial proliferation [36]. On the other hand, we can verify that the level of antiinflammatory cytokine IL-10 also increased in the infected group treated with thyme (Fig. 5a (III)), when compared to the group with no microorganism (Fig. 5b (III)). This suggests that the plant product can provide immunoregulatory effect in the production of inflammatory cytokines. Interleukin 10 is responsible for controlling the duration and degree of inflammatory response by blocking the expression of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6; additionally, IL-10 can inhibit the cell migration to the infected site [37].

In the infection by *P. aeruginosa*, there was production of IL-1β in the group treated with thyme (Fig. 5c (I)) above the control group, demonstrating that the extract contributed for the elevation of the level of this cytokine, which in this case was beneficial for the control of infection. In addition, the basal level of IL-1β was also higher in the macrophage group exposed to the plant extract and free of infection (Fig. 5d (I)). The extract also contributed to increase the basal level of IL-10 (Fig. 5d (III)), which during the infection remained similar (Fig. 5c (III)). This proves that the extract can also act as a modulating agent in infection by *P. aeruginosa*. The phytocompound increased the level of TNF-α during this infection (Fig. 5c (II)), compared to the non-infected group (Fig. 5d (II)), being significantly higher. Tumor necrosis factor alpha is a key cytokine, capable of stimulating the production of other cytokines and also its own secretion, besides being involved in the control of leukocyte migration [38]. Elevation of proinflammatory cytokines is important for the activation of macrophages in order to control infection; however, after elimination of the infectious agents, the levels of these cytokines should be stabilized by antiinflammatory mediators, such as IL-10 [35]; this scenario was observed in the present study.

Macrophages infected by *C. albicans* and treated with thyme produced more IL-1β (Fig. 5e (I)) and IL-10 (Fig. 5e (III)) in comparison to the control groups. Likewise, in non-infected groups, the exposure to the extract provided higher levels of these cytokines (Fig. 5f (I, II)). It can be observed that in the infection by *C. albicans*, the thyme acted as a regulator of the production of inflammatory cytokines. As what happened in the infection by *P. aeruginosa* (Fig. 5c (II), d(II)), the level of TNF-α in the infection by *C. albicans* was higher in the infected group and treated with thymol, demonstrating that this phytocompound can also act as a stimulator of the production of this cytokine, in order to control the fungal infection. It is noteworthy that the macrophages infected or not by the yeast tended to seek equilibrium in the immune response, as verified in the regulation of the production of pro- and antiinflammatory cytokines in the groups treated with the plant products.

The generation of NO in infection by *S. aureus* (Fig. 6a (I)) and *P. aeruginosa* (Fig. 6a (II)) was significantly higher in the groups treated with thyme, even in non-infected groups (Fig. 6 b (I, II)). However, in macrophages infected by *C. albicans*, the production of NO was inexpressive (Fig. 6a (III)), being also similar to the non-infected groups (Fig. 6b (III)). These results demonstrated that the plant products can contribute to the production of NO in cells infected by *S. aureus* and *P. aeruginosa*, and effectively act on the control these infections. Nitric oxide is an important immunoregulatory molecule produced in response to a microbial invasion, for example [39]. The production of NO is performed by induced nitric oxide synthase (iNOS) previously stimulated by the presence of microorganisms or their constituents of membrane and cell wall [40]. Thereby, occurring the control of these stimuli by antiinflammatory mediators, the level of NO soon reaches concentrations that are not capable of causing significant immune response, providing reduction of damage.

According to the results obtained in the present study, thyme and thymol effectively assisted in the control of in vitro infections by *S. aureus* and *P. aeruginosa* in murine macrophages (RAW 264.7). In the case of infection by *C. albicans*, no significant effect was demonstrated. The lysosomal activity of the macrophages was increased in infected groups and treated with the plant products, in bacterial infections, demonstrating aid in the control of these microorganisms after phagocytosis; however, in the infection by *C. albicans*, this cellular activation was similar to the non-infected groups. The plant products acted as immunoregulators of inflammatory cytokines in infections by *S. aureus*, *P. aeruginosa*, and *C. albicans*. Additionally, the thyme extract contributed for the generation of NO in bacterial infections. In this way, the effectiveness of these plant products can be verified in the control of in vitro microbial infections.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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