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DENISAR PALMITO DOS SANTOS

AVALIAÇÃO DE NOVOS FOTOSSENSIBILIZADORES PARA A TERAPIA FOTODINÂMICA NO TRATAMENTO DA INFECÇÃO INTRADÉRMICA POR *Staphylococcus aureus* EM MODELO MURINO

VITÓRIA DA CONQUISTA - BA

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Orientador: Prof. Dr. **Robson Amaro Augusto da Silva**

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À Deus, por sempre iluminar meus caminhos,
À minha mãe, Dina, pelo imenso amor, força e carinho,
À minha querida irmã Denize, pelo amor e exemplo de pessoa,
À Jéssica por ser tão companheira e amorosa comigo,
Ao Professor Robson por tudo e aos amigos do laboratório 106,

Aos animais.

PALMITO, Denisar dos Santos. **Avaliação de novos fotossensibilizadores para a Terapia Fotodinâmica no tratamento da infecção intradérmica por *Staphylococcus aureus* em modelo murino** (Tese de Doutorado) – Instituto Multidisciplinar de Saúde, Universidade Federal da Bahia, Vitória da Conquista, 2019.

Introdução: Infecções bacterianas são um problema de saúde pública devido a grande variedade de microrganismos patogênicos. Entre estes, *Staphylococcus aureus* resistentes à meticilina (*MRSA*) é o principal agente causador de infecções bacterianas no mundo e diversas cepas deste agente já são capazes de criar resistência a terapia medicamentosa convencional. Nesse contexto, a terapia fotodinâmica antimicrobiana (*antimicrobial photodynamic therapy; aPDT*) aparece como uma ferramenta promissora por meio da inativação microbiana com o uso da luz. Em geral, a *aPDT* é aplicada em tratamentos envolvendo fotossensibilizadores capazes de gerar radicais livres de oxigênio que vão levar a depuração bacteriana. Assim, este trabalho propõe de maneira pioneira o uso do Resveratrol e do extrato de *Myrciaria cauliflora* como fotossensibilizadores na terapia antimicrobiana contra *MRSA*. **Métodos:** Testes *in vitro* foram realizados para determinar a atividade antibacteriana dos fotossensibilizadores fotoativados com luz LED azul, bem como experimentos com ácido úrico para verificação da formação de oxigênio *singlete* . Possíveis alterações estruturais do Resveratrol foram avaliadas por HPLC. Nos ensaios *in vivo*, o modelo de bolsão de ar foi realizado em camundongos C57Bl/6 para avaliar a atividade antimicrobiana do Resveratrol fotoativado, além da avaliação da migração celular por técnicas histológicas e produção de citocinas pela técnica de ELISA. Em outra análise, um modelo de infecção intradérmica, na orelha esquerda foi realizado para avaliação dos dois fotossensibilizadores, separadamente, em camundongos Balb/c. Após a infecção e eutanásia, a orelha foi coletada e utilizada a técnica de imunohistoquímica para a marcação de Mieloperoxidase (MPO) e E-caderina. O linfonodo dos animais foi retirado para avaliação da carga bacteriana e produção de citocinas. **Resultados e discussão:** O Resveratrol fotoativado exibiu um aumento na marcação para MPO sendo sua ação antibacteriana, possivelmente causada pela formação de oxigênio *singlete* . No modelo de bolsão de ar, foram produzidas citocinas TNF-α e IL-17A, diminuindo a carga bacteriana e, consequentemente, diminuindo inflamação após 24 horas de infecção. A diminuição do número de celulas no ambiente inflamatório foi associada menor inflamação, juntamente com maior produção de IL-10. Além disso, foi observado na derme da orelha dos animais, que o Resveratrol fotoativado promoveu um aumento

produção de MPO com redução da carga bacteriana no linfonodo drenante. Em relação a *Myrciaria cauliflora* foi observada a redução na carga bacteriana nos testes *in vitro*, com aumento da produção de TNF-α, 17A no linfonodo drenante e MPO no sítio indradérmico de infecção. **Conclusão:** A partir dos dados analisados, é possível predizer que o Resveratrol e o extrato de *Myrciaria cauliflora* são fotossensibilizadores promissores para a terapia fotodinâmica antimicrobiana, sobretudo para o controle da carga bacteriana em infecções intradérmicas causadas por *MRSA*

Palavras-chave: Terapia fotodinâmica antimicrobiana; Resveratrol; *Myrciaria cauliflora; Staphylococcus aureus*

PALMITO, Denisar dos Santos - **Evaluation of new photosensitizers for photodynamic therapy in the treatment of intradermal *Staphylococcus aureus* infection in a murine model** (doctoral thesis) - Instituto Multidisciplinar de Saúde, Universidade Federal da Bahia, Vitória da Conquista, 2019.

Introduction: Bacterial infections are a public health problem due to the wide variety of pathogenic microorganisms. Among these, *Staphylococcus aureus* is the main causative agent of bacterial infections in the world and several strains of this agent are already able to resist conventional drug therapy. In this context, antimicrobial photodynamic therapy (aPDT) appears as a promising tool through microbial inactivation with the use of light. In general, aPDT is applied in treatments involving photosensitizers capable of generating oxygen free radicals that will lead to bacterial clearance. Thus, this work has pioneered the use of Resveratrol and *Myrciaria cauliflora* extract as photosensitizers in antimicrobial therapy against *S. aureus*. **Methods:** *In vitro* tests were performed to determine the antibacterial activity of blue LED light-activated photosensitizers, as well as experiments with uric acid to verify singlet oxygen formation. Possible structural changes of Resveratrol were evaluated by HPLC. In *in vivo* assays, the air pocket model was performed in C57Bl/6 mice to evaluate the antimicrobial activity of photoactivated Resveratrol, as well as to evaluate cell migration by histological techniques and cytokine production by ELISA. In another analysis, a model of intradermal infection in the left ear was performed to evaluate the two photosensitizers separately in Balb/c mice. After infection and euthanasia, the ear was collected and the immunohistochemistry technique was used to mark Myeloperoxidase (MPO) and E-cadherin. The lymph node of the animals was removed to evaluate the bacterial load and cytokine production. **Results and Discussion**: Photoactivated Resveratrol exhibited an increase in MPO labeling and its antibacterial action, possibly caused by singlet oxygen formation. In the air pocket model, TNF-α and IL-17A cytokines were produced, decreasing bacterial load and, consequently, decreasing inflammation after 24 hours of infection. Decreased number of cells in the inflammatory environment was associated with resolution of inflammation along with higher IL-10 production. In addition, it was observed in the ear dermis that photoactivated Resveratrol promoted an increase in MPO production with reduction of bacterial load in the draining lymph node. Regarding *Myrciaria cauliflora*, a reduction in bacterial load was observed in *in vitro* tests, with increased production of TNF-α, 17A in

the lymph node drainage and MPO in the indradermal site of infection. **Conclusion:** From the analyzed data, it is possible to predict that Resveratrol and *Myrciaria cauliflora* extract are promising photosensitizers for antimicrobial photodynamic therapy, especially for the control of bacterial load in intradermal infections caused by *S. aureus*.

Keywords: Antimicrobial Photodynamic Therapy; Resveratrol; *Myrciaria cauliflora*; *Staphylococcus aureus*

LISTA DE ABREVIATURAS

µl	Microlitros
µg/mL	Microgramas por mililitros
µm	Micrômetros
aPDT	*antimicrobial photodynamic therapy*,
ATCC	*American Type Culture Collection*
BHI	*Brain Heart Infusion*
CEUA	Comitê de Ética em Uso Animal
CLAE	Cromatografia líquida de alta eficiência
cm	Centímetros
ELISA	*Enzyme-Linked Immunosorbent Assay*
IL	Interleucina
IL-17A	Interleucina 17A
IL-1β	Interleucina 1 Beta
IL-4	Interleucina 4
INF-γ	Interferon gama
J/cm^2	Joules por centímetro ao quadrado
LED	*Light Emitting Diode*
mg/mL	Miligramas por mililitros
mL	Mililitros
mmHg	Milímetros de mercúrio
MPO	Mieloperoxidase
MRSA	*Methicillin-resistant Staphylococcus aureus*
MSSA	*Staphylococcus aureus* sensíveis a meticilina
mW/cm^2	Miliwatts por centímetro ao quadrado
nm	Nanometros
PBS-*Tween*	*Phosphate buffered saline- Tween*
PDT	*Photodynamic therapy*
pH	Potencial hidrogênionico
S. aureus	*Staphylococcus aureus*
TMB	Tetrametilbenzidina

TNF-α	Fatores de Necrose Tumoral Alfa
UFBA-IMS/CAT	Universidade Federal da Bahia - Instituto Multidisciplinar de Saúde - instalações do *Campus* Anísio Teixeira
UFC	Unidades formadoras de colônias
UV-VIS	Ultravioleta visível

LISTA DE SÍMBOLOS

%	porcentagem
ºC	graus Celsius

SUMÁRIO

1. INTRODUÇÃO

Infecções bacterianas ocorrem em todo mundo e são um importante problema de saúde pública. Em meados do século passado, essas infecções levavam a morte de um grande número de pacientes (VENTOLA, 2015). Com a introdução dos antimicrobianos, a sobrevida de pessoas infectadas aumentou. Contudo, com o passar dos anos, algumas cepas de microrganismos apresentaram resistência a principal classe de antimicrobiano utilizada: as penicilinas (LEHMAN et al., 2019). Dentre estes patógenos, *Staphylococcus aureus* é o principal agente causador de doenças bacterianas no mundo. Essa bactéria leva a morte de milhões de pessoas anualmente, sobretudo por mecanismos de virulência cada vez mais aprimorados (SUKUMARAN; SENANAYAKE, 2016).

Os isolados de *S. aureus* que apresentam resistência à meticilina são denominados MRSA (*Methicillin-resistant Staphylococcus aureus)*. A meticilina foi o segundo antimicrobiano utilizado para tratamento das patologias causadas por este microrganismo após o surgimento da resistência a penicilina. Assim, surtos de infecções comunitárias por cepas resistentes apresentam ampla distribuição no globo terrestre e o aumento destas ressalta a necessidade do desenvolvimento de novas estratégias para prevenir infecções invasivas por *MRSA* (DONKOR; DAYIE; TETTE, 2019; REGEV-YOCHAY, 2019).

Além disso, MRSAs são comuns em hospitais e estão presentes também na comunidade. As penicilinas resistentes à penicilinase (flucloxacilina, dicloxacilina) continuam sendo os antibióticos de escolha para o tratamento de infecções graves por *S. aureus* sensíveis a meticilina (MSSA). As cefalosporinas de primeira geração (cefazolina, cefalotina e cefalexina), clindamicina, lincomicina e eritromicina têm papéis terapêuticos importantes em infecções leves por MSSA, como na pele e tecidos moles, ou em pacientes com hipersensibilidade à penicilina, embora as cefalosporinas sejam contraindicadas em pacientes com hipersensibilidade imediata à penicilina (devido as reações adversas como urticária, angioedema, broncoespasmo ou anafilaxia) (LOWY, 1998; GELATTI et al., 2009).

Portanto, novas terapias antimicrobianas são fundamentais. Nesse contexto, a terapia fotodinâmica antimicrobiana (*antimicrobial photodynamic therapy*, *aPDT*) segue princípios semelhantes aos da terapia fotodinâmica (*photodynamic therapy*, *PDT*), que é mais amplamente conhecida por sua aplicação em doenças não cancerosas e lesões cancerígenas (HANAKOVA et al., 2014). Esta técnica é usada em tratamentos que envolvem fotossensibilizadores capazes de gerar radicais livres de oxigênio quando

ativados em um determinado comprimento de onda pela luz emitida por um laser (GOIS et al., 2010; VATANSEVER et al., 2013).

Assim, este trabalho apresenta o uso do Resveratrol e do extrato da casca do fruto de *Myrciaria cauliflora* como fotossensibilizadores. O Resveratrol é especialmente presente na uva, promove benefícios à saúde através de sua atividade antiangiogênica, anti-inflamatória e antimicrobiana (CHAN, 2002; CHALAL et al., 2014; LANCON; FRAZZI; LATRUFFE, 2016; BOSTANGHADIRI et al., 2017; DE SA COUTINHO et al., 2018; ZHOU et al., 2018). Nos estudos iniciais, foi caracterizado como uma fitoalexina. Desde então, vários estudos começaram a apontar sua correlação com a prevenção ou redução da progressão tumoral, condições clínicas cardiovasculares, entre outros. Trabalhos recentes mostraram que esse polifenol é capaz de interagir com fontes de luz, inclusive alterando sua estrutura (YANG et al., 2012; RODRIGUEZ-CABO et al., 2015).

A *Myrciaria cauliflora* pertencente à família Myrtaceae, é uma planta com frutos semelhantes à ameixa-preta-roxa, conhecida popularmente como jabuticaba, que cresce diretamente ao redor do caule e dos ramos principais. Esta planta é nativa do Brasil e está distribuída por todo o bioma da Mata Atlântica. A fruta tem aparência e textura semelhante à uva, mas com uma pele mais espessa, mais dura e roxa, sendo esse um pigmento natural marcante da espécie. Rica em constituintes fenólicos, incluindo resorcinol, ácido p-hidroxibenzóico, antocianinas, ácidos hidroxicinâmicos, flavonóides e cumarinas (BOARI LIMA ADE et al., 2008). O extrato dos frutos e do caule é utilizado como agente cicatrizante além de anti-inflamatório e antimicrobiano.

Já foi demonstrado que a *aPDT* é capaz de inativar cepas de MRSA através de dois processos fotodinâmicos distintos (DE OLIVEIRA et al., 2017; ARAUJO et al., 2018; CAI et al., 2019; MA et al., 2019; MAHMOUDI et al., 2019). O primeiro, denominado tipo I, radicais livres são formados devido à incidência do laser, sendo responsáveis por reagir com lipídios e proteínas, levando a célula bacteriana a enfrentar um estado de oxidação celular que culmina em sua morte. No tipo II, o laser excita um fotossensibilizador que é responsável por transferir seus elétrons para o oxigênio molecular, gerando um estado de livre oxidação, com a produção de oxigênio *singlete* (DING et al., 2011).

Neste contexto, o oxigênio *singlete* após produzido *in vivo* possui como moléculas alvo proteínas, ácidos nucléicos, além de sistemas organizados como membranas. Tais reações levam, via de regra, a produtos oxidados que perdem suas propriedades gerando

disfunções (DI MASCIO et al., 2019). Diante disso, na literatura estão presentes trabalhos em que a *aPDT* é eficaz no controle de infecções causadas por *MRSA* devido não apenas à inativação fotodinâmica, como também através da inativação de seus fatores de virulência como enterotoxinas e coagulase (BARTOLOMEU et al., 2016). Sendo assim, é importante ter uma ampla biblioteca de fotossensibilizadores para serem utilizados nas diversas terapias antimicrobianas (DOS SANTOS et al., 2019a; DOS SANTOS et al., 2019b).

Diante da crescente resistência já apresentada, pesquisas que procuram desenvolver ou utilizar novos fotossenbilizadores se tornam fundamentais, pois quanto mais compostos fotoativos maiores serão as possibilidades para o combate a infecção causada por *MRSA*. Vale ressaltar que a *aPDT* não exclui a utilização da terapia antimicrobiana convencional, podendo atuar tanto de forma isolada em infecções leves, quanto em associação com os medicamentos antimicrobianos. Outro fator que chama a atenção é o baixo relato de resistência desenvolvida pelos microrganismos a essa técnica. Tal fato ocorre devido a geração de radicais livres durante a fotobioestimulação do fotossensibilizador, essas substâncias podem ter como alvos biológicos qualquer biomolécula da célula bacteriana, o que dificulta a geração de resistência pela bactéria .

Sendo assim, este trabalho teve como objetivo desenvolver novos compostos fotossensibilizadores para serem utilizados na terapia fotodinâmica antimicrobiana. Além disso, objetivamos elucidar os mecanismos principais de ação destes novos compostos em modelo de estudo desenvolvido *in vivo*.

2. JUSTIFICATIVA DO ESTUDO

MRSA é um importante patógeno humano associado a uma variedade de doenças como endocardite e sepse, entre outras. Após o surgimento da resistência aos atuais antibióticos utilizados, o interesse em desenvolver uma medida terapêutica contra a infecção têm sido crescente. Diversos estudos apontam o potencial da Terapia Fotodinâmica na redução significativa do número de unidade formadora de colônia (UFC) em biofilmes e infecções experimentais.

Muitos fotossensibilizadores, têm sido igualmente testados a fim de se padronizar os tempos e concentrações de incubação. Entretanto, as pesquisas experimentais nesta área referem-se em sua maior parte às infecções ósseas e análises *in vitro* relativas à susceptibilidade do *S. aureus* à terapia, utilizando-se a curcumina e outros compostos

como fotossensibilizadores. Diante da resistência crescente aos antimicrobianos clássicos utilizados na terapia convencional, torna-se fundamental a análise de novos fotossensibilizadores, bem como testes *in vivo* para a comprovação da eficácia destes novos compostos.

Neste contexto, a aPDT é fundamental, pois trata-se de uma técnica de baixo custo com alta efetividade. Além do mais, o desenvolvimento de um fotossensibilizador é fundamentalmente mais rápido que a geração dos medicamentos antimicrobianos tradicionais. Outro fato relevante, é o não relato até o momento de resistência bacteriana a essa técnica, o que a torna ainda mais promissora.

3. CÓPIAS DOS TRABALHOS PUBLICADOS

3.1. Artigo I – *Efficacy of photoactivated Myrciaria cauliflora extract against Staphylococcus aureus infection - A pilot study*

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Efficacy of photoactivated *Myrciaria cauliflora* extract against *Staphylococcus aureus* infection – A pilot study

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ABSTRACT

Staphylococcus aureus is one of the major microorganisms that cause human diseases, leading from mild skin infections to serious diseases. With the use of semi-synthetic penicillins, methicillin-resistant strains called Methicillin-resistant *Staphylococcus aureus* (MRSA) have emerged, whose resistance pattern extends to other beta-lactam antibiotics. It has already been shown that photodynamic therapy is capable of inactivating MRSA as the laser excites the photosensitizer responsible for transferring its electrons to the molecular oxygen, generating extremely reactive molecules, such as singlet oxygen, being these reactive components the chemicals that promote the bacterial clearance. Thus, the research aiming at the development of new photosensitizers becomes important, especially to increase the amount of therapeutic resources available for the treatment of persistent infections related to this bacterium. In this context, *Myrciaria cauliflora* is a plant that has antimicrobial action and there are no reports of the use of its crude extract as a photosensitizer in antimicrobial photodynamic therapy. In that way, this work conveys an innovative way of the use of *M. cauliflora* extract as a photosensitizer, comprising its use as an antimicrobial agent when activated by light, against *S. aureus*. *In vitro* tests were performed where it was observed that after the photoactivation with blue LED light, the extract presented an augment in its antimicrobial activity, together with production of singlet oxygen. In the model of intradermal infection in Balb/c mice, a reduction in bacterial load was also detected, with raised expression of TNF-α, IL-17A, and MPO. Here, we demonstrate that the extract of *M. cauliflora* has photosensitizing action, promoting the production of singlet oxygen, besides IL-17A, TNF-α, and MPO.

1. Introduction

Staphylococcus aureus is one of the major microorganisms that cause human diseases, leading from mild skin to serious infections such as endocarditis, septic arthritis, osteomyelitis, and sepsis [1,2]. Its pathogenic capacity is attributed to the large repertoire of toxins produced by this microorganism. Staphylococci are gram-positive, spherical bacteria with a diameter varying from 0.5 to 1.5 μm, are immobile, maintain grouped in irregular masses like cells adhered to each other, chained together in pairs, and forming bunches of grapes [3].

The pathogen can be found in the normal skin microbiota, mucous membranes of humans, and is related to several infections, mainly occurring in nurseries and intensive care units. With the use of semi-synthetic penicillins, methicillin-resistant strains called Methicillin-resistant *Staphylococcus aureus* (MRSA) have emerged, due to the acquisition of the *mec*A gene, whose resistance pattern extends to other beta-lactam antibiotics [4,5].

Infections caused by this microorganism are extending worldwide, with > 90% of them initiating through the skin. For these illnesses, the physical barriers and, especially, the host immune system will be directly related to infection control [6,7]. However, *S. aureus* is able to evade immunoprotective defenses due to the expression of virulence factors such as adhesins and proteins in order to neutralize both components of innate and acquired immunity. Then, outbreaks of community-based infections generated by resistant strains present a wide distribution in the terrestrial world, and the intensification on these facts stresses the necessity for developing new strategies to prevent and treat infections by this pathogen [8,9].

It has already been shown that photodynamic therapy (PDT) is capable of inactivating MRSA strains through two distinct

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photodynamic processes [10,11]. The first, called type I, free radicals are formed due to the excitation caused by laser, being responsible for reacting with lipids and proteins, leading the bacterial cell to face a state of cellular oxidation that culminates in its death. In type II, the laser excites a photosensitizer that is responsible for transferring its electrons to the molecular oxygen, generating a state of free oxidation such as singlet oxygen, being these reactive components responsible for bacterial clearance [12].

Thus, the research on discovering of new photosensitizers becomes important, especially to enlarge the amount of therapeutic resources available for the treatment of persistent infections evoked by this bacterium [13,14]. There is no report in literature of resistance to PDT, which sets this technique even more promising, since, nowadays, the arising of bacterial resistance is much greater than the development of new antimicrobial drugs [15,16].

In this context, jaboticaba (*Myrciaria cauliflora*), belonging to the family *Myrtaceae*, is a plant with like-plum-black-purple fruits that grow directly around the stem and main branches. This plant is native to Brazil, and is distributed throughout the Atlantic Forest biome. The fruit has a grape-like appearance and texture, but with a thicker, harder and purple skin, and is rich in phenolic constituents, including resorcinol, p-hydroxybenzoic acid, anthocyanins, hydroxycinnamic acids, flavonoids, and coumarins [17].

Its use as an antimicrobial agent has already been demonstrated in literature [18]. The crude extract holds antimicrobial activity against gram-positive and gram-negative strains. Among the evaluated microorganisms that were sensitive to the crude extract it has *Escherichia coli, Pseudomonas fluorescens, Listeria monocytogenes, Pseudomonas aeruginosa, Bacillus cereus, Salmonella Enteritidis, Salmonella Typhimurium, and Staphylococcus aureus* [19]. Justified by this broad spectrum of antimicrobial action, this plant was chosen for this work.

There are no reports of the use of *M. cauliflora* crude extract as a photosensitizer in antimicrobial photodynamic therapy. This technique displays of several dyes in the treatment of bacterial infections. Such substances are excellent for adhering to the patient's skin and, consequently boosting the bactericidal capacity when they are photo-stimulated. *M. cauliflora* also possesses the property of being a natural dye, due to the presence of anthocyanins in its composition, among others, as previously mentioned [20,21]. That was another important factor for the decision to use this plant species in this our study.

Hence, this work conveys an innovative way of the use of *M. cauliflora* extract as a photosensitizer, comprising its use as an antimicrobial agent when activated by light, against *S. aureus*.

2. Materials and Methods

2.1. Obtaining the Myrciaria cauliflora Extract

In order to produce the extract, the raw material used was the residue of the processed fruit bark, which have the purple pigmentation. The jaboticaba was acquired in the commerce of Vitória da Conquista, Bahia, Brazil. Three and a half kilograms of fresh jaboticaba were prepared and a bleaching was performed by immersing in boiling water for 5 min to inactivate enzymes and reduce the number of microorganisms on the fruit surface. The remaining total residue was 1.57 kg which were packed and frozen at −18 °C.

After thawing in a refrigerator at 4 ± 1 °C for 12 h, the pigment extraction was carried out with the addition of distilled water in the ratio of 1:3 (residue: water), in the absence of light and under controlled mechanical agitation at 6500 rpm for six hours. The obtained fluid was then filtered with the aid of an 18 cm diameter sieve with holes of 0.2 cm^2. The extract was concentrated to 1/3 of its initial volume using a rotary evaporator (TE-102, Tecnal, Brazil) at 60 °C coupled to a vacuum pump (TE-058, Tecnal, Brazil) operating at 600 mmHg, generating 1.5 g of concentrated extract that was stored in Eppendorf and frozen at −80 °C.

2.2. Determination of Bacterial Load

The ATCC 43300 MRSA strain was acquired from the collection of the Institute of Biomedical Sciences at the University of São Paulo. The samples were stored in a freezer at −80 °C (Equilam, São Paulo, Brazil). At the time of culture, samples were thawed at room temperature, plated on BHI (Brain Heart Infusion, pH 7.4, HIMEDIA), and taken to the incubator (Prolab, São Paulo, Brazil) for 24 h at 37 °C.

The bacterial load was determined by spectrophotometry. The inoculum was made through direct suspension, carried out in a previously sterilized laminar flow (Prolab, São Paulo, Brazil) by removing from 3 to 5 colonies from the culture plates, and diluting in 1 mL of sterile saline. Afterwards, an aliquot of the solution was placed in quartz cuvette for reading in spectrophotometer (Prolab, São Paulo, Brazil). At this moment, some parameters were observed: 0.135 absorbance at 660 nm (0.5 on McFarland scale, equivalent to 1.5×10^8 CFU/mL), in order to reach the quantity of 10^8 CFU (colony forming units) of *S. aureus* [22].

2.3. Protocol for Extract Photoactivation

The extract was solubilized in propylene glycol (0.75 μg/mL) in a sterile environment and later activated with blue light. *In vitro* photoactivation was performed with the incident light by a prototype device number 1.012960–3 (MM Optics, São Carlos, São Paulo, Brazil) that have five blue LEDs with a wavelength of 450 ± 20 nm. The application was performed in a continue way lighted with incidence time of 5 min, intensity of 75 mW/cm^2, for a total energy dose delivered of 54 J/cm^2.

In the living model, the *M. cauliflora* extract was injected in mice in the same place where the infection was carried out (it will be described later in the *in vivo* experimental design) and after 30 min, waiting in a dark environment, the photoactivation occurred in the same way as *in vitro*. The light was applied at a distance of 1 cm from the tip of the machine above the surface of the infected region.

2.4. Evaluation of In vitro Antibacterial Activity of Myrciaria cauliflora Extract

In vitro experiments followed models described in literature, with adaptations [22–24]. In a 24-well plate, 10^7 CFU of MRSA were placed in each well in 1 mL of BHI broth. Later, different concentrations of *M. cauciflora* extract (0.5 μg/mL, 1 μg/mL, 1.5 μg/mL and 2 μg/mL) were tested. Curcumin (100 μg/mL) was used as the standard photosensitizer for comparison of results, and a group containing only MRSA completed the wells. The plate was incubated in the dark for 5 min and after this period of pre-irradiation, it was irradiated following the protocol described above. The controls were not irradiated by blue LED light.

Immediately after irradiation, 5 μL of each well were cultured in BHI plates and oven-dried for 24 h at 37 °C. The technique used for sowing was the pour plate, thus facilitating the quantification of the number of colonies formed after the culture time. The quantification of CFU was performed with the aid of a colony counter (CP-600 Plus, Phoenix).

2.5. Analysis by UV-VIS Spectrophotometry

In order to verify if after the photoactivation protocol the *M. cauliflora* extract led to the formation of singlet oxygen, a solution containing the extract together with uric acid was produced. *M. cauliflora* extract (0.75 μg/mL) and a solution of uric acid 30 μg/mL (Bioclin, São Paulo, Brazil) were analyzed by spectrophotometry (UV-1800 - Shimadzu Scientific Instruments), at room temperature. This spectrophotometer operates in the spectral range of 200 to 700 nm. Quartz cuvettes (Analítica web, São Paulo, Brazil) with optical path of 1.0 cm and capacity of 4 mL were used.

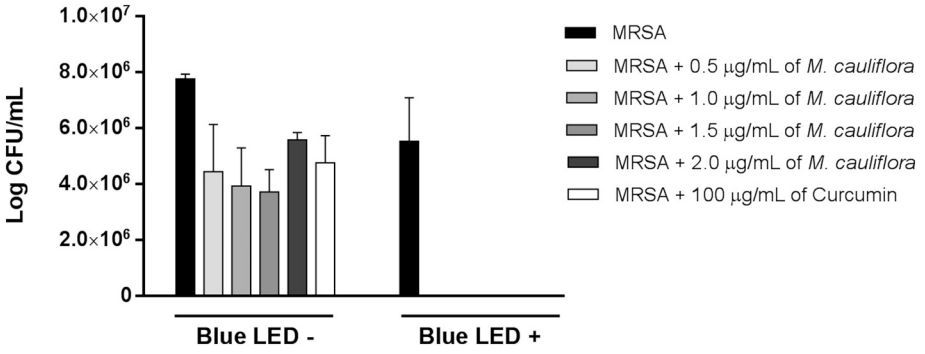


Fig. 1. Microbicidal action of *M. cauliflora* extract against *MRSA* (Log_{10} UFC/mL) - In a 24-well plate, 10^7 CFU of MRSA were placed in each well in 1 mL of BHI broth. Later, different concentrations of *M. cauciflora* extract (0.5 μg/mL, 1 μg/mL, 1.5 μg/mL and 2 μg/mL) were tested. Curcumin (100 μg/mL) was used as the standard photosensitizer for comparison of results and a group containing only MRSA. (n = 4); (BHI = Brain Heart Infusion; MRSA: Methicillin-resistant *Staphylococcus aureus*).

The photoactivation protocol was the same as previously described. Absorbance analysis of the solution containing uric acid and *M. cauliflora* was proceeded at every 60 s of irradiation.

2.6. Animals

Mice Balb/c aged six to eight weeks were obtained from the Federal University of Bahia - Multidisciplinary Institute in Health - Anísio Teixeira *Campus* facilities. The animals were maintained under controlled conditions of temperature with free access to water and food. All procedures involving animals were approved by the Ethics Committee on Animal Use (CEUA) IMS-CAT UFBA, under protocol number 042/2017. The animal challenge was performed using the ear infection model [25].

2.7. Experimental Design in vivo

The animals were infected by the intradermal route in the left ear with the quantity of 10^7 CFU of MRSA, as described in the literature, with adaptations [25]. In order to establish the infection, after inoculation of the bacterium, a waiting time of 12 h was set for the treatment with the extract together with the photodynamic therapy could be done afterwards. The extract was solubilized in 10 μL of propylene glycol.

The groups were formed as following: animals challenged with propylene glycol (Control, group 1), infected animals with 10^7 CFU of MRSA (MRSA, group 2), infected animals with 10^7 CFU of MRSA and 0.5 μg/mL of *M. cauliflora* extract (MRSA + *M. cauliflora*, Group 3), and finally, infected animals with 10^7 CFU of MRSA and 0.5 μg/mL of *M. cauliflora* extract followed by the photoactivation protocol (MRSA + *M. cauliflora* photoactivated, group 4), (n = 6/group/time). The irradiation with blue LED light was executed 5 min after the inoculation of the extract in the animals' ears and adhered to the same photoactivation protocol of the *in vitro* assay.

The animals' euthanasia of the animals occurred 24 h after the treatment and was carried out through the inoculation of ketamine (Vetnil, São Paulo, Brazil) and xylazine (Vetnil, São Paulo, Brazil), at doses of 400 mg/kg and 40 mg/kg, respectively, by intraperitoneal route.

2.8. Histopathology

After euthanasia, the left ears were cut and branched for histopathology and immunohistochemistry. Thus, the tissue fragments were soaked in 10% formalin (Isofar, Duque de Caxias, RJ, Brazil) for 24 h and then embedded in paraffin (Casa Álvarez Material Científico S.A, Madrid, Spain). Portions of 5 mm were prepared and stained using a standard protocol for staining with hematoxylin (Laborclin, Pinhais, PR, Brazil) and eosin (EA36, Laborclin, Pinhais, PR, Brazil).

The stained sections were visualized in light microscopy (L-200A, Bioval, São Paulo, SP, Brazil) and the morphometric analysis was executed in photomicrographs through the software AnalySIS getIT! 5.1 (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). After getting the photomicrographs, differential leukocyte count was performed.

2.9. Obtaining the Draining Lymph Node

The draining lymph node, near the left ear of animals, was removed and macerated in 1 mL of sterile saline (Vita e saúde, São Paulo, Brazil). After the maceration, 5 μL were cultured on BHI plates and incubated for 24 h at 37 °C. The technique used for seeding was the pour plate, thus facilitating the quantification of the number of colonies formed after the culture time. The quantification of the CFU was performed after 24 and 48 h of culture with the aid of a colony counter (CP- 600 Plus, Phoenix).

Photos of the plates were taken after the incubation period, and afterwards the measurement of the inhibition halos. In addition, in the remaining macerate, the cytokine production was assessed by ELISA (Enzyme-Linked Immunosorbent Assay).

2.10. Determination of Cytokines

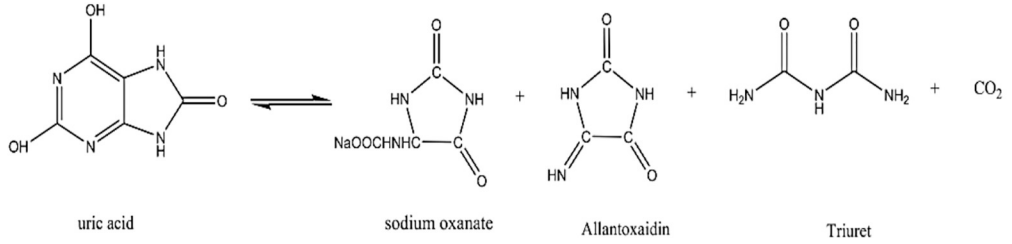
A 96-well ELISA plate was sensitized with 9.6 mL of capture antibody (100 μL/well) specific for a given cytokine, which could be TNF-α, IL-1β, IL-5, IL-4, IL-17A or IL-10, and was incubated overnight at 4 °C. On the next day, the plate was washed with 50 mL PBS Tween (Invitrogen™) to remove the free antibodies, and was blocked for 2 h with 200 μL/well of 1 × assay diluent (Assay Diluent, BD Biosciences). It was then, washed one more time and samples of the macerate, 100 μL per well, were added, where the antigens that bound to the antibodies were fixed to the plates, and again, were incubated overnight at 4 °C.

Following, the plates were cleaned, the detection antibodies (100 μL/well) were added, and they were kept in reaction for 1 h. The wells were washed again and the free conjugated secondary antibody (100 μL/well) was added. A solution of Tetramethylbenzidine (TMB) was added to the wells (100 μL/well) and the color change pattern was observed. The stop reaction solution was added to each plate (50 μL/well) and the color intensity was mensurated on an automatic plate reader (ThermoPlate) at 450 nm. All procedures followed the manufacturer's recommendations.

2.11. Evaluation of Myeloperoxidase and E-Cadherin Expression

The animals' ears were cut and fixed in 10% formaldehyde (Loja Agropecuária, São Paulo, Brazil), after, the samples were embedded in paraffin (Parafina Santa Cruz, São Paulo, Brazil), and histological sections (4 μm) were made for the preparation of slides. Next, the cuts were dewaxed in xylol and rehydrated in decreasing graduations of alcohol to distilled water. For myeloperoxidase immunohistochemistry, the antigen was retrieved by heating the slides in a pressure cooker in Tris-buffered saline with 0.075% Tween-20 (pH 7.6) for 10 min.

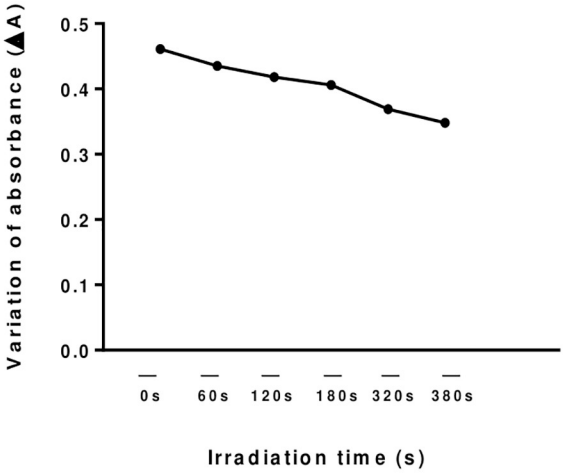
A



B

Irradiation time	Uric acid
	293 nm
0 second	0.461 A
60 seconds	0.435 A
120 seconds	0.418 A
180 seconds	0.406 A
320 seconds	0.369 A
380 seconds	0.348 A

C



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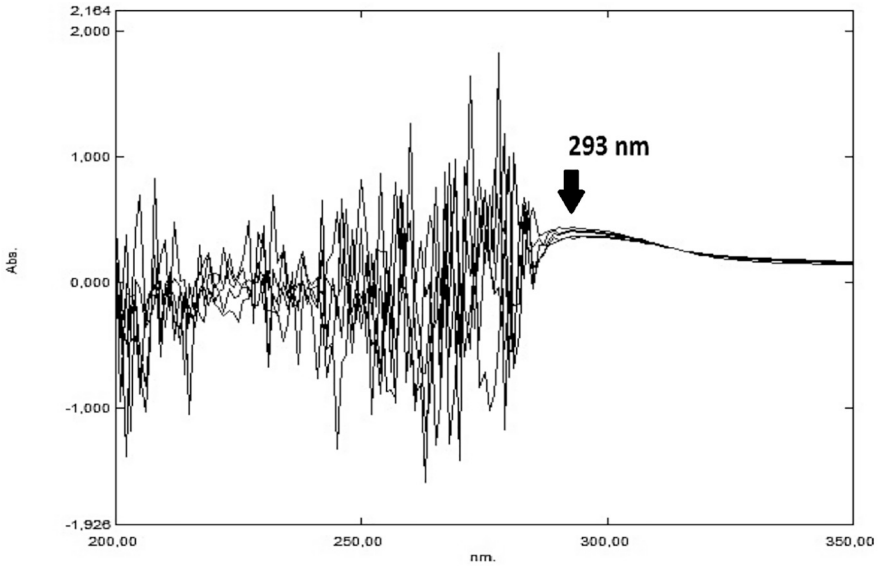

Fig. 2. Oxidation of uric acid - A solution of *M. cauliflora* at the concentration of 0.001 mg/mL and uric acid 30 μg/mL was prepared for analysis. The absorption peak of uric acid is at 293 nm, when it is oxidized (Fig. 2A) the band intensity is diminished. After the photoactivation protocol, a reduction in the solution absorbance as a function of time (Fig. 2B) was observed. The variation of the absorbances of the two solutions as a function of time (Fig. 2C) was calculated. By observation of the spectral behavior, it is possible to note the decrease of uric acid band at 293 nm (Fig. 2D).

Endogenous peroxidase activity was assessed by incubation in 0.3% *v*/v H_2O_2 in methanol for 20 min at room temperature. The sections were also incubated at room temperature for 30 min with polyclonal rabbit anti-human myeloperoxidase antibody diluted in the proportion 1:1500.

For E-cadherin, a streptavidin-biotin immunoperoxidase complex procedure was used for staining. Formalin-fixed, paraffin-embedded samples (4 μm) were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol (Prolab, São Paulo, Brazil) for 30 min. Antigen retrieval was carried out by microwave treatment in sodium citrate buffer (0.01 M, pH 6.0) for 10 min. Incubation with primary antibody was carried out for E-cadherin (clone M3612, diluted 1:400; DAKO) during 2 h, followed by incubation with biotinylated secondary antibody for 30 min at room temperature. Slides were produced with diaminobenzidine (Sigma, USA). Finally, sections were counterstained with hematoxylin, dehydrated, cleared, and

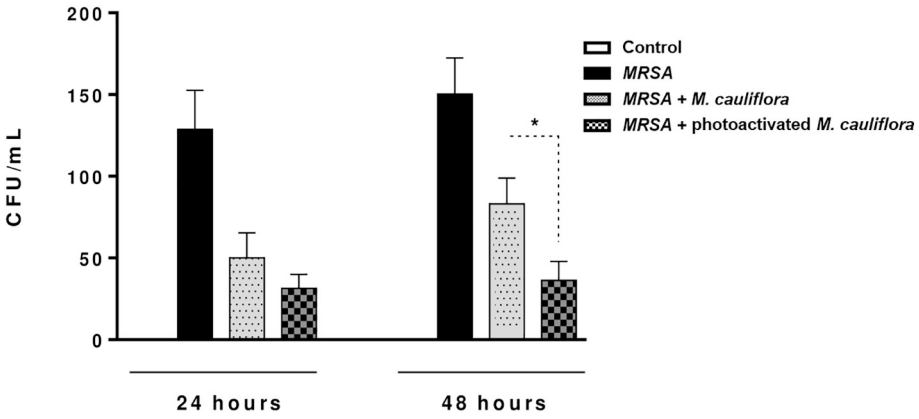

Fig. 3. Determination of bacterial load in the draining lymph node - The animals were euthanized 24 h after the challenge. In sterile environment, the draining lymph node was removed, macerated in 1 mL sterile saline solution, and 5 μL were cultured in BHI medium. CFUs were quantified after 24 and 48 h of culture. (n = 6); (BHI = Brain Heart Infusion); (CFU: Colony Forming Unit) (*$p < 0.05$).

mounted.

Pictures from each animal sample were taken using analySIS (version 5.2, Olympus Soft Imaging Solutions, Münster, NRW, Rhine-Westphali, Germany). Then, the labeled cells in the immunohistochemistry were counted in each field with the help of Image J software (version 1.50b, National Health Institute, USA).

2.12. Statistical Analysis

Statistical analyses of the experiments were developed by using the Kruskal-Wallis test through GraphPad Prism software (version 5.0, GraphPad Program Inc., San Diego, CA, USA) and Dunn's as post-test. For other outcomes, in order to compare the lineages, the Mann-Whitney test to make comparisons between groups was used. Statistical differences were considered significant at p values < 0.05.

3. Results

3.1. M. cauliflora Increased Antibacterial Action when Stimulated by Blue LED Light

After the *in vitro* assay, it was observed that photoactivation of the extract increased its antimicrobial activity (Fig. 1).

3.2. Photoactivated M. cauliflora Promotes the Oxidation of Uric Acid

With the objective of evaluating the possible mechanism of antimicrobial action of the photoactivated extract, it was done the experiment in association with uric acid (Fig. 2).

3.3. M. cauliflora Extract Increased In vivo Antimicrobial Action when Photoactivated

In view of the augment in antimicrobial activity *in vitro*, tests were carried out to verify whether it would occur *in vivo*. As a result, the treated animals exhibited a reduction in bacterial load in the draining lymph node (Fig. 3).

3.4. Photoactivation of M. cauliflora Extract Promotes Augment in the Release of TNF-α in the Draining Lymph Node

Animals treated with the photoactivated extract showed higher production of TNF-α and decreased in IL-10 quantity, and there was also a decline in all other evaluated cytokines (Fig. 4).

3.5. Photoactivation of M. cauliflora Extract Enhances the Expression of Myeloperoxidase

Through the differential count, it was possible to observe that animals treated with photoactivated *M. cauliflora* extract presented a higher number of neutrophils when compared to the group that received the extract without photoactivation (Fig. 5A). After analysis by immunohistochemistry, it could be detected that animals treated with the photoactivated extract exhibited an increase in the expression of MPO (Fig. 5B). However, when analyzing E-cadherin, there was no difference between the groups of animals treated with the photoactivated and non-photoactivated extract (Fig. 5A).

4. Discussion

The development of new photosensitizers is essential due to the increasing bacterial resistance to the main classically used antimicrobials. Thus, the expansion of the treatment resources of these infections beyond traditional medicines has been gaining more space. Therefore, the application of photodynamic therapy along with the use of photosensitizers have extensively been studied. This work conveys the first literary report of the association between the *M. cauliflora* extract and the photodynamic therapy.

After the photoactivation protocol, an increment in the antimicrobial activity of the *M. cauliflora* extract (Fig. 1) was observed. Obtaining compounds of plant origin is important because the development of an ideal photosensitizer requires some characteristics like being easy to extract, with fast synthesis, high yield, among others, since the more expensive the process of discovering and acquiring the chemical, the higher the value of the final product [26]. In this context, the *M. cauliflora* extract presents great advantages as it is a very popular species in Brazil and is easy to purchase, besides the preparation of the extract is extremely simple and requires few financial resources.

Furthermore, photodynamic therapy is typically associated with natural dyes such as curcumin, with excellent results in studies evaluating antimicrobial activity, especially against the pathogen presented here [25,27,28]. This plant was chosen for this research due to the fact it is also a well-known natural dye and its antimicrobial potential have already been mentioned in literature [18,19].

In the crude extract of *M. cauliflora* can be found hydrolysable tannins, such as pedunculagin, castalagin and vescalagin. Besides these, several phenolic compounds like gallic acid, cauliflorin, cyanidin-3-O-glucoside, among others. This work brings preliminary results on the use *M. cauliflora* in the PDT therapy, but other studies should be carried out with the objective of determining which compound(s) of the crude extract are responsible for the antimicrobial action after the photoactivation with blue LED light, and further, determine whether these compounds possess action alone or synergistically. However, after the analysis of our results, we believe that the extract of *M. cauliflora* has a photosensitizing action and possibly its compounds have a synergistic action after the activation with blue LED light.

Seeking to understand the mechanism of antimicrobial action of the extract after photoactivation, it was associated with uric acid. This

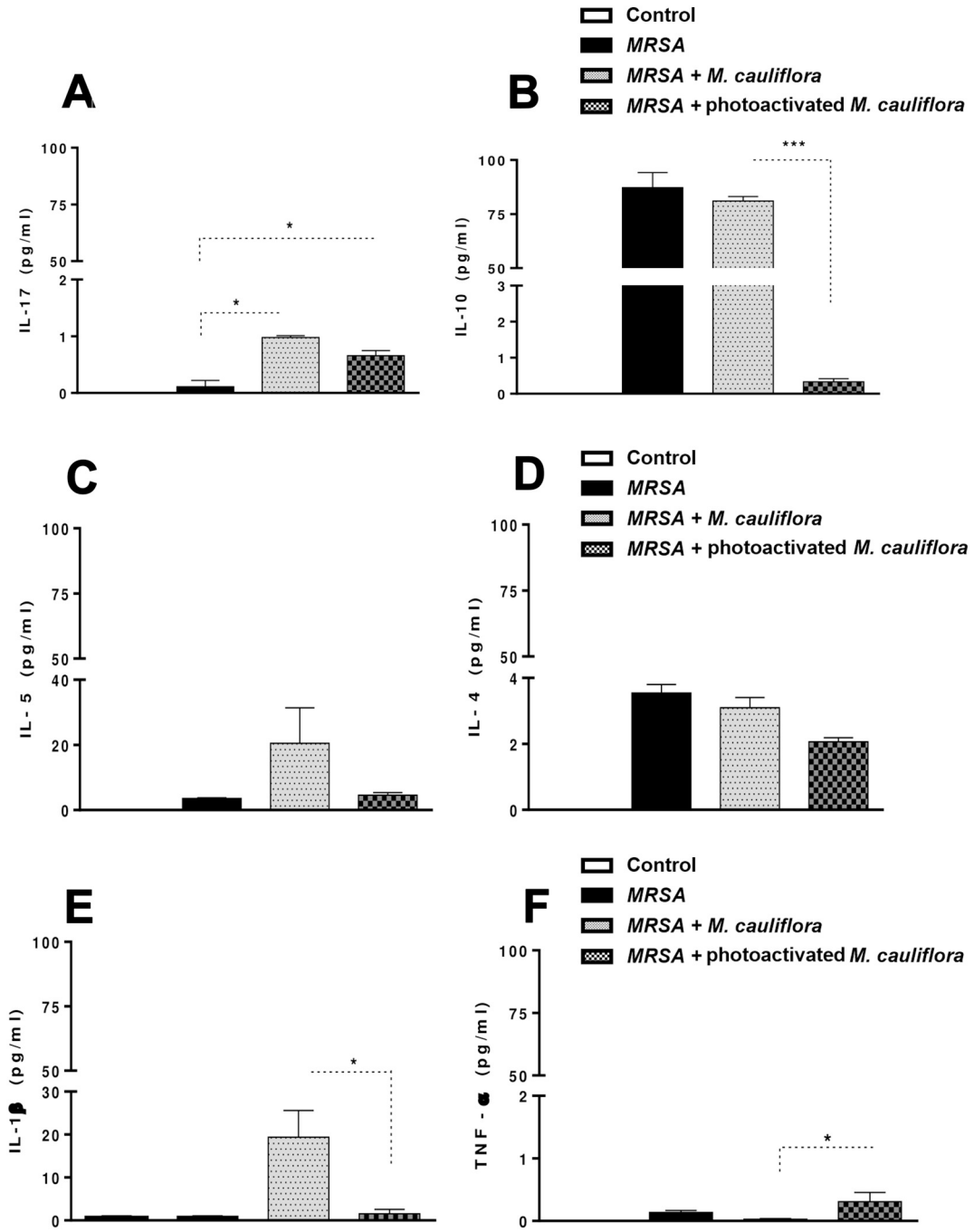

Fig. 4. Quantification of cytokines in the draining lymph node (pg/mL)- The animals were euthanized 24 h after the challenge. In a sterile environment, the draining lymph node was removed, macerated in 1 mL of sterile saline, and evaluated by ELISA for the presence of IL-17A (A), IL-10 (B), IL-5 (C), IL-4 (D) IL-1β (E) and TNF-α (F). (n = 6); (ELISA: Enzyme Linked Immuno Sorbent Assay); (IL: Interleukin); (*$p < 0,05$, **$p < 0,01$).

compound was selected for its high reactivity to singlet oxygen and oxyradicals, *in vitro* stability to autoxidation, low light absorptivity, and good photochemical stability in the region of interest [29–31]. In this assay, the rate of reactive oxygen species production was determined by spectrophotometry, evaluating the kinetics of the decay of uric acid UV-absorbance at 293 nm. It was detected that *M. cauliflora* extract possesses as a mechanism of action, the production of singlet oxygen, as shown in Fig. 2.

The production of singlet oxygen by a photosensitizer is important as there is no microbial resistance to this compound, so it does not matter if the strain is resistant to one or many classes of antibacterial agents since the photosensitizer is captured by the microorganism. Moreover, the reactivity of ROS with organic molecules is not specific, any macromolecule within the cell may be a potential target for photodynamic therapy. Here, a strain resistant to several classes of antimicrobials, MRSA, was applied and it was observed that photoactivated *M. cauliflora* reduced the growth of this bacterium *in vitro* (Fig. 1) and presented a bacteriostatic action when used in a living model (Fig. 3).

For the animal model, the intradermal infection was chosen as route, this model is based on the fact that *S. aureus* is the main agent of skin infections, with reports in the literature such as impetigo, scalded skin syndrome, boils, among others [7,32,33]. Moreover, it is known that photodynamic therapy is a technique directed to superficial areas of the skin and mucous membranes as the light is of low intensity, thus

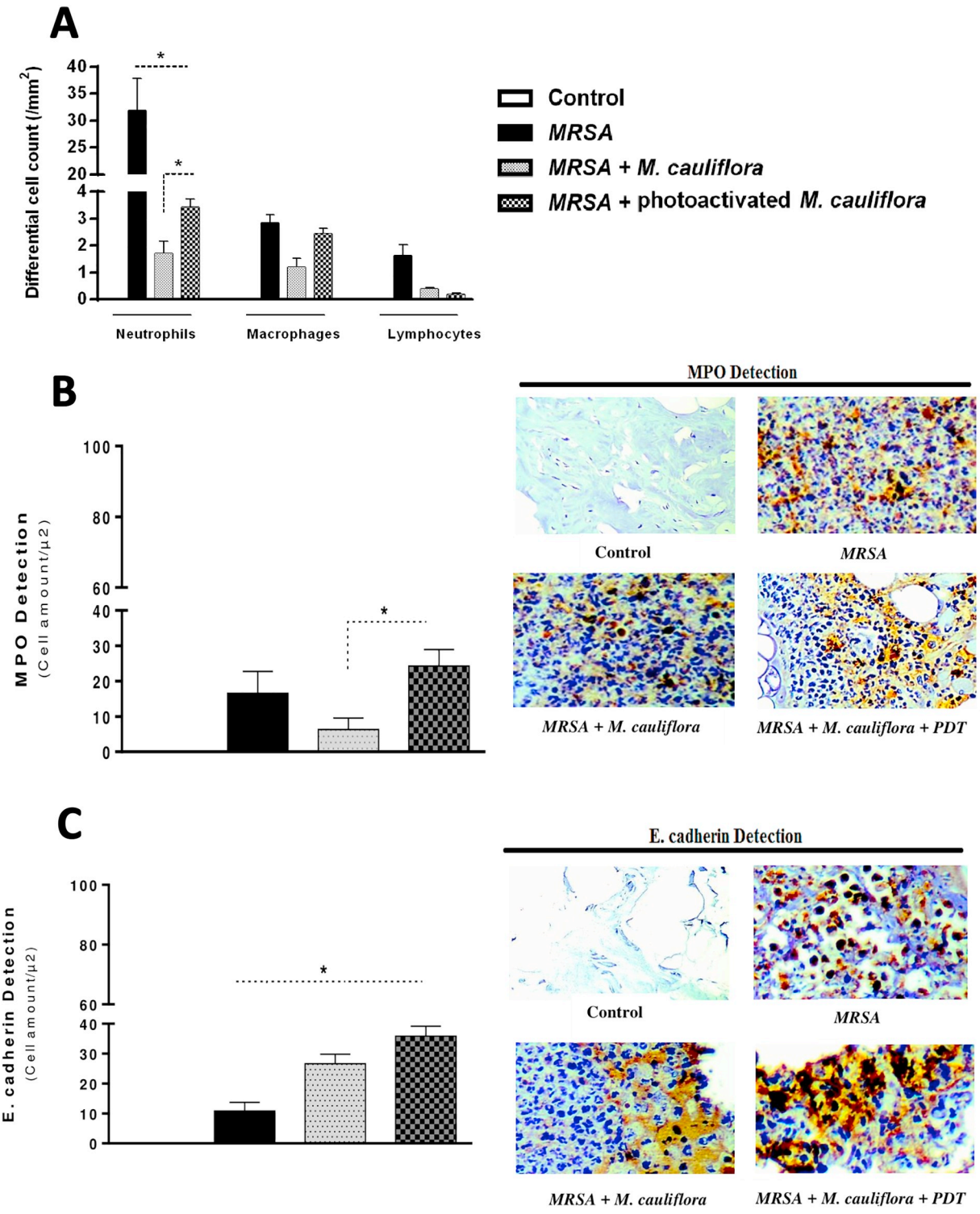

Fig. 5. Determination of Myeloperoxidase and E-cadherin expression - The animals' ears were cut, embedded in paraffin, histological sections were made, and the differential count of leukocytes in the inflammatory environment was carried out (A). In addition, the immunohistochemical technique for the expression of Myeloperoxidase (B) and E-cadherin (C) were proceeded. (n = 6); (*$p < 0,05$, **$p < 0,01$).

presenting a low penetration potential through the skin. After several tests, it was observed that the ideal volume for application to the animals' ears was 10 µL since it did not spill and is possible to notice the liquid at the application site, demonstrating that the procedure was performed correctly. Additionally, the literature displays of works that applied similar infection model as the one used here, also applied the same volume of their substances [25,34].

Another fact that has to be highlighted is that as the production of cytokines is higher in infected animals that did not underwent photoactivation, they produce more IL-10 in an attempt to balance the inflammatory process (Fig. 4B). The IL-10 is a potent anti-inflammatory mediator that plays a crucial role in limiting host immunopathology during bacterial infections by controlling effector T cell activation [35]. *S. aureus* has previously been shown to manipulate the IL-10 response

as a mechanism of immune evasion in models of infection. Our findings demonstrate that induction of IL-10 has a major influence on the outcome infection by *S. aureus* [35–37]. An excess in IL-10 expression, on the other hand, may suppress protective T cell responses, thus facilitating persistence of the bacteria [35].

Recent studies have demonstrated that the recruitment of neutrophils into the skin of animals infected with *S. aureus* is dependent on the action of TNF-α [38]. Here, the animals treated with the photoactivated extract had a greater expression of TNF-α together with a superior number of neutrophils in the inflammatory environment. The role of neutrophils in the epicutaneous skin infection model was assessed. The results exposed that neutrophils play a critical function in the containment of *S. aureus* skin infections in this model. Furthermore, in literature, and also in this study, the increase of TNF-α occurred in the draining lymph node, being this organ fundamental for the control of the infection by the production of cytokines. *In vivo* neutrophil depletion revealed that these cells play a protective role in preventing bacterial dissemination and fatal invasive infection [39].

Another relevant finding is the evaluation of the IL-17 participation against the *S. aureus* infection. There was an augment in the expression of this cytokine (Fig. 4A). Thus, this is the first report regarding the induction of IL-17A production by *M. cauliflora*. There are few reports that address the importance of IL-17A profile after infectious processes by MRSA [22,40,41]. It has been shown that knockout mice for IL-17 are unable to effectively eliminate *S. aureus*. Therefore, immune responses associated with Th17 could be a strategic target to eliminate persistent infections in humans. Many studies have described that this cytokine is produced by T lymphocytes, but most of this component in inflammatory processes is secreted by cells of the innate immunity [42,43]. The release of IL-17A by neutrophils has been described by different authors.

As well as for IL-17, there was also a higher IL-1 production in infected animals treated with the extract alone. Monocytes and macrophages are the main source of IL-1. Among the primordial biological activities, it is responsible for the stimulation of $CD4^+$ cells to secrete IL-2 and produce receptors for IL-2, cell proliferation and activation, intensifying chemotactic and phagocytic activities [44]. However, it is known that this bacterium has the ability to evade these mechanisms of innate immunity response, a fact that explains why a decline in bacterial load in this group did not occur significantly [45]. In this work, it was noticed that only the IL-1 elevation, without singlet oxygen, TNF-α and MPO, was not sufficient to cause bacterial load reduction.

In the determination of the mechanism of bacteriostatic action of the photoactivated *M. cauliflora* extract, it was noticed that, besides the action by the induction of singlet oxygen, there was an increment in the expression of MPO. It is supported by the fact that in the animals treated with the extract activated by light there was greater MPO expression in the ear (Fig. 5B). MPO is a lysosomal hemoprotein localized in polymorphonuclear leukocyte granules, which in the presence of hydrogen peroxide (H_2O_2) and halogen ions constitute a potent bactericidal system [46]. During the oxidative burst occurs the electronic transference of O_2 in superoxide anion that is reduced to H_2O_2. MPO has catalytic action in the formation of highly reactive radicals, such as hypochlorous acid, from H_2O_2 and halogen ions, which plays an important role in the mechanisms of intracellular killing of bacteria, possibly through protein halogenation [47].

Authors have already reported the important role of the superoxide dismutase and myeloperoxidase enzymes in the oxidative process of *S. aureus* death within the neutrophils, and concluded that their main function is to act for the myeloperoxidase-dependent pathway. However, there are several papers that report the ability of *S. aureus* to evade the action of MPO by many mechanisms like the inhibition of the action of this enzyme through the production of virulence factors [48].

In contrast, the presence of neutrophils in the inflammatory environment will not necessarily lead to bacterial clearance. In this case, it is necessary that the polymorphonuclear be modulated to fight infection. Thus, the components TNF-α and IL-17 are cytokines known to modulate polymorphonuclear cells and boost their mechanism of antibacterial action. This feature was observed in this study. The group of animals challenged with only the bacterium showed higher neutrophil counts (Fig. 5A), however, it did not obtain greater clearance of the pathogen (Fig. 3), because they did not produce TNF-α and IL-17 cytokines in a significant number (Fig. 4A and F). Concerned to the other cytokines analyzed (IL-5 and IL-4), no significant results were observed.

From the *in vitro* and *in vivo* data, a hypothesis that photoactivated *M. cauliflora* promoted the reduction of the bacterial quantity in the ear can be casted, a fact that is noticed through the maintenance of the levels of E-cadherin in the animals treated with the photoactivated compound (Fig. 5C). E-cadherin promotes cell adhesion in the tissue, thus, the APCs reduce the expression of this glycoprotein to migrate to the draining lymph node. This finding occurred in all the animals infected in this experiment, but was not observed in the mice treated with the extract photoactivated [49]. Then, the sustenance of E-cadherin expression in the animals treated with the photoactivated compound was due to the bacterial load that led to a lower need for cell migration to the draining lymph node.

Several works that used vegetal extracts associated with photodynamic therapy are presented in literature showing promising results [50–55]. Here, it was demonstrated that the extract of *M. cauliflora* has photosensitizing action, promoting the production of singlet oxygen, TNF-α and MPO. In view of this, a patent with process number BR1020170246930 was deposited. As this research is a pilot experiment, the development of further studies with this extract, in order to characterize it as well as the to deepen the understanding of its mechanism of action is encouraged.

Disclosure of Conflict of Interests

The authors have no conflicts of interests to declare.

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3.2. Artigo II – *Photoactivated resveratrol against Staphylococcus aureus infection in mice*

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Photoactivated resveratrol against *Staphylococcus aureus* infection in mice

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ABSTRACT

Background: Among the pathogens, *Staphylococcus aureus* is the main causative agent of bacterial diseases in the world. In this context, antimicrobial photodynamic therapy (aPDT) appears as a promising tool by means of microbial inactivation with the use of light. aPDT is applied in treatments involving photosensitizers capable of generating oxygen free radicals. Thus, this work proposes the use of resveratrol as a photosensitizer.

Methods: *In vitro* tests were performed to determine the antibacterial activity of photoactivated resveratrol with blue LED light, as well as uric acid experiments for verification of singlet oxygen formation. Possible resveratrol structural changes were evaluated by HPLC. In the *in vivo* assays, the air pouch model was performed in mice for antimicrobial activity and cytokine production.

Results: The photoactivated resveratrol exhibited an increase in its antibacterial action and it is possibly brought about by the singlet oxygen formation. In the air pouch model, TNF-α and IL-17A cytokines were produced, diminishing the bacterial load, and consequently, reducing inflammation after 24 h of infection. Cellular number decrease in the inflammatory environment was associated with resolution of inflammation along with greater IL-10 production.

Conclusion: It is the first time that resveratrol has been associated with aPDT. It was demonstrated in this work that resveratrol activated by blue LED light can be a promising photosensitizer. This compound, after the light stimulus, produces singlet oxygen, in addition to having effects on the immune system triggering TNF-α and IL-17A production, aiding in the clearance of several bacteria, including *S. aureus*.

1. Introduction

Bacterial infections occur worldwide and constitute a major public health problem [1]. With the introduction of antimicrobials, the survival of infected people has increased, however, over the years, some strains have become resistant to traditional compounds used in the therapeutic practice [2,3].

Among the pathogens, *Staphylococcus aureus* is the main causative agent of bacterial diseases. Infections by this microorganism have led to the death of millions of people annually, especially due to its advanced mechanisms of virulence [4]. Isolates of this infectious agent that are resistant to methicillin are called MRSA (Methicillin-resistant *Staphylococcus aureus)* [5]. Therefore, outbreaks of community-based infections by resistant strains present a wide distribution in the terrestrial environment resulting in increase in the necessity for developing new strategies to prevent and treat such disorders.

Most *S. aureus* strains are now resistant to penicillin, MRSAs are common in hospitals and are emerging in the community. Penicillinase-resistant penicillins (flucloxacillin, dicloxacillin) remain the antibiotics of choice for the management of serious methicillin-susceptible *S. aureus* (MSSA) infections. First generation cephalosporins (cefazolin, cephalothin and cephalexin), clindamycin, lincomycin, and erythromycin have important therapeutic roles in mild MSSA infections such as in skin and soft tissue, or in patients with penicillin hypersensitivity, although cephalosporins are contraindicated in patients with immediate penicillin hypersensitivity (urticaria, angioedema, bronchospasm or anaphylaxis) [5,6].

Therefore, new antimicrobial therapies are critical. In this context, antimicrobial photodynamic therapy (aPDT) follows similar principles to the photodynamic therapy (PDT) ones, which is more widely known for its application in non-cancerous diseases and cancerous lesions therapeutic [7]. This technique is used in treatments involving

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photosensitizers capable of generating oxygen free radicals when activated at a given wavelength by light emitted through a laser [8,9].

It has already been shown that aPDT is capable of inactivating MRSA strains through two distinct photodynamic processes [10,11]. Upon light activation, an excited photosensitizer can undergo type I (electron transfer) and/or type II (energy transfer) reactions to produce highly reactive oxygen species (ROS). Type I reactions generate radical anion species, while type II reactions produce singlet oxygen [12–14].

Hence, the research on new photosensitizers development becomes important, especially for providing alternative therapeutic resources for the persistent infections healing process. In addition, there is no report of resistance to the aPDT, and the financial investment required for the new photosensitizer studies is lower when compared to a traditional antimicrobial drug [15].

Thus, this work presents the use of photoactivated resveratrol by light. This polyphenol, especially present in grape, promotes health benefits through its anti-angiogenic, anti-inflammatory and antimicrobial activity [16–21]. In the initial studies, it was characterized as a phytoalexin. Since then, several studies have begun to point out its correlation with the prevention or reduction of tumor progression, cardiovascular clinical conditions, among others. Recent works have shown that this polyphenol is capable of interacting with light sources, even changing its structure [22,23].

Faced with the promising ability of resveratrol, several studies correlated this compound and the immune system. In many of them, it was observed its interaction with cells and inflammatory response factors, mainly by regulating the proinflammatory cytokines production [24–26]. There are no reports on the resveratrol use in photodynamic therapy in the treatment of bacterial infections, being this a pilot study.

In this context, this work aimed to evaluate the use of photoactivated resveratrol by blue LED light in the treatment of *S. aureus* in an air pouch model of infection in mice.

2. Materials and methods

2.1. MRSA strains

The MRSA ATCC 43300 strain was obtained from the Federal University of Bahia –Multidisciplinary Institute in Health - Anísio Teixeira *Campus*. The samples were stored in a freezer at −80 °C (Equilam, São Paulo, Brazil). At the time of culture, they were thawed at room temperature, plated on Brain Heart Infusion agar (BHI, pH7.4, HIMEDIA), and taken to the incubator (Prolab, São Paulo, Brazil) for 24 h at 37 °C.

2.2. Determination of bacterial inoculum

The determination of the bacterial load was executed by spectrophotometry. It was performed by means of the direct suspension, and carried out in previously sterilized laminar flow (Prolab, São Paulo, Brazil), through the removal of 3 to 5 colonies from the culture plates and their dilution in 1 mL of sterile saline. Subsequently, an aliquot of this solution was placed in quartz cuvettes for reading on the spectrophotometer (Prolab, São Paulo, Brazil).

At this time, the following parameters have to be reached: 0.135 absorbance at 660 nm (0.5 in the McFarland scale, equivalent to 1.5×10^8 CFU/mL), in order to obtain 10^8 CFU of MRSA. The suspension was seeded on the surface of the Brain Heart Infusion agar (BHI, pH 7.4, HIMEDIA).

2.3. Resveratrol photoactivation

The resveratrol (PharmaNostra, Campinas, São Paulo) was obtained from the Federal University of Bahia – Multidisciplinary Institute in Health – Anísio Teixeira *Campus*. For *in vitro* and *in vivo* antimicrobial activity assays, the compound was solubilized in propylene glycol in a sterile environment, and used at a concentration of 2 mg/mL.

The resveratrol photoactivation was performed through the incident light by a prototype device number 1.012960-3 (MM Optics, São Carlos, São Paulo, Brazil) that have five blue LEDs with a wavelength of 450 ± 20 nm. Irradiation procedure was carried out for 5 min, power density of 75 mW/cm^2, for a total fluence delivered of 54 J/cm^2.

2.4. In vitro antimicrobial activity evaluation

The *in vitro* antimicrobial evaluation test was carried out following the literature, with adaptations [27]. In a 24-well plate, 10^8 CFU of MRSA were added in 1 mL of BHI broth (BHI, pH 7.4, HIMEDIA) in each well. Subsequently, the formation of groups were performed: six wells were left in the dark and received no treatment (Group 1 – MRSA), six wells were treated with only blue LED light (Group 2 – MRSA + Blue LED light), six wells received twenty microlitres of resveratrol and were kept in the dark (Group 3 – MRSA + resveratrol), finally, six wells received twenty microlitres of resveratrol and blue LED light (Group 4 – MRSA + photoactivated resveratrol). The concentration of resveratrol and the photoactivation protocol were the same as described above.

After the execution period of the photoactivation protocol, five microliters from each well were seeded on a BHI medium plate. The tests were performed in triplicate and the CFU counts were performed after the time of 6, 12, 18 and 24 h. Free open source software, ImageJ (NIH), was used to count CFUs.

2.5. Analysis by UV–vis spectroscopy

For the spectrophotometric and high performance liquid chromatography (HPLC) techniques, a mass of 100 mg of resveratrol, after dissolution in an aqueous ethanol solution (Merckmillipore, Darmstadt, Germany), was transferred to a volumetric flask of 1000 mL. Then, the volume of the flask was filled with the aqueous ethanol solution (95%) until a stock solution containing resveratrol at a concentration of 100 mg/L. Afterwards, 1 mL of this solution was transferred to a 100 mL volumetric flask. The volume of the flask was quenched with ethanol until an intermediate solution containing resveratrol at the concentration of 0,001 mg/mL.

Uric acid was used to verify if resveratrol photoactivation produces reactive oxygen species. In order to reach that, the solutions were analyzed before and after the photoactivation. The light activation protocol was the same as described above. Therefore, the resveratrol solution described and a solution of 30 µg/mL of uric acid (Bioclin, São Paulo, Brazil) were analyzed by spectrophotometry (UV-1800 - Shimadzu Scientific Instruments), at room temperature. This spectrophotometer operates in the Spectral range of 200–900 nm. Quartz cuvettes (Analiticaweb, São Paulo, Brazil) with Optic path of 1.0 cm and capacity of 4 mL were used. The same resveratrol solution was used in the (HPLC) technique.

2.6. HPLC instrumentation and conditions

The HPLC system consisted of a Gynkotek M 580 GT pump, Rheodyne 8125 injector (20-µL loop) (Cotati, CA), and a Gynkotek M 340S UV diode-array detector (Gynkotek GmbH, Germering, Germany). A column (250 × 4.6 mm) packed with 6 µm particle size C18 material was used for the separations. Chromeleon data management software (Dionex Corp., Sunnyvale, CA) was used for equipment control and for data evaluation.

A multistep gradient method was applied using methanol–water–acetic acid (10:90:1, v/v) mixture as solvent A and methanol–water–acetic acid (90:10:1, v/v) mixture as solvent B at a flow rate of 1.5 cm^3/min (the reagents supplier was Quimisul, Santa Catarina, Brazil). The gradient profile was: 0.0–18.0 min, from 0% to 40% B; 18.1–25.0 min, from 40% to 100% B; and 25.1–27.0 min, 100% B. Chromatographic separations were monitored at 306 nm. Chromatographic peaks were

identified by comparing the samples retention time with the ones belonging to the standard compounds in the literature.

2.7. Animals

C57BL/6 mice aging from six to eight weeks were obtained from the UFBA-IMS/CAT facilities. The animals were maintained under controlled conditions of temperature with free access to water and food. The animal challenge was performed using the air pouch model. All procedures involving animals were approved by the Ethics Committee on Animal Use (CEUA) IMS-CAT UFBA, under protocol number 042/2017.

2.8. The air pouch model

The manufacture of the air pouch was carried out as described in the literature, with variations [28–30]. Initially, the animals were anesthetized with 50 mg/kg of ketamine (Vetnil, São Paulo, Brasil) and 10 mg/kg of xylazine (Vetnil, São Paulo, Brasil). In each mouse 3 mL of sterile air was inoculated into the dorsal subcutaneous space. Mice challenged with MRSA received 10^7 CFU of inoculum resuspended in 100 µL of saline.

In the *in vivo* experiments, resveratrol was solubilized in sterile propylene glycol (Quimisul, Santa Catarina, Brazil). Thus, groups of animals challenged with sterile propylene glycol (**Control, group 1**), animals challenged with 10^7 CFU of MRSA (***MRSA*, group 2**), animals challenged with 10^7 CFU of MRSA and 100 µL of Resveratrol (2 mg/mL) (***MRSA* + Resveratrol, group 3**), and finally, animals challenged with 10^7 CFU of MRSA and 100 µL of photoactivated Resveratrol (2 mg/mL) (**MRSA + Photoactivated Resveratrol, group 4)** were set up. After photoactivation, resveratrol was immediately inoculated into the air pouch in the treated animals. The concentration and volume were the same as that in the *in vitro* assay.

Then, 24 h post infection, the animals were euthanized (n = 6/group). The euthanasia of the animals was performed by deep anesthesia with intraperitoneal administration of ketamine and xylazine at doses of 400 mg/kg and 40 mg/kg, respectively.

2.9. Inflammatory cell influx

The lavages of air pouch were performed with 5 mL of sterile saline and stored at 4 °C. Later, the fluid was centrifuged at the conditions of 300 g for 10 min at 4 °C. The total cell count was done in a Neubauer chamber (Prolab, São Paulo, Brazil). The differential cell counts were performed by cytocentrifuge (Cytopro®) in Panoptic stained slides and analyzed by light microscopy (Olympus, Münster, NRW, Germany). The remaining fluid was stored at −80 °C for later quantification of cytokines by Enzyme-Linked Immunosorbent Assay (ELISA).

2.10. Bacterial load

Five microliters of lavages were seeded on BHI plates and incubated at 37 °C for 24 h. The technique used was the pour plate, thus facilitating the quantitation of the number of colonies formed after culture time [30]. The CFU quantification was performed with the aid of a colony counter (CP-600 Plus, Phoenix), at 12 and 24 h.

2.11. Quantification of inflammatory cytokines in the air pouch lavages

The supernatant extracted from the air pouch lavages were used to quantify TNF-α, IL-1β, IL-10 or IL-17A cytokines by sandwich ELISA using the ELISA Ready-SET-GO® kit (*Bioscience*), according to the manufacturer. A standard curve was obtained, and the cytokines in the samples were calculated according to the manufacturer´s instructions.

2.12. Histological study

Samples of the air pouch skin were collected, fixed in 10% formalin, and embedded in paraffin. These samples were sectioned at 5 µm, and stained by Hematoxilin and Eosin (Laborclin, Pinhais, Brazil). Histological slides of each animal were analyzed through light microscopy using an optical microscope Olympus® (Olympus, Münster, NRW, Germany) coupled to a camera Olympus SC30 (Olympus, Münster, NRW, Germany) for pathological alterations evaluation. Pictures from each animal sample were taken using analysis getIT® (version 5.2, Olympus Soft Imaging Solutions, Münster, NRW, Rhine-Westphali, Germany).

2.13. Statistical analysis

Statistical analyses of the experiments were performed using the *Kruskal-Wallis* test through GraphPad Prism® software (version 5.0, GraphPad Program Inc., San Diego, CA, USA), and Dunn´s as post-test. For other outcomes, we used the *Mann-Whitney* test to make comparisons between groups. Statistical differences were considered significant at p value < 0.05.

3. Results

3.1. Photoactivated resveratrol promotes increase in the inhibition of bacterial growth in vitro

The Fig. 1 demonstrates that there was greater inhibition of bacterial growth in the culture plates treated with the photoactivated form of Resveratrol.

3.2. Resveratrol has photodynamic activity generating singlet oxygen

To analyze the mechanism of antimicrobial action of the compound formed after resveratrol stimulation with blue led light, an uric acid

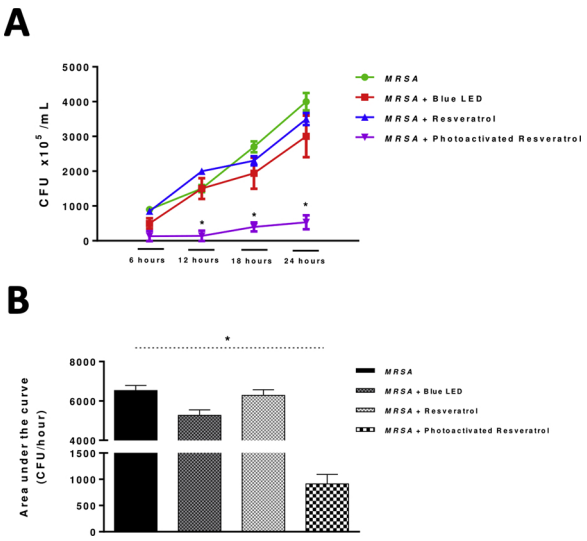

Fig. 1. Bacterial Load of *Staphylococcus aureus* (CFUx10^5 /mL) - In a 24-well plate, 10^8 CFU of MRSA were added in 1 mL of BHI broth. Subsequently, six wells were left in the dark and received no treatment (Group 1 - MRSA), six wells were treated with only blue LED light (Group 2 - MRSA + Blue LED light), six wells received twenty microlitres of resveratrol and were kept in the dark (Group 3 MRSA + resveratrol), finally, six wells received twenty microlitres of resveratrol and blue LED light (Group 4 – MRSA + photoactivated resveratrol). After the period of execution of the photoactivation protocol, five microliters from each well were seeded on a BHI medium plate and the counts of the CFUs were performed after the time of 6, 12, 18 and 24 h (A) and the area under the curve was measured (B). (* $p < 0.05$).

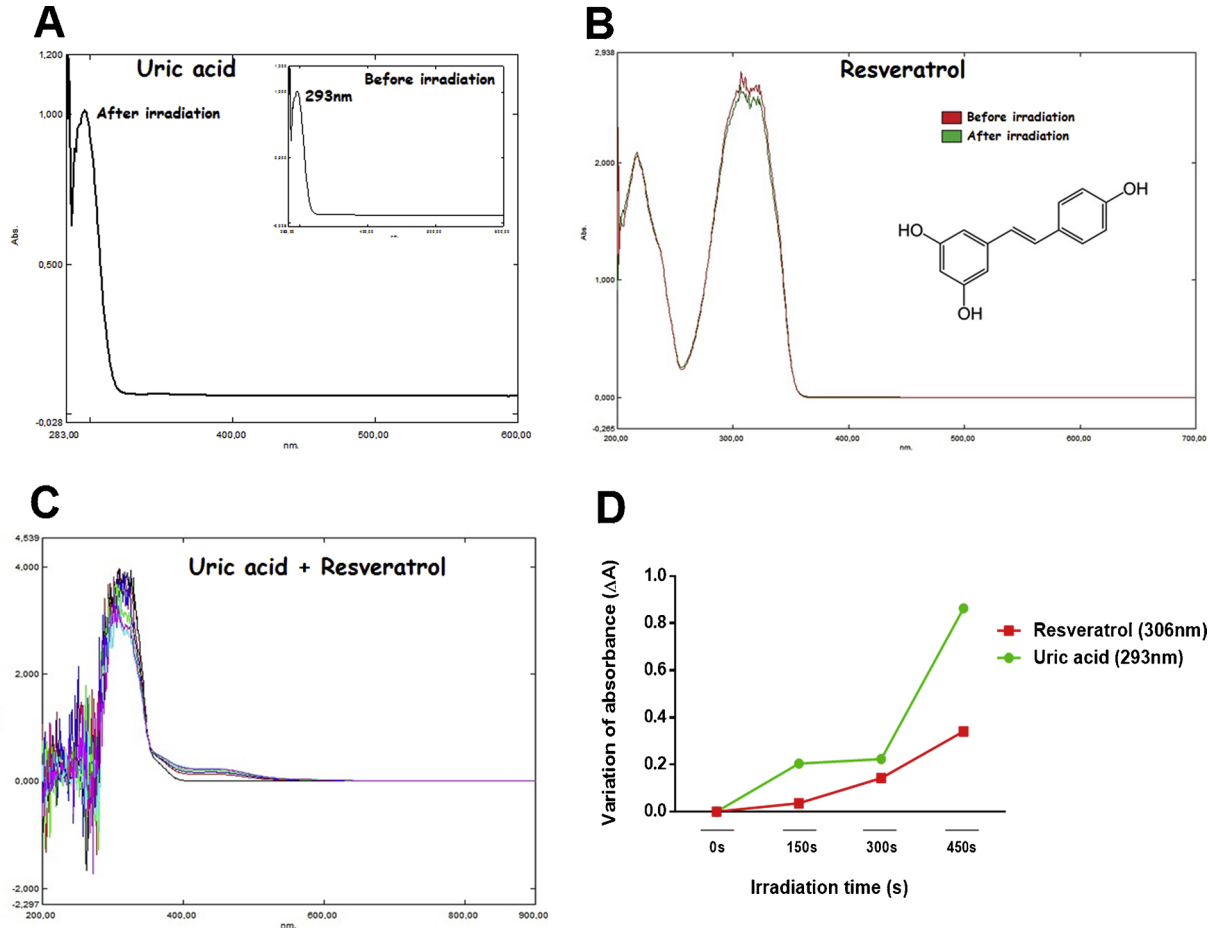

Fig. 2. UV/Visible Spectroscopy analysis of a resveratrol and uric acid solution – The solutions of 0001 mg/mL resveratrol and 30 μg/mL uric acid were prepared for analysis. Uric acid did not change absorbance after being submitted to the photoactivation protocol (A). The resveratrol used in this experiment showed its characteristic absorbance at 306 nm (B). After mixing the solutions of resveratrol and uric acid followed by the photoactivation protocol, the reduction in the absorbance of the solution as a function of time (C) was observed. In view of this, the variation of the absorbances of the two solutions as a function of time (D) was calculated.

solution was photoactivated along with the resveratrol solution described above. The uric acid absorbance variation was observed as a function of the irradiation time due to a uric acid photo-oxidation *via* singlet oxygen produced by photoactivated resveratrol (after photoactivation).

3.3. Photoactivated resveratrol exhibits change in retention time in HPLC analysis

To analyze whether the photoactivation causes some type of alteration in resveratrol, HPLC (Fig. 3) analysis was performed. The tests were conducted for both the photoactivated and no photoactivated form.

3.4. Therapy using photoactivated resveratrol as a photosensitizer reduces the number of inflammatory cells in the air pouch

To assess whether there were quantitative differences in inflammatory cells in the air pouch, the inflammatory environment lavage was analyzed for total and differential count of inflammatory cells. As seen in Fig. 4, data obtained demonstrate that there was a reduction in total inflammatory cells in the animals treated with the photoactivated resveratrol. There was also a reduction in the number of cells in the air pouch skin, as it can be seen in the images analysis.

3.5. Photoactivated resveratrol enhances the release of TNF-α and IL-17 into the air pouch

With the objective of analyzing animals treated with photoactivated resveratrol, a production profile on differentiated IL-1β, IL-10, TNF-α, and IL-17A cytokines was conducted. The air pouch lavages were analyzed by ELISA. Animals treated with the photoactivated compound presented greater TNF-α and IL-17A production (Fig. 5).

3.6. Animals treated with photoactivated resveratrol demonstrated greater levels of bacterial clearance in the air pouch

The Fig. 6 presents the bacterial growth behavior after 24 h of cultivation. It was observed that C57Bl/6 mice treated with photoactivated resveratrol had lower formation of CFU than the non-treated group.

4. Discussion

S. aureus infections constitute a public health problem due to the large numbers of resistance cases of this pathogen to the traditional antimicrobials, additional to the fact the medicines production is a rather slow process, and consequently, the development of new active compounds does not follow such growth. This work brings a new proposal for the treatment of these infections by using resveratrol activated with blue light as an alternative.

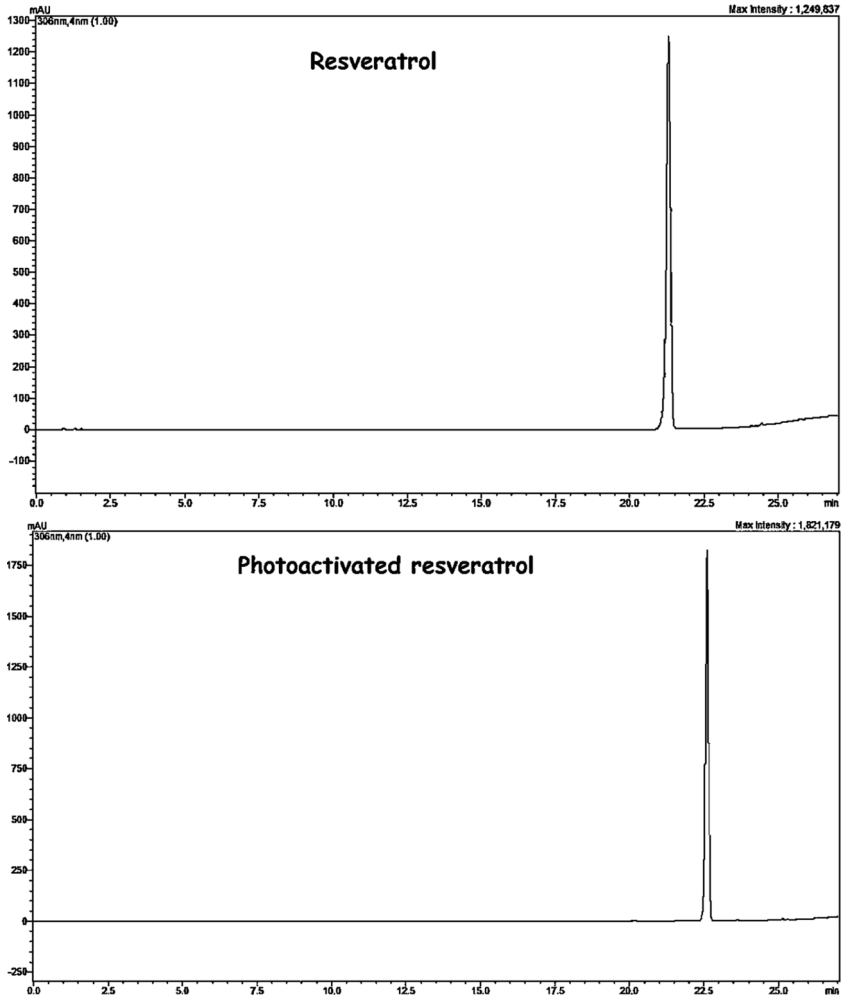

Fig. 3. HPLC chromatograms of Resveratrol - A 0.001 mg/mL resveratrol solution was prepared for analysis. Analyses of photoactivated and no photoactivated compounds were performed. Chromatographic separations were monitored at 306 nm.

It was observed that this association was able to reduce bacterial growth, both *in vitro* and *in vivo*, showing lower local inflammation and higher levels of cytokines production, such as TNF-α and IL-17, besides the generation of singlet oxygen. In addition, it is possible that the light irradiation had caused structural changes in the resveratrol molecule, since the compound presented a change in its retention time in the HPLC analysis after the photoactivation. In that way, it is likely the resveratrol absorbs photons and undergoes photochemical, and chemical reactions that lead it to become a biologically active compound with bacteriostatic activity.

The current literature conveys works presenting the resveratrol photostimulation under several different light sources. When exposed to the sunlight, this compound undergoes a molecular rearrangement whose structure consists of two fused aromatic rings attached to a linear chain containing a carbonyl group conjugated to a double bond, in which the authors termed the molecule obtained as "Resveratrone" [22,23]. With LED light, authors have reported the glycosylation of resveratrol. The use of blue LEDs as light source for the resveratrol biotransformation has remarkably improved the yield of its β-D-glycosides and changed the composition of products, the 3-O-β-D-glucoside was the major product. [31].

Once it is a pilot study, it is not possible to say that resveratrone or 3-O-β-D-glucoside was formed in our experiment, however, it can be affirmed that resveratrol had the ability to interact with blue LED light, modifying its structure (Fig. 3), and exhibiting antimicrobial action (Figs. 1 and 6).

The uric acid is an efficient singlet oxygen sensor acting as a chemical dosimeter for the quantitative determination of the photodynamic action of a compound [32,33]. The Fig. 2 shows its optical absorption spectrum and photo-oxidation. Initially, the light stimulation of uric acid was performed in the wavelength range studied for this study. Therefore, as noted in Fig. 2A, the wavelength range used herein was not able to change the absorbance of the compound, which ensures that the interaction with the light is restricted to the resveratrol in the experiments of determination of photodynamic activity.

Fig. 2D shows the absorbance variation of the uric acid bands (293 nm) and resveratrol (306 nm) as a function of the irradiation time. The photo-oxidation reaction of uric acid *via* singlet oxygen is well-known, and lead to the formation of products such as triurea, alantoxaidin, oxanate ion and CO_2, occurring the suppression of singlet oxygen by uric acid with the capture of the triplet state energy of the resveratrol molecule in the excited state by the uric acid molecule. Further, this highly reactive oxygen athom initiates oxidative reactions in the proximate environment, like the bacterial cell wall, lipid membranes, enzymes, or nucleic acids [34].

When we associate resveratrol with uric acid with subsequent photoactivation we noticed a reduction in the absorbance profiles of the two molecules (Fig. 2D). The literature classifies this effect as photobleaching, being a characteristic directly related to the therapeutic dose of the compound with photodynamic activity [35]. Accordingly, here it was presented a decay of the characteristic band signal of uric acid at 293 nm, indicating that the decomposition reaction of this molecule

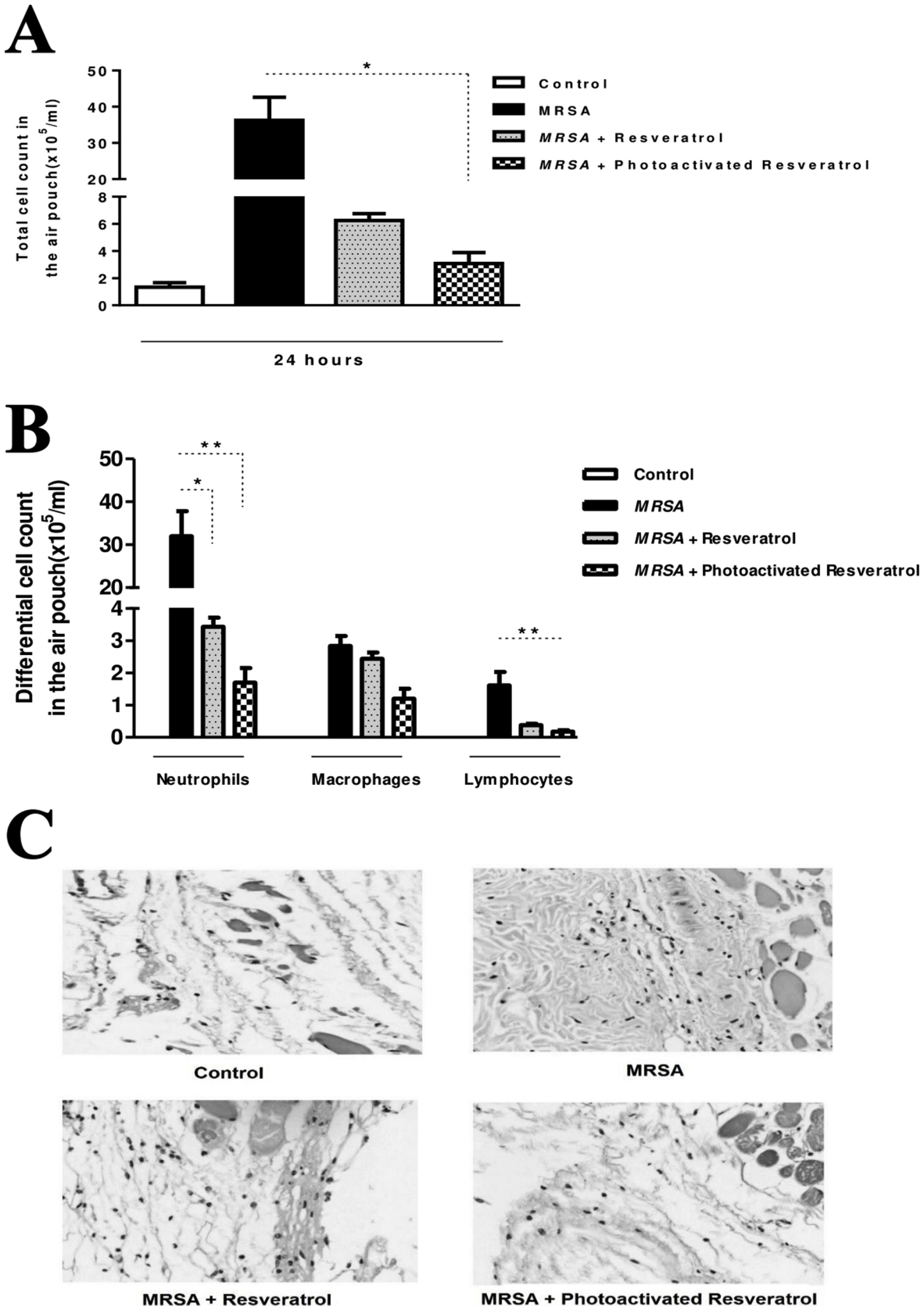

Fig. 4. Total and differential counts of leukocytes in the air pouch lavages and visualization of inflammation in the air pouch skin (x10^5/mL) - Skin samples from the air pouch were removed (collected), fixed in 10% formalin, and stained by hematoxilin and eosin. After slides production, the total (A) and differential (B) counts of the leukocytes were performed, as well as the representative images of the histological slides (C). (n = 6); (BHI = brain heart infusion; MRSA: Methicillin-resistant *Staphylococcus aureus*); (* $p < 0.05$, ** $p < 0.01$).

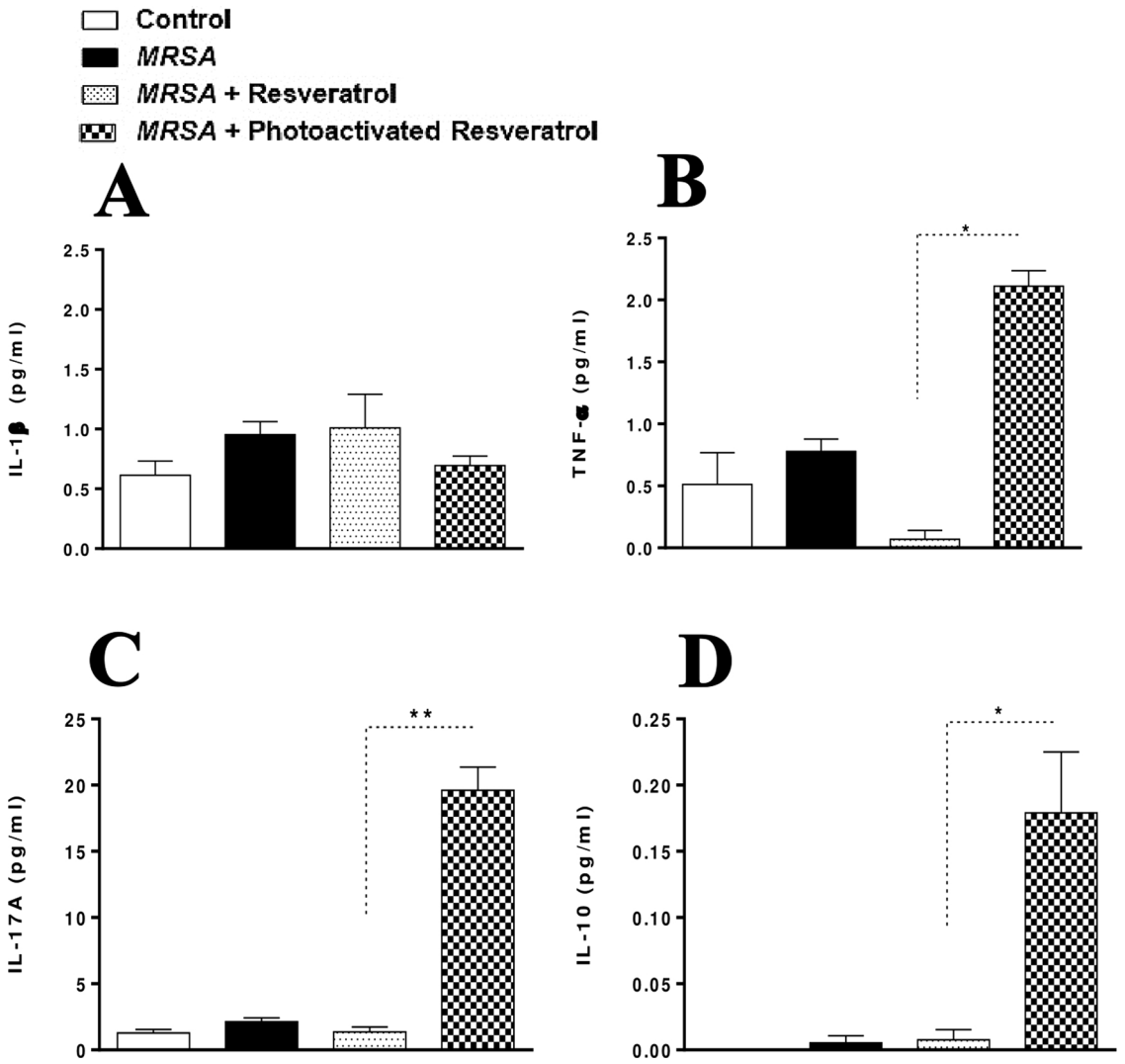

Fig. 5. Quantification of cytokines in the inflammatory environment (pg/mL) - Lavages from air pouch were collected later and evaluated by ELISA for the presence of IL-17A, IL-10, IL-1β and TNF-α. (n = 6); (BHI = brain heart infusion; MRSA: Methicillin-resistant *Staphylococcus aureus*);(* $p < 0.05$, ** $p < 0.01$).

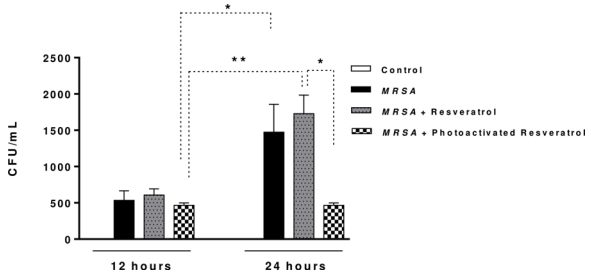

Fig. 6. Bacterial Load of *Staphylococcus aureus* in the air pouch (CFUx10^5 /mL) – Lavages from air pouch were collected later and cultured in BHI. The CFUs were quantified after 24 h. (n = 6); (BHI = brain heart infusion; MRSA: Methicillin-resistant *Staphylococcus aureus*);(* $p < 0.05$, ** $p < 0.01$).

occurs through the singlet oxygen formed by the excited resveratrol.

These results support raising the theory on that the photostimulated resveratrol antimicrobial action is by the generation of reactive oxygen species. The products generated from the reactions are cytotoxic, but singlet oxygen has been considered the main responsible for the antimicrobial effect of aPDT. The antimicrobial effect of the photosensitizer can happen by producing sufficient quantity of singlet oxygen nearby the outer membrane of the bacteria, so that it can diffuse into the cellular interior, culminating in lethal damage. As a consequence, the photosensitizer must be in close contact with the target cell in such a way the singlet oxygen generated can exert its antimicrobial effect, since it presents a small diffusion distance (20 nm) and short half-life time [36–38].

The great difficulty in developing an effective treatment against *S. aureus* is for the reason this pathogen is part of the human microbiota, and is in understanding which type of inflammatory response is effective in the clearance of this microorganism. Several studies in the attempt to develop immunoprotection have failed to develop a prophylactic method against infections by this pathogen [39–41]. Thus, works that seek complementary forms of traditional antimicrobial therapies present promising results. PDT has been used to combat staphylococcal infections for a long time [42–45]. Many of these works use curcumin as a photosensitizing agent [11,46].

However, even if there are few reports of resistance to aPDT, it is possible that the development of resistant strains occur. Since it is a new therapy, with expansion in its use it cannot be assured that resistance will not come out [47]. aPDT has short half-life time and acts based on the attack of multiple cellular targets by photo-generated ROS; then, a prior concept is that the development of resistance to the aPDT is unlikely [48]. However, there are reports in literature indicating that some *S. aureus* strains are more tolerant to the aPDT than others, and the findings that some clinical isolates demonstrated decreased susceptibility of to the aPDT after photodynamic exposure, point out a

need for detailed investigations on bacterial adaptive responses to photodynamic treatment [48–51].

Therefore, it is necessary to increase the photosensitizers availability, as it is necessary to develop new antimicrobials. To date, studies have sought to determine adequate parameters for clinical application of aPDT, which involves assessing different photosensitizers, light sources and doses, and drug concentrations [52,53]. On that account, researches have aimed to identify photosensitizers with antimicrobial activity when associated with LED light, a lower cost light source and simpler technology in comparison to the laser devices, facilitating the clinical application of this therapy [54].

In addition, when compared to other antimicrobial therapies, aPDT has many advantages such as higher specificity (the photosensitizer can be delivered to the cell and can be highly focused to the site of injury in cases of cutaneous treatment), and fewer undesirable side effects [55,56]. Given these facts, we chose to use Resveratrol activated by blue light because of its antimicrobial activity, additionally to its structural similarity as other photosensitizers as phenolic groups, like the curcumin.

Moreover, the resveratrol in the UV/Visible spectrum sweep presents an absorption band at 306 nm. Then, the light emission is close to the maximum absorption range of the molecule. However, this is not a rule, since there are studies that stimulate photosensitizers in several bands of wavelength and not only in the maximum absorption range [57–61]. In this work, we photoactivated propylene glycol, and found that this compound did not exhibit inhibition of bacterial growth *in vitro*. Taking this into consideration, we used propylene glycol as a solvent for resveratrol in the experiments.

The photoactivation of the solutions were performed outside the body environment of the C57Bl/6 animals, since the melanin could be an interfering agent in the aPDT [51]. Therefore, here we are proposing an adaptation of aPDT for the treatment of infections in black patients, since in the literature, there are few studies evaluating this technique in these patients [62]. After photoactivation, the solutions were immediately applied to the animals. The air pouch model is a convenient *in vivo* model to study localized inflammation without systemic effects [63]. In this context, it was possible to observe the effects of photoactivated resveratrol against a MRSA infection in a live model.

What can be inferred from the results obtained through this study is that the interaction of photoactivated resveratrol with the immune system becomes evident (Fig. 4). Notably the stimulation of the release of important cytokines such as TNF-α and IL-17 (Fig. 5). This is fundamental for the clearance of *S. aureus*, which have already been reported in several works [64,65]. The stimulation of their release is a determining factor for the treatment, especially in infections by resistant strains.

It has been shown that knockout mice for IL-17 are unable to effectively eliminate *S. aureus*. In that way, immune responses associated with Th17 cells could be a strategic target to eradicate persistent infections in humans [66]. Many studies have described that this cytokine is produced by T lymphocytes, but most of this component in inflammatory processes is secreted by cells of the innate immunity [67]. The release of IL-17A by neutrophils has been described by different authors [68,69]. Mice deficient in IL-17A or IL-17F have proved prone to skin infections caused by gram-positive bacteria such as *S. aureus* [70]. Experimental models using immunogens associated with a Th17 response against pathogens such as *S. aureus* and *Candida albicans* demonstrated an increase in recruitment and activation of phagocytes at infection sites, in addition to the more effective clearance of these pathogens in tissues [71]. In this context, the IL-17 production in the induction of effector mechanisms against *S. aureus* and the presence of cells that produce this cytokine is fundamental for the infection control.

Increased release of TNF-α is an interesting finding, since there are conflicting data in literature on the Resveratrol ability to alter the production of this cytokine. We found that in the our group where this polyphenol was not photoactivated, actually occurred a reduction in the release of TNF-α. Different studies have shown the importance of the TNF-α release, a pro-inflammatory cytokine, with the reduction of MRSA infection associated with laser therapy [72–75]. Furthermore, aPDT stimulates macrophages to release TNF-α in great quantities, leading to an increase of this cytokine at the site of inflammation [76–79]. The leukocytes activated by TNF-α produce reactive species of oxygen and nitrogen, essential for the elimination of microorganisms. In that way, the immune system cells activation by TNF-α is essential for the infections control [80,81].

Quantitatively, the number of inflammatory cells in the resveratrol-treated groups was lower than in non-treated ones (Fig. 4A). This data corroborates with several works showing that after the treatment of animal models with this polyphenol, there was a local inflammation reduction [82]. However, in our data, even with a reduction in the number of cells in the site, the inhibition of bacterial growth was greater (Fig. 6), a fact, since the exacerbation of the local inflammation can be harmful to the organism. Moreover, it is possible that even in a smaller quantity, the inflammatory cells are directed by the synergism of IL-17A and TNF-α [83,84], resulting in a greater) reduction of the bacterial load, even with a lower cell number.

On the other hand, it is possible that 24 h after treatment with photoactivated resveratrol, time analyzed by our work, the decrease in number of inflammatory cells has already occurred due to the bacterial clearance. This fact would also explain the higher levels of IL-10 production (Fig. 5D), an anti-inflammatory cytokine, secreted to modulate inflammatory responses [85]. No differences were observed between the groups regarding IL-1β production. In addition to these data, the air pouch model was one of the factors that aided in the effective visualization of inflammation, since it is easy to manipulate and obtain clinical samples that help in the understanding of the local inflammatory response.

Considering the results obtained in this work, we can see that resveratrol activated by blue LED light can be a promising photosensitizer to be used in antimicrobial photodynamic therapy. This compound, after the light stimulus, produces singlet oxygen, a potent antimicrobial, combined to the fact it was able to exert effects on the immune system with TNF-α and IL-17A production, which are pro-inflammatory cytokines, aiding in the clearance of several bacteria, including *S. aureus*. Since it is a pioneering work, more studies are necessary to allow a clear elucidation of the mechanism of action of photoactivated resveratrol.

Disclosure of conflict of interests

The authors have no conflicts of interests to declare.

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3.3. Artigo III – *Photoactivated Resveratrol Controls Intradermal infection by Staphylococcus aureus in mice*

Photoactivated resveratrol controls intradermal infection by *Staphylococcus aureus* in mice – a pilot study

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ABSTRACT

Staphylococcus aureus is one of the main causative agent of infections acquired in both community and hospital environment. In this context, photodynamic therapy (PDT) consists in using a photosensitizer that, activated by light, evokes the formation of reactive oxygen species (ROS), which lead to the death of microorganisms due to oxidative damage; it is useful tool since this action, harmful to pathogens, does not significantly injure human cells. In view of this, this work proposes a more in-depth study on the use of resveratrol (RSV) as a possible photosensitizer. It was observed, in the intradermal infection model in animals' ear dermis, that photoactivated resveratrol promotes an increase in myeloperoxidase expression with reduced bacterial load in the draining lymph node. Besides that, the draining lymph node of the animals treated with photoactivated RSV controls inflammation through IL-10 production. These are pioneers data and this work being a pilot study, then, other works must be conducted with the objective of elucidate the photoactivated resveratrol mechanism of action.

Keywords: *Staphylococcus aureus*; photodynamic therapy; photosensitizers; resveratrol

ABSTRACT

Staphylococcus aureus is one of the main causative agent of infections acquired in both community and hospital environment. In this context, photodynamic therapy (PDT) consists in using a photosensitizer that, activated by light, evokes the formation of reactive oxygen species (ROS), which lead to the death of microorganisms due to oxidative damage; it is useful tool since this action, harmful to pathogens, does not significantly injure human cells. In view of this, this work proposes a more in-depth study on the use of resveratrol (RSV) as a possible photosensitizer. It was observed, in the intradermal infection model in animals' ear dermis, that photoactivated resveratrol promotes an increase in myeloperoxidase expression with reduced bacterial load in the draining lymph node. Besides that, the draining lymph node of the animals treated with photoactivated RSV controls inflammation through IL-10 production. These are pioneers data and this work being a pilot study, then, other works must be conducted with the objective of elucidate the photoactivated resveratrol mechanism of action.

Keywords: *Staphylococcus aureus***;** photodynamic therapy; photosensitizers; resveratrol

INTRODUCTION

Staphylococcus aureus is one of the main causative agent of infections acquired in both community and hospital environment [1]. The vast majority of disorders caused by *S. aureus* result from asymptomatic carriers, in which the individual can be colonized for short or long periods, that can lead to a disease in case of the immune system has some impairment[2-4].

Therefore, serious disorders provoked by this microorganism can often tackle hospitalized patients and trigger worrying consequences, especially concerned to antibiotic therapy [5]. Since MRSA (Multiresistant *Staphylococcus aureus*) was first described as one of the leading hospital pathogens in 1960s, the incidence of this microorganism's infections continues to broaden in health care institutions, and recently, in worldwide community[2].

In this context, photodynamic therapy (PDT) consists in using a photosensitizer that, activated by light, evokes the formation of reactive oxygen species (ROS), leading to the death of microorganisms due to oxidative damage [6,7]. In view of this, several *in vitro* studies have demonstrated that some coloring substances can be light-activated and produce antimicrobial effect against several types of pathogens [7-10]. However, the number of dyes available today is limited and many of them need further study on their antimicrobial mechanism of action.

In view of this, this work proposes a more in-depth study on the use of resveratrol (RSV) as a possible photosensitizer. This polyphenol, especially present in grape, promotes health benefits through anti-angiogenic, anti-inflammatory, and antimicrobial activity [11]. When an animal is selected for research on a human disease, previous procedures are often necessary to adapt the disease to the animal. An adapted infection protocol for such animals was applied in this research. Most infections caused by *S. aureus* occur through the skin [3,4]. Therefore, an intradermal infection model in mice was performed.

Regarding the great need for new therapeutic options for the treatment of infections caused by MRSA strains combined with the lack of *in vivo* studies on the application of antimicrobial photodynamic therapy, this study's objective was to evaluate the antimicrobial action of photoactivated RSV in MRSA intradermal infections in mice.

MATERIALS AND METHODS

MRSA strains

MRSA ATCC 43300 strain was obtained from the Anísio Teixeira *Campus*, Multidisciplinary Institute in Health, Federal University of Bahia (UFBA-IMS/CAT). The samples were stored in freezer at -80°C (Equilam, São Paulo, Brazil). At the time of culture, they were thawed at room temperature, plated on Brain Heart Infusion agar (BHI, pH7.4, HIMEDIA), and taken to the incubator (Prolab, São Paulo, Brazil) for 24 hours at 37°C.

Determination of Bacterial Inoculum

The determination of bacterial load was executed by spectrophotometry. It was performed through direct suspension, and carried out in previously sterilized laminar flow (Prolab, São Paulo, Brazil), by removing 3 to 5 colonies from the culture plates, and diluting them in 1 milliliter (mL) of sterile saline. After that, an aliquot of this solution was placed in quartz cuvettes for reading on spectrophotometer (Prolab, São Paulo, Brazil).

At this time, the following parameters have to be reached: 0.135 absorbance at 660 nm (0.5 in the McFarland scale, equivalent to $1.5x10^8$ colony forming unit/mL), in order to obtain 10^8 colony forming unit (CFU) of MRSA. The suspension was seeded on the surface of the Brain Heart Infusion agar (BHI, pH 7.4, HIMEDIA).

Resveratrol Photoactivation

The resveratrol (PharmaNostra, Campinas, São Paulo) was obtained from Anísio Teixeira *Campus*, Multidisciplinary Institute in Health, Federal University of Bahia. For *in vitro* and *in vivo* antimicrobial activity assays, the compound was solubilized in propylene glycol in a sterile environment, and utilized at a concentration of 2 mg/mL.

Resveratrol photoactivation was performed through incident light by a prototype device number 1.012960-3 (MM Optics, São Carlos, São Paulo, Brazil) that have five blue LEDs with a wavelength of 450 ± 20 nm. Irradiation procedure was carried out for 5 minutes, with power density of 75 mW/cm^2, for a total fluence delivered of 22.5 J/cm^2.

Experimental design *in vivo*

Balb/c mice aging from six to eight weeks were acquired from the UFBA-IMS/CAT facilities. The animals were maintained under controlled conditions of temperature, free access to water and food. All procedures involving animals were approved by the Ethics Committee on Animal Use, under protocol number 042/2017.

The intradermal infection protocol followed the literature, with adaptations [12,7,2]. Therefore, mice were infected intradermally in the left ear with the amount of 10^7 CFU of MRSA, 24 hours before treatment. The bacterium was inoculated into the mouse dermis using 29G needles (Farmadelivery, São Paulo, Brazil) in a 10 microliters volume of sterile propylene glycol solution.

Thus, the groups were formed as follows: animals treated with Propylene Glycol (Control, group 1), animals infected with 10^7 CFU of MRSA (MRSA, group 2), animals infected with 10^7 CFU of MRSA and soon thereafter 10 microliters of non-photoactivated RSV (MRSA + RSV, Group 3), and finally, animals infected with 10^7 CFU of MRSA and then 10 microliters of photoactivated RSV (MRSA + RSV + Blue LED, group 4), (n = 6 / group / time). Prior to irradiation with the blue LED light the animals were in a dark environment for 30 minutes [13].

Animals' euthanasia occurred 24 hours after the treatment and was carried out by inoculation of ketamine (Vetnil, São Paulo, Brasil) and xylazine (Vetnil, São Paulo, Brasil), at dosages of 400 mg/kg and 40 mg/kg respectively, intraperitoneally.

Obtaining the draining lymph node

After euthanasia, the clinical specimens were collected. The draining lymph node was removed, and macerated in 1mL of sterile saline (Vitaesaude, São Paulo, Brazil). This material was applied in determination of bacterial load and cytokine production.

Following the maceration, 5 µL were cultured on BHI plates and incubated for 24 hours at 37°C. The technique used for sowing was the pour plate, thus facilitating the number of colonies quantification originated after the culture time. CFU quantification was done after 24 and 48 hours of culture, with the aid of a colony counter (*CP- 600 Plus, Phoenix*). In addition, in the remaining macerate, cytokine production was assessed by ELISA (Enzyme-Linked Immunosorbent Assay).

Determination of cytokines

A 96-well ELISA plate was sensitized with 9.6 mL of capture antibody (100 µL/ well) specific for a given cytokine, which could be TNF-α, IL-1β, IL-17A, or IL-10, incubated overnight at 4°C. All procedures were followed according to manufacturer's recommendations.

Evaluation of Myeloperoxidase and E-cadherin expression

Tissue sections (4- to 5-µm thick) were cut from formalin-fixed (Loja Agropecuária, São Paulo, Brazil) paraffin-embedded (Parafina Santa Cruz, São Paulo, Brazil) ears. Sections were deparaffinized and rehydrated by diving in a graded series of ethanol (Prolab, São Paulo, Brazil) and distilled water. For myeloperoxidase immunohistochemistry, the antigen was retrieved by heating the slides in a pressure cooker in Tris-buffered saline with 0.075% Tween-20 (pH 7.6) for 10 minutes. Endogenous peroxidase activity was quenched by incubation in 0.3% v/v H_2O_2 in methanol for 20 minutes at room temperature. Sections were incubated at room temperature for 30 minutes with polyclonal rabbit anti-human myeloperoxidase antibody diluted 1:1500.

For E-cadherin, a streptavidin-biotin immunoperoxidase complex procedure was utilized for staining. Formalin-fixed, paraffin-embedded samples (4 µm thick) were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol (Prolab, São Paulo, Brazil) for 30 minutes. Antigen retrieval was carried out by microwave treatment in sodium citrate buffer (0.01 M, pH 6.0) for 10 minutes. Incubation with primary antibody was performed for E-cadherin (clone M3612, diluted 1:400; DAKO) for 2 hours, followed by incubation with biotinylated secondary antibody for 30 minutes at room temperature. Slides were developed with diaminobenzidine (Sigma, USA). Finally, sections were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Pictures from each animal sample were taken using optical microscopic getIT™ coupled to a camera device (version 5.2, Olympus Soft Imaging Solutions, Münster, NRW, Rhine-Westphali, Germany). Then, the labeled cells in the immunohistochemistry were counted in each field with aid of Image J software (version 1.50b, National Health Institute, USA).

Statistical analysis

Considering the small numbers of animals per group (n = 6), it was applied the normality test of *Kolmogorov-Smirnov*, by which it was verified that the data did not pass normality test. Therefore, experiments statistical analyses were performed using the *Kruskal-Wallis* test through GraphPad Prism® software (version 5.0, GraphPad Program Inc., San Diego, CA, USA), and *Dunn´*s as *post-test*. For other outcomes, *Mann-Whitney* test was applied in order to make comparisons between the groups. Statistical differences were considered significant at p value <0.05.

RESULTS

Photoactivated Resveratrol promotes an increase in the myeloperoxidase expression in the ear dermis

Figure 1 shows that after photoactivation, resveratrol was able to increase MPO and E-cadherin expressions in the mouse ears treated with the activated compound. It can also be verified that there is no raised expression when the light stimulus does not occur. Thus, a higher influx of MPO producing cells was observed in the presence of the treatment with photoactivated resveratrol.

Figure 1: Determination of Myeloperoxidase and E-cadherin expression - The animals' ears were cut, embedded in paraffin, histological sections were made, the immunohistochemical technique for the expression of Myeloperoxidase (**A**; * p = 0,0352) and E-cadherin (**B**; * p = 0,0436) were proceeded (n = 6).

Mice treated with photoactivated RSV present reduced bacterial load in the draining lymph node

Bacteria traffic from the ear to the lymph node was lower in the animals that were treated with photoactivated RSV (Figure 2) at the evaluated culture times of the macerate

supernatant in the draining lymph node. In this context, bacterial clearance in the ear of animals was higher in animals treated with antimicrobial photodynamic therapy.

Figure 2 : Determination of bacterial load in the draining lymph node - The animals were euthanized 24 hours after the challenge. In sterile environment, the draining lymph node was removed, macerated in 1 mL sterile saline solution, and 5 µL were cultured in BHI medium. CFUs were quantified after 24 hours (* p = 0,031 ; ** p = 0,0022) and 48 hours (* p = 0,026 ; ** p = 0,0026) of culture. (n = 6); (BHI = Brain Heart Infusion); (CFU: Colony Forming Unit)).

The draining lymph node of the animals treated with photoactivated RSV controls inflammation through the IL-10 production

After 24 hours of administration, the cytokine pattern in animals treated with photoactivated RSV was altered (figure 3). It was possible to observe a reduction in pro-inflammatory cytokine production, such as TNF-α (Fig 3A) and neutrophil chemoattractant cytokines as IL-17A (fig 3E). However, a higher level of IL-10 production (fig. 3F), an inflammatory modulating cytokine, was observed. No significant differences were detected for the other assessed cytokines.

Figure 3: Quantification of cytokines in the inflammatory environment (pg/mL) - In a sterile environment, the draining lymph node was removed, macerated in 1 mL of sterile saline, and evaluated by ELISA for the presence of TNF-α (**A**; * p = 0,0319), IL-1β (**B**), IL-4 (**C**), IL-5 (**D**), IL-17A (**E**; * p =0,0428) and IL-10 (**F**; * p = 0,0392). (n = 6); (BHI = *brain heart infusion*; MRSA: Methicillin-resistant *Staphylococcus aureus*).

DISCUSSION

This work presented the resveratrol associated with photodynamic therapy in the treatment of intradermal *S. aureus* infection model, being a pilot study. A greater bacterial clearance was observed in the animals treated with the photoactivated compound, with an increase in MPO and E-cadherin expression in the ear dermis, besides reduction of bacterial discharge to the draining lymph node, and an augment in IL-10 expression.

Even if the light absorption peak of resveratrol is at 306 nm, in literature are presented works that demonstrate that it interacts with longer wavelengths, like those used in our study [14-16]. Therefore, papers already report that after photoactivation, RSV produces singlet oxygen [17], a potent antimicrobial agent. Here, we are reporting yet another important effect

of photoactivated RSV: MPO production. These data together may help elucidate the complete new photosensitizer mechanism of action. MPO is an enzyme that plays a fundamental role in bactericidal mechanisms, being distributed particularly in neutrophils. Moreover, it is directly related to oxygen-dependent bactericidal mechanisms, being a catalyst for the production of highly reactive radicals, and therefore, is an important defense mechanism against pathogenic microorganisms [18].

After bacterial phagocytosis, MPO catalyzes a reaction with chloride and hydrogen peroxide to produce hypochlorous acid, a potent oxidant and microbicide, as well as byproducts such as chloramines, hydroxyl radical, and singlet oxygen [19,20]. Here, we believe that bacterial clearance (Fig.2) occurred in two ways. First by photoactivated RSV itself generating reactive oxygen species [17], and the second, by increasing MPO expression (fig. 1A) in animals treated with the photoactivated compound.

Literature corroborates with the aforementioned statement, since the pharmacological inhibition of MPO leads to a decrease in the neutrophil capacity to kill bacteria [21]. Neutrophils from patients with MPO deficiency retained microbicidal activity against various bacterial pathogens, although at a lower level compared to neutrophils from normal donors, implying a reduced response [22].

Besides that, when analyzing E-cadherin expression (Fig. 1B), we noticed that animals treated with activated RSV had higher levels of this glycoprotein. Studies have shown that *S. aureus* is able to reduce the E-cadherin expression by releasing α-hemolysin which in its turn intensifies metalloproteases activity, resulting in cleavage of E-cadherin [23]. The cleavage is associated with disruption of epithelial barrier function, contributing to pathogenesis by facilitating bacterial penetration into infected tissue. Here we observed that the photoactivated RSV prevented this possible mechanism of bacterial virulence. However, we do not have enough information to assure such proposes, so that we encourage other authors to conduct researches on this subject for data collaboration and elucidation.

What can be asserted is that fewer cells migrated to the draining lymph node in the animals treated with the photoactivated compound, which resulted in a lower hyperplasia of this organ. Thus, it is possible to say that the resolution of inflammation occurred mostly in the animal's own ear, where the highest proportion of bacterial clearance. Due to the thinness of mice tissue in the area of inoculation, it is supposed that the surface layers of skin have not acted as a screen, so that almost all the irradiated light has hit resveratrol. This data is strengthened when we look at the bacterial amount in the draining lymph node of the groups studied here, in which the animals not treated with the photoactivated RSV had a higher

bacterial load. Moreover, of these untreated animals there was lower E-cadherin expression, therefore, there was a higher cell detachment and migration to the draining lymph nodes.

Also, in the lymph nodes of animals non-treated with the photoactivated RSV, a greater expression of pro-inflammatory cytokines such as TNF-α and IL-17A was observed (Fig. 3A and 3E). This fact demonstrates that inflammation in these groups is still happening, as TNF-α is produced especially by activated macrophages or monocytes [24]. Other cell types such as natural killer cells, mast cells, endothelial cells, keratinocytes, and smooth muscle cells, are also able to produce TNF-α [25-27].

This inflammatory cytokine is responsible for a variety of functions in the immune system, such as elevated expression of adhesion molecules in endothelial cells, leading to accumulation of leukocytes at the site of inflammation, polymorphonuclear activation, stimulation of other cytokines production such as interleukins 1, 6, and TNF-α itself by several immune system cells, among others [28-30].

Besides TNF-α, an augment in IL-17A in untreated animals is important since it promotes neutrophil chemotaxis for cells like $CD4^+$ T, $CD8^+$ T, neutrophils, eosinophils, among others. IL-17RA receptor activation often results in the induction of other proinflammatory cytokines through the NF-κB activation (nuclear factor kappa B) [31]**.** Therefore, we can see that the inflammatory process is still occurring in animals not treated with photoactivated RSV.

Otherwise, when analyzing the cytokines expression in the lymph nodes of the animals treated with photoactivated RSV, we did not observe the TNF-α and IL-17A expression in similar levels to the other groups, but in smaller proportions. Moreover, IL-10 expression occurred at levels much higher, this data is relevant since it demonstrates that was happening modulation of the inflammation in these animals [32,33]. As IL-10 is a cytokine with broad anti-inflammatory properties, which include inactivation of macrophages, dendritic cells, decreased expression of proinflammatory cytokines (including IL-1α and β, IL-6, IL-12, IL -18 and TNF-α) and chemokines (MCP-1, MCP-5, RANTES, IL-8, IP-10 and MIP-2) [34,35]. Thus, through IL-10 quantification in mice treated with the photoactivated RSV, we can verify a resolution of the inflammation in these animals**.**

CONCLUSION

Despite many data remain obscure, our work is pioneer and we exhibit promising results regarding the use of RSV in photodynamic therapy. Given the increasing resistance

to antimicrobials used in conventional therapies, promoting complementary forms of treatment is extremely important. Here we show that RSV is an important promising agent when associated with photodynamic therapy in intradermal infections with the induction of MPO expression, greater bacterial clearance and infection control by IL-10 production. However, further studies should be conducted in order to elucidate the mechanism of action of this activated compound and to corroborate with our data.

Compliance with ethical standards: The study followed the guidelines of the Animal Experimentation Code of Ethics of the Brazilian College of Animal Experimentation and was approved by the Ethics Committee on the use of animals (CEUA, Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira) at which the studies were conducted, under protocol number 042/2017.

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

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4. RESUMO DOS RESULTADOS E DISCUSSÃO GERAL

4.1. Experimento I – Eficácia do extrato fotoativado de *Myrciaria cauliflora* contra a infecção por *Staphylococcus aureus* - um estudo piloto

4.1.1. A atividade antimicrobiana da *M. cauliflora* é intensificada pela estimulação com luz LED azul

O desenvolvimento de novos fotossensibilizadores é essencial devido ao aumento da resistência bacteriana aos principais antimicrobianos usados classicamente. Assim, a expansão dos recursos de tratamento dessas infecções para além dos medicamentos tradicionais vem ganhando mais espaço. Portanto, a aplicação da terapia fotodinâmica juntamente com o uso de fotossensibilizadores tem sido extensivamente estudada. Este trabalho apresenta o primeiro relato literário da associação entre o extrato de *M. cauliflora* e a terapia fotodinâmica.

Após o ensaio *in vitro*, foi observado que a fotoativação do extrato aumentou sua atividade antimicrobiana (Fig. 1).

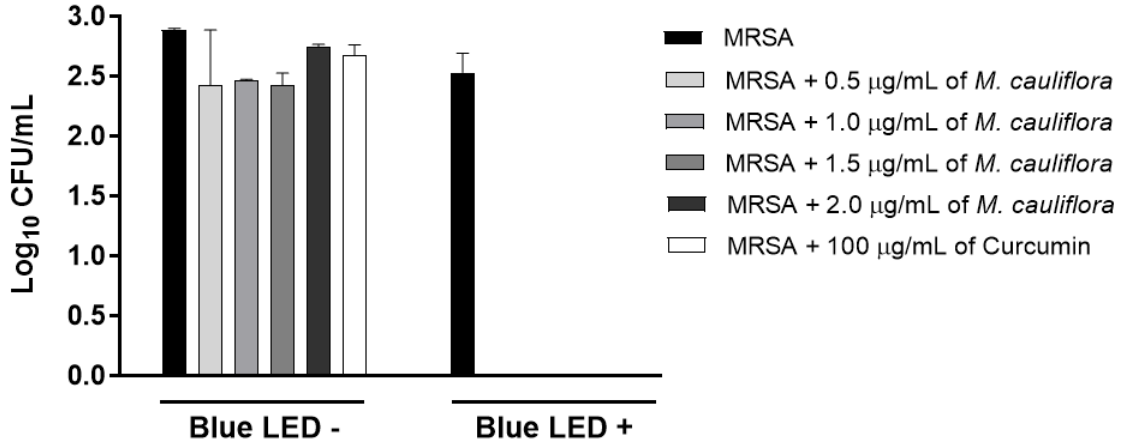


Figura 1: Ação microbicida do extrato de *M. cauliflora* contra MRSA (Log_{10} UFC/mL) - Em uma placa de 24 poços, 10^7 UFC de MRSA foram plaqueados em cada poço em 1 mL de caldo BHI. Posteriormente, diferentes concentrações de extrato de *M. cauciflora* (0.5 μg/mL, 1 μg/mL, 1.5 μg/mL e 2 μg/mL) foram testados, além disso, a Curcumina (100 μg/mL) foi utilizada como fotossensibilizador padrão para comparação de resultados. Somados a estes grupos supracitados, também houve grupo contendo apenas MRSA. (n=4); (BHI = *Brain Heart Infusion*; MRSA: Methicillin-resistant *Staphylococcus aureus*).

A obtenção de compostos de origem vegetal é importante, pois o desenvolvimento de um fotossensibilizador ideal requer algumas características, como fácil extração, com síntese rápida, alto rendimento, entre outras, pois quanto mais caro o processo de

descoberta e aquisição do ativo químico, maior o valor do produto final (YOO; HA, 2012). Deste modo, o extrato de *M. cauliflora* apresenta grandes vantagens, pois é uma espécie muito popular no Brasil sendo fácil sua aquisição, além de o preparo do extrato ser extremamente simples e requerer poucos recursos financeiros.

Além disso, a terapia fotodinâmica está tipicamente associada a corantes naturais, como a curcumina, com excelentes resultados em estudos que avaliam a atividade antimicrobiana, especialmente contra o patógeno aqui apresentado (RIBEIRO, A. P. et al., 2013; YE; LI; FANG, 2014; ALMEIDA et al., 2017). Esta planta foi escolhida para esta pesquisa devido ao fato de ser também um corante natural bem conhecido e seu potencial antimicrobiano já ter sido mencionado na literatura (BALDIN et al., 2016; HACKE et al., 2016).

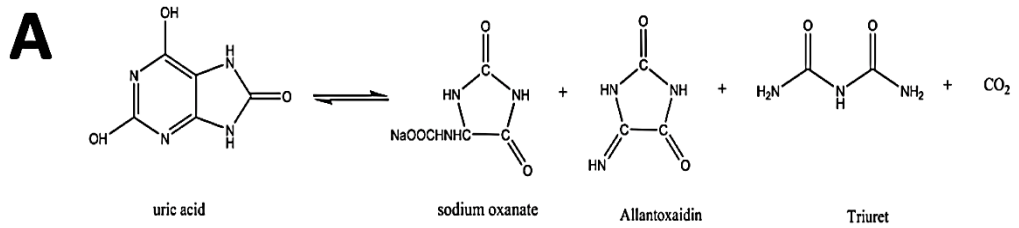
No extrato bruto de *M. cauliflora* podem ser encontrados taninos hidrolisáveis, como pedunculagina, castalagina e vescalagina. Além destes, vários compostos fenólicos como ácido gálico, cauliflorina, cianidin-3-O-glucósido, entre outros. Porém, ainda devem ser realizados estudos com o objetivo de determinar quais compostos do extrato bruto são responsáveis pela ação antimicrobiana após a fotoativação com luz azul LED, além de determinar ainda, se esses compostos possuem ação isolada ou sinérgica.

4.1.2. *M. cauliflora* fotoativada promove a oxidação do ácido úrico

Com o objetivo de avaliar o possível mecanismo de ação antimicrobiana do extrato fotoativado, foi realizado o experimento em associação com ácido úrico (Fig. 2). Este, foi selecionado por suas características de alta reatividade ao oxigênio *singlete* e oxirradicais, estabilidade *in vitro* à autoxidação, baixa absorção de luz e boa estabilidade fotoquímica na região de interesse (AMES et al., 1981; DUNLAP et al., 1998; BREGNHOJ et al., 2018). Neste ensaio, a taxa de produção das espécies reativas de oxigênio (EROs) foi determinada por espectrofotometria, avaliando a cinética do decaimento da absorção de raios ultravioleta (UV) do ácido úrico a 293 nm. Sendo assim, foi detectado que o extrato de *M. cauliflora* possui como possível mecanismo de ação a produção de oxigênio *singlete*, como mostra a Fig. 2.

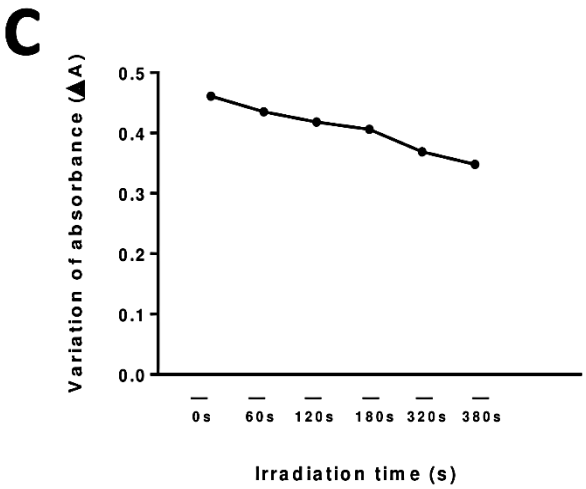
A produção de oxigênio *singlete* por um fotossensibilizador é importante, pois não há resistência microbiana a esse composto, portanto, não importa se a cepa é resistente a uma ou várias classes de agentes antibacterianos, pois o fotossensibilizador é capturado

pelo microrganismo. Além disso, a reatividade das EROs com moléculas orgânicas não é específica, qualquer macromolécula dentro da célula pode ser um alvo potencial para terapia fotodinâmica. Aqui, foi utilizada uma cepa resistente a várias classes de antimicrobianos, e observou-se que *M. cauliflora* fotoativada reduziu o crescimento dessa bactéria *in vitro* (Fig. 1) e apresentou ação bacteriostática quando utilizada em modelo vivo (Fig. 3).



B

Irradiation time	Uric acid
	293 nm
0 second	0.461 A
60 seconds	0.435 A
120 seconds	0.418 A
180 seconds	0.406 A
320 seconds	0.369 A
380 seconds	0.348 A



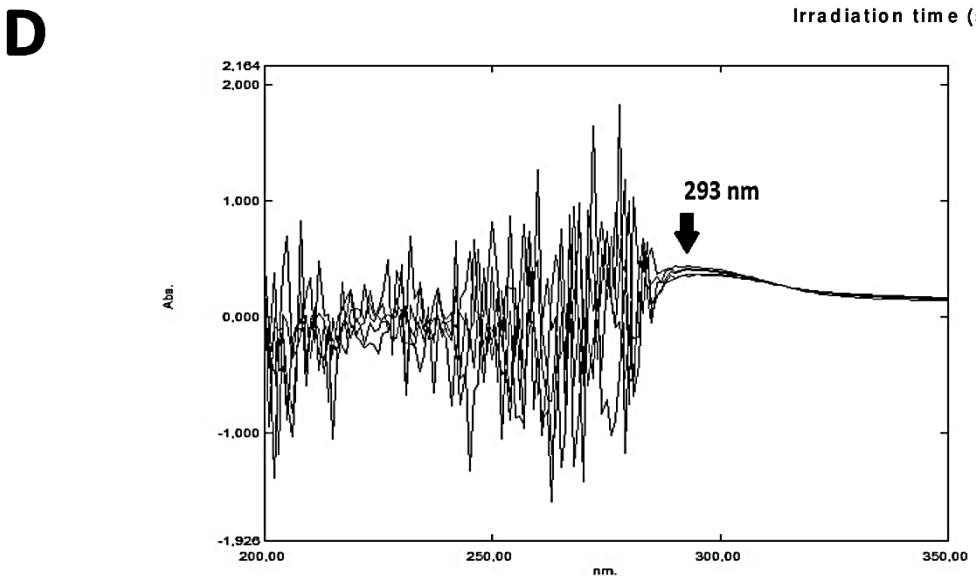


Figura 2: Oxidação do ácido úrico - Uma solução de *M. cauliflora* na concentração de 0,001 mg/mL e ácido úrico 30 µg/mL foi preparada para análise. O pico de absorção do ácido úrico é em 293 nm, quando

é oxidado (**A**) a intensidade da banda é diminuída. Após o protocolo de fotoativação, houve redução da absorbância da solução em função do tempo (**B**). A variação das absorbâncias das duas soluções em função do tempo (**C**) foi calculada. Pela observação do comportamento espectral, é possível notar a diminuição da banda de ácido úrico em 293 nm (**D**).

4.1.3. *M. cauliflora* aumenta a ação antimicrobiana *in vivo* quando fotoativada

Tendo em vista o aumento da atividade antimicrobiana *in vitro*, foram realizados testes para verificar se ocorreria também *in vivo*. Como resultado, os animais tratados exibiram uma redução na carga bacteriana no linfonodo drenante (Fig. 3).

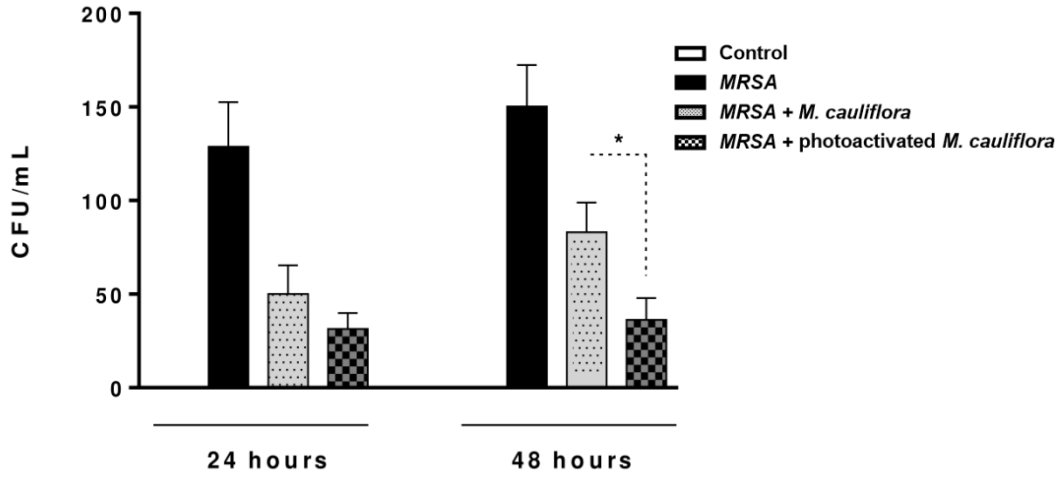


Figura 3: Determinação da carga bacteriana no linfonodo drenante - Os animais foram eutanasiados 24 horas após o desafio. Em ambiente estéril, o linfonodo drenante foi removido, macerado em 1 mL de solução salina estéril e 5 µL foram cultivados em meio BHI. As UFCs foram quantificadas após 24 e 48 h de cultura. (n=6); (BHI=*Brain Heart Infusion*); (CFU: *Colony Forming Unit*) (*$p < 0.05$).

Para este modelo animal, a infecção intradérmica foi escolhida como via, sendo que este modelo baseia-se no fato de que *S. aureus* é o principal agente de infecções de pele, com relatos na literatura como impetigo, síndrome da pele escaldada, furúnculos, entre outros (BASSETTI; CARNELUTTI; RIGHI, 2017; CLAEYS et al., 2018; PIMENTEL DE ARAUJO et al., 2018). Além disso, sabe-se que a terapia fotodinâmica é uma técnica direcionada a áreas superficiais da pele e mucosas, pois a luz é de baixa intensidade, apresentando baixo potencial de penetração através da pele. Após vários testes, observou-se que o volume ideal para aplicação nas orelhas dos animais foi de 10 µL, pois não extravasou e é possível notar o líquido no local da aplicação, demonstrando

que o procedimento foi realizado corretamente. Além disso, a literatura mostra trabalhos que aplicaram modelo de infecção semelhante ao usado aqui, também aplicaram o mesmo volume de suas substâncias (CORTES et al., 2010; ALMEIDA et al., 2017).

4.1.4. Fotoativação do extrato de *M. cauliflora* promove o aumento da liberação de TNF-α no linfonodo drenante

Animais tratados com o extrato fotoativado apresentaram maior produção de TNF-α e diminuição da quantidade de IL-10, além disso, também houve declínio em todas as outras citocinas avaliadas (Fig. 4).

Outro fato que deve ser destacado é que, como a produção de citocinas é maior em animais infectados que não foram submetidos ao tratamento de fotoativação com o extrato, aparentemente eles produzem mais IL-10 na tentativa de equilibrar o processo inflamatório (Fig. 4B). A IL-10 é um potente mediador anti-inflamatório que desempenha um papel crucial na limitação da imunopatologia do hospedeiro durante infecções bacterianas, controlando a ativação efetiva das células T (LEECH et al., 2017). Foi demonstrado anteriormente que *o MRSA* modula a resposta da IL-10 como um mecanismo de evasão imune em modelos de infecção. Nossos resultados demonstram que a indução de IL-10 tem uma grande influência no resultado da infecção por *S. aureus* (WANG; RODERIQUEZ; NORCROSS, 2012; PARCINA et al., 2013; LEECH et al., 2017). Um excesso na expressão da IL-10, por outro lado, pode suprimir as respostas protetoras facilitando a persistência das bactérias (LEECH et al., 2017).

Estudos recentes demonstraram que o recrutamento de neutrófilos na pele de animais infectados com *S. aureus* depende da ação do TNF-α (LIU, C. et al., 2018). Aqui, os animais tratados com o extrato fotoativado apresentaram maior expressão de TNF-α (Fig. 4F) junto com um número superior de neutrófilos no ambiente inflamatório (Fig. 5A). Os resultados revelaram que os neutrófilos desempenham uma função crítica na contenção de infecções cutâneas por *S. aureus* neste modelo. Além disso, na literatura e também neste estudo, o aumento do TNF-α ocorreu no linfonodo drenante, sendo este órgão fundamental para o controle da infecção pela produção de citocinas. A depleção de neutrófilos *in vivo* revelou que essas células desempenham um papel protetor na prevenção da disseminação bacteriana e infecção invasiva fatal (PRABHAKARA et al., 2013).

Outro achado relevante é a avaliação da participação da IL-17 contra a infecção por *S. aureus*. Houve um aumento na expressão desta citocina quando os animais foram tratados com o extrato (Fig. 4A). Assim, este é o primeiro relato sobre a indução da produção de IL-17A por *M. cauliflora*. Existem poucos relatos que abordam a importância do perfil da IL-17A após processos infecciosos por MRSA (CHO et al., 2010; NAKAGAWA et al., 2017; DOS SANTOS et al., 2018). Foi demonstrado que camundongos *knockout* para IL-17 são incapazes de eliminar efetivamente *S. aureus* (ARCHER; HARRO; SHIRTLIFF, 2013). Portanto, as respostas imunes associadas ao Th17 podem ser um alvo estratégico para eliminar infecções persistentes em humanos. Muitos estudos descreveram que essa citocina é produzida pelos linfócitos T, mas a maior parte desse componente nos processos inflamatórios é secretada pelas células da imunidade inata (KORN et al., 2009; SOUZA et al., 2017).

Assim como para IL-17, houve também uma maior produção de IL-1 em animais infectados tratados apenas com o extrato (Fig. 4E). Monócitos e macrófagos são a principal fonte de IL-1. Dentre as atividades biológicas primordiais, esta citocina é responsável pela estimulação das células $CD4^+$ para secretar IL-2 e produzir receptores para IL-2, proliferação e ativação celular, intensificando as atividades quimiotáticas e fagocíticas (ARANGO DUQUE; DESCOTEAUX, 2014). No entanto, sabe-se que esta bactéria tem a capacidade de evitar esses mecanismos de resposta imune inata, fato que explica porque um declínio na carga bacteriana nesse grupo não ocorreu significativamente (RYU et al., 2014). Neste trabalho, observou-se que apenas a elevação da IL-1, sem oxigênio *singlete*, TNF-α e MPO, não foi suficiente para causar redução da carga bacteriana.

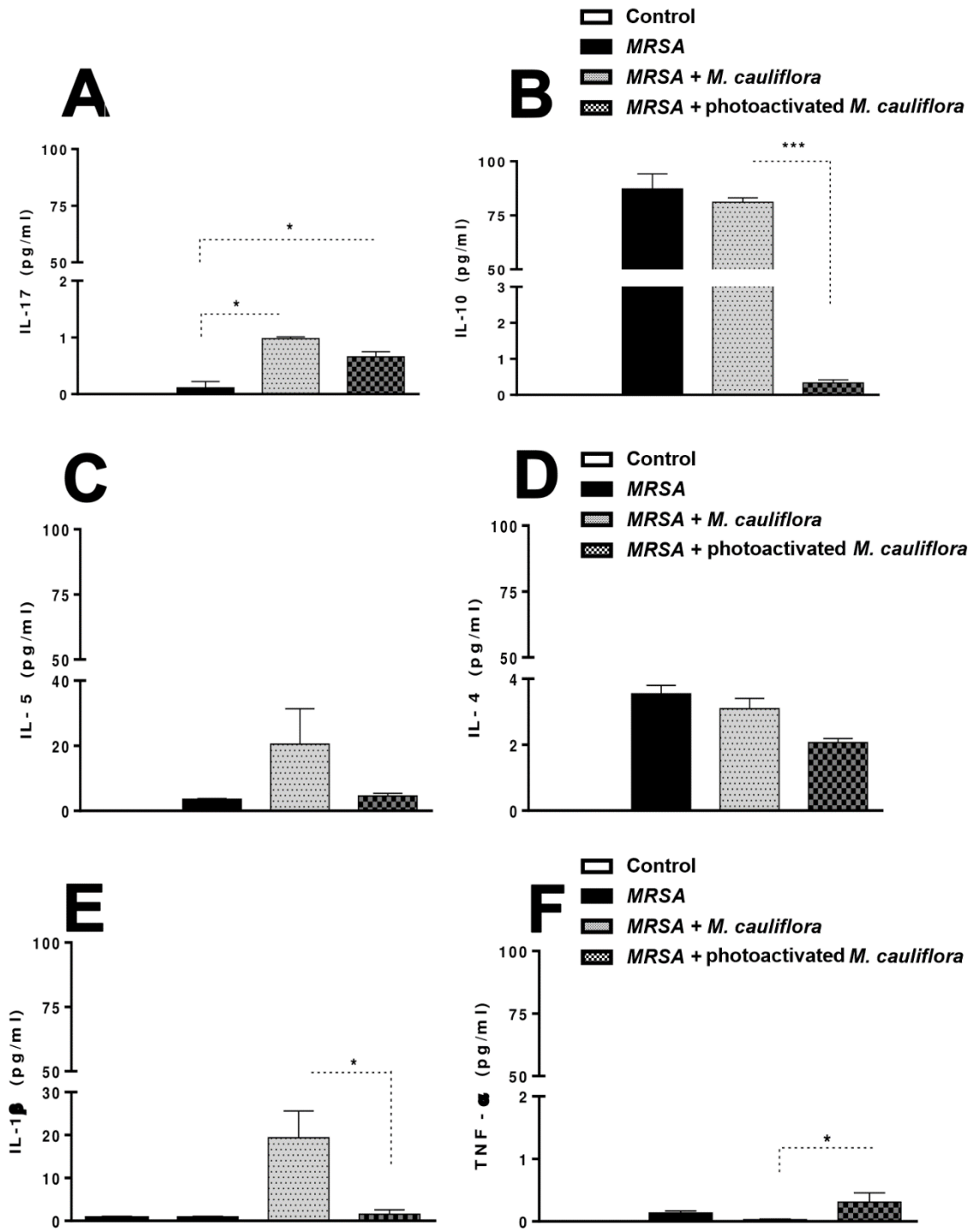


Figura 4: Quantificação de citocinas no linfonodo drenante (pg/mL) - Os animais foram eutanasiados 24 horas após o desafio. Em ambiente estéril, o linfonodo drenante foi removido, macerado em 1 mL de solução salina estéril e avaliado por ELISA para a presença de IL-17A (A), IL-10 (B), IL-5 (C), IL-4 (D) IL-1β (E) e TNF-α (F). (n=6); (ELISA: *Enzyme Linked Immuno Sorbent Assay*); (IL: *Interleukin*); (*$p <$ 0,05, **$p < 0,01$).

4.1.5. Fotoativação do da *M. cauliflora* aumenta a expressão da Mieloperoxidase

Ainda na determinação do mecanismo de ação bacteriostática do extrato fotoativado de *M. cauliflora*, observou-se que, além da ação pela indução de oxigênio *singlete,* houve um incremento na expressão da MPO (Fig. 5B). Esta enzima é uma hemoproteína lisossômica localizada em grânulos de leucócitos polimorfonucleares, que na presença de peróxido de hidrogênio (H_2O_2) e íons halogênios constituem um sistema bactericida potente (HAMPTON; KETTLE; WINTERBOURN, 1996).

Durante o *burs*t oxidativo a atuação antimicrobiana do neutrófilo ocorre graças a formação de espécies reativas de oxigênio dependentes da participação de enzimas oxidativas proteolíticas. A MPO possui ação catalítica na formação de radicais altamente reativos, como o ácido hipocloroso, a partir de íons H_2O_2 e halogênio, que desempenham um papel importante nos mecanismos de morte intracelular de bactérias, possivelmente através da halogenação de proteínas (PARKER et al., 2012). A literatura corrobora com a afirmação acima mencionada, uma vez que a inibição farmacológica da MPO leva a uma diminuição na capacidade de neutrófilos de eliminar bactérias (KLEBANOFF et al., 2013). Neutrófilos de pacientes com deficiência de MPO se mostraram capazes de manter atividade microbicida contra vários patógenos bacterianos, embora em um nível mais baixo em comparação aos neutrófilos de doadores normais, implicando uma resposta reduzida (DOMINGUES-FERREIRA et al., 2017).

Alguns autores já relataram o importante papel das enzimas superóxido dismutase e mieloperoxidase no processo oxidativo da morte de *S. aureus* nos neutrófilos e concluíram que sua principal função é atuar na via dependente da mieloperoxidase. No entanto, existem vários artigos que relatam a capacidade do *S. aureus* de escapar à ação da MPO por muitos mecanismos, como a inibição da ação dessa enzima através da produção de fatores de virulência (DE JONG et al., 2018).

Por outro lado, a presença de neutrófilos no ambiente inflamatório não levará necessariamente à depuração bacteriana. Nesse caso, é necessário que o polimorfonuclear seja modulado para combater a infecção. Assim, os componentes TNF-α e IL-17 são citocinas conhecidas por modular células polimorfonucleares e aumentar seu mecanismo de ação antibacteriana (DAS, S.; KHADER, 2017; GUERRA et al., 2017). Esta característica foi observada neste estudo. O grupo de animais desafiados apenas com a bactéria apresentou maior contagem de neutrófilos (Fig. 5A), no entanto, não obteve

maior depuração do patógeno (Fig. 3), por não produzirem citocinas TNF-α e IL-17 em uma quantidade significativa (Fig. 4A e Fig. 4F). Em relação às demais citocinas analisadas (IL-5 e IL-4), não foram observados resultados significativos.

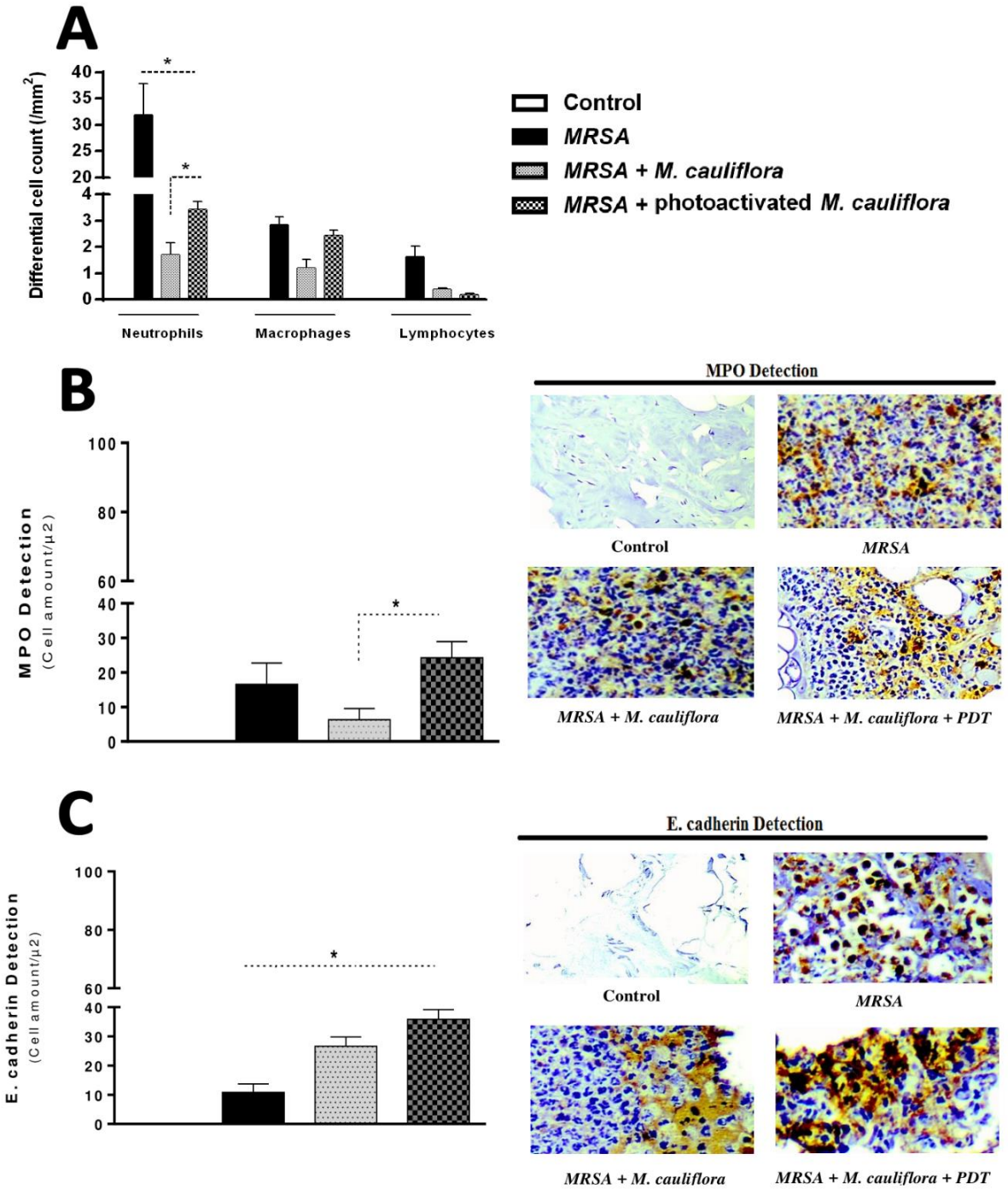


Figura 5: Determinação da expressão de mieloperoxidase e E-caderina - As orelhas dos animais foram coletadas, embebidas em parafina, cortes histológicos foram feitos e a contagem diferencial de leucócitos

no ambiente inflamatório foi realizada (**A**). Além disso, a técnica imunohistoquímica foi utilizada para avaliar a expressão de Mieloperoxidase (**B**) e E-caderina (**C**). (n = 6); (* p <0,05, ** p <0,01).

A partir dos dados *in vitro* e *in vivo*, pode-se lançar uma hipótese que *M. cauliflora* fotoativada promove a redução da quantidade bacteriana na orelha dos animais, fato observado pela manutenção dos níveis de caderina-E nos animais tratados com o composto fotoativado (Fig. 5 C). A E-caderina promove a adesão celular no tecido, portanto, as células apresentadoras de antígenos reduzem a expressão dessa glicoproteína para migrar para o linfonodo drenante. Esse achado ocorreu em todos os animais infectados neste experimento, mas não foi observado nos camundongos tratados com o extrato fotoativado (VAN ROY; BERX, 2008). Em seguida, o manutenção da expressão da caderina-E nos animais tratados com o composto fotoativado pode ser correlacionado à redução da carga bacteriana sendo associada a uma menor necessidade de migração celular para o linfonodo drenante.

4.2. Experimento II – Resveratrol fotoativado contra infecção por *Staphylococcus aureus* em camundongos

4.2.1. Resveratrol fotoativado promove redução do crescimento bacteriano *in vitro*

A figura 6 demonstra que houve maior inibição do crescimento bacteriano nas placas de cultura tratadas com a forma fotoativada do Resveratrol. Além do uso da *M. cauliflora*, este trabalho também traz uma nova proposta para o tratamento dessas infecções usando o Resveratrol ativado com luz azul como alternativa.

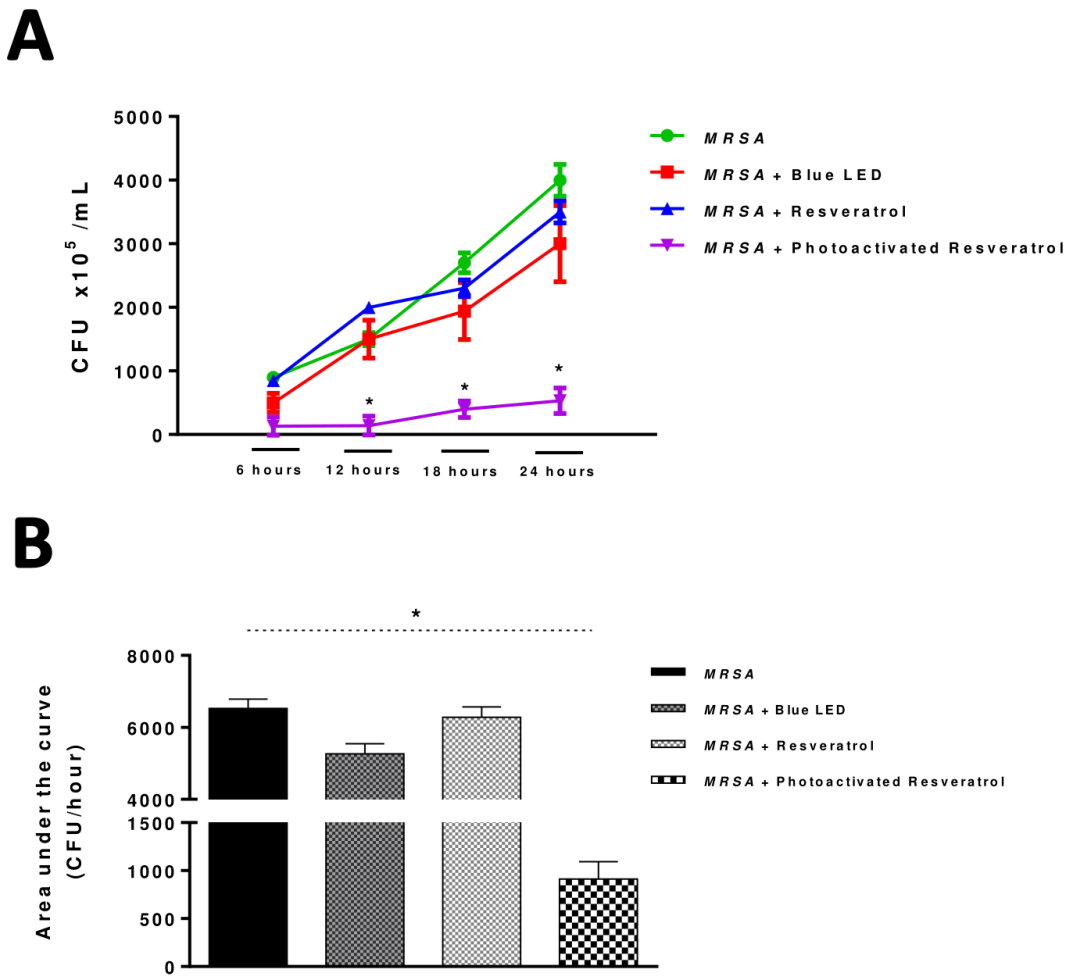


Figura 6: Carga bacteriana de *Staphylococcus aureus* (UFCx10^5 /mL) - Em uma placa de 24 poços, 10^8 UFC de MRSA foram adicionados em 1 mL de caldo BHI. Posteriormente, seis poços foram deixados no escuro e não receberam tratamento (**Grupo 1** - MRSA), seis poços foram tratados apenas com luz LED azul (**Grupo 2 -** MRSA + luz LED azul), seis poços receberam 20 microlitros de Resveratrol e foram mantidos no escuro (**Grupo 3 -** MRSA + Resveratrol), finalmente, seis poços receberam 20 microlitros de Resveratrol e luz LED azul (**Grupo 4 -** MRSA + Resveratrol fotoativado). Após o período de execução do protocolo de fotoativação, cinco microlitros de cada poço foram semeados em uma placa média BHI e as contagens das UFCs foram realizadas após o tempo de 6, 12, 18 e 24 horas (**A**) e a área sob a curva foi medida (**B**). (* p <0.05).

4.2.2. O Resveratrol tem atividade fotodinâmica gerando oxigênio *singlete*

Para analisar o mecanismo de ação antimicrobiana do composto formado após a estimulação do Resveratrol com luz LED azul, uma solução de ácido úrico foi fotoativada juntamente com a solução de Resveratrol. A variação da absorbância do ácido úrico foi observada em função do tempo de irradiação devido à foto-oxidação do ácido úrico via oxigênio *singlete* produzido pelo Resveratrol fotoativado (após fotoativação).

O Resveratrol (3,5,4'-trihidroxiestilbeno) é um polifenol natural com uma estrutura de estilbeno. Na literatura, os autores descrevem que sua estrutura química foi caracterizada no século passado, em componentes isolados da raiz de *Veratrum grandiflorum* (TIMMERS; AUWERX; SCHRAUWEN, 2012; GAMBINI et al., 2015). No entanto, tem sido apresentado em preparações medicinais há mais de 2000 anos. Sua estrutura básica consiste em dois anéis fenólicos unidos por uma ligação dupla de estireno, que forma o 3,5,4 '(Fig. 7B). Essa ligação dupla é responsável pelas formas cis isométrica e trans do Resveratrol. Vale ressaltar que o isômero trans é o mais estável do ponto de vista estérico (GAMBINI et al., 2015). Neste trabalho, usamos o isômero trans.

A literatura atual traz trabalhos que apresentam a fotoestimulação do Resveratrol sob várias fontes de luz diferentes. Quando exposto à luz solar, esse composto sofre um rearranjo molecular cuja estrutura consiste em dois anéis aromáticos fundidos ligados a uma cadeia linear contendo um grupo carbonil conjugado a uma ligação dupla, na qual os autores denominaram a molécula obtida como "resveratrone" (YANG et al., 2012; RODRIGUEZ-CABO et al., 2015). Com luz LED, os autores relataram a glicosilação do Resveratrol. O uso de LEDs azuis como fonte de luz para a biotransformação do Resveratrol melhorou notavelmente o rendimento de seus β-D-glicosídeos e alterou a composição dos produtos, o 3-O-β-D-glucosídeo foi o principal produto (UESUGIA; HAMADA; SHIMODA, 2016).

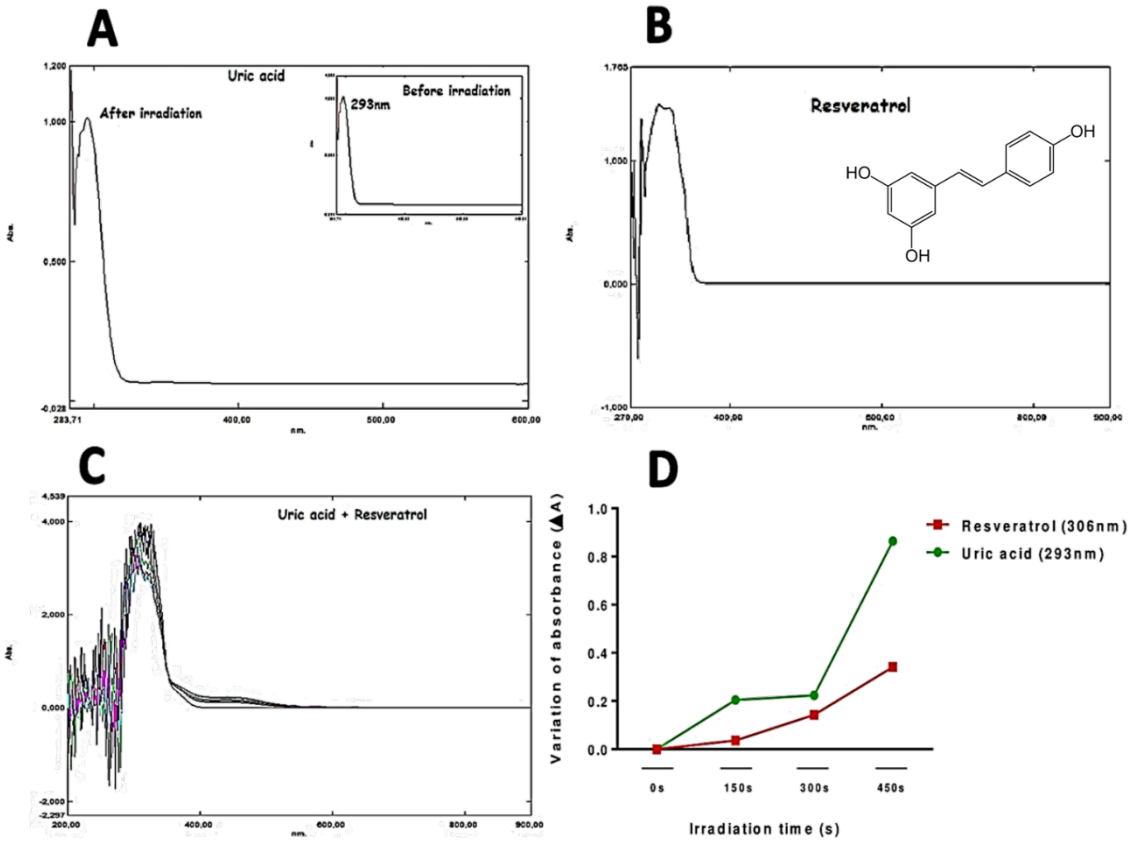


Figura 7: Análise de Espectroscopia UV/Visível de uma solução de Resveratrol e ácido úrico – As soluções de 0,001 mg/mL de Resveratrol e 30 μg/mL de ácido úrico foram preparadas para análise. O ácido úrico não alterou a absorbância após ser submetido ao protocolo de fotoativação (**A**). O Resveratrol usado neste experimento mostrou sua absorbância característica a 306 nm (**B**). Após misturar as soluções de Resveratrol e ácido úrico seguidas do protocolo de fotoativação, a redução da absorbância da solução em função do tempo (**C**) foi observada. Em vista disso, a variação das absorbâncias das duas soluções em função do tempo (**D**) foi calculada.

Uma vez que se trata de um estudo piloto, não é possível afirmar que o resveratrone ou o 3-O-β-D-glucosídeo foi formado em nosso experimento, no entanto, pode-se afirmar que o Resveratrol teve a capacidade de interagir com a luz azul do LED, modificando sua estrutura (Fig. 8) e exibindo ação antimicrobiana (Fig. 6 e Fig. 11).

Como citado anteriormente, o ácido úrico é um sensor de oxigênio *singlete* (FISCHER et al., 1998; IIDA et al., 2017). A Figura 7 mostra seu espectro de absorção óptica e foto-oxidação. Inicialmente, a estimulação leve do ácido úrico foi realizada na faixa de comprimento de onda estudada para este estudo. Portanto, como observado na Figura 7A, a faixa de comprimento de onda usada aqui não foi capaz de alterar a

absorvância do composto, o que garante que a interação com a luz seja restrita ao Resveratrol nos experimentos de determinação da atividade fotodinâmica

A Figura 7D mostra a variação da absorbância das bandas de ácido úrico (293 nm) e Resveratrol (306 nm) em função do tempo de irradiação. A reação de foto-oxidação do ácido úrico via oxigênio *singlete* é bem conhecida e leva à formação de produtos como triúria, alantoxaidina, íon oxanato e CO_2 (Fig. 2A), ocorrendo a supressão do oxigênio *singlete* pelo ácido úrico com a captura do estado tripleto energia da molécula de Resveratrol no estado excitado pela molécula de ácido úrico. Além disso, esse átomo de oxigênio altamente reativo inicia reações oxidativas no ambiente próximo, como a parede celular bacteriana, membranas lipídicas, enzimas ou ácidos nucleicos (MAISCH et al., 2007).

Quando associamos o Resveratrol ao ácido úrico com a fotoativação subsequente, notamos uma redução no perfil de absorvância das duas moléculas (Fig 7 D). A literatura classifica esse efeito como fotodegradação, sendo uma característica diretamente relacionada à dose terapêutica do composto com atividade fotodinâmica (AL-OMARI; ALI, 2009). Nesse sentido, aqui foi apresentado um decaimento do sinal característico da banda do ácido úrico a 293 nm, indicando que a reação de decomposição dessa molécula ocorre através do oxigênio *singlete* formado pelo Resveratrol excitado.

Esses resultados corroboram a teoria de que a ação antimicrobiana do Resveratrol fotoestimulado ocorre pela geração de espécies reativas de oxigênio e o oxigênio *singlete* tem sido considerado o principal responsável pelo efeito antimicrobiano do aPDT. O oxigênio *singlete* reage rapidamente com uma ampla variedade de macromoléculas celulares, incluindo proteínas, lipídios, DNA e RNA, e assim outras substâncias reativas, incluindo peróxidos orgânicos e sulfóxidos, são formadas (GLAESER et al., 2011). Consequentemente, o fotosensibilizador deve estar em contato com a célula-alvo de forma que o oxigênio *singlete* gerado possa exercer seu efeito antimicrobiano (DAHL; MIDDEN; HARTMAN, 1987; RAGAS et al., 2013; JIANG; LI; CUI, 2017).

Entre as grandes dificuldades no desenvolvimento de um tratamento eficaz contra *S. aureus*, está o fato patógeno fazer parte da microbiota humana além do entendimento de qual tipo de resposta inflamatória é eficaz na depuração desse microrganismo. Vários estudos na tentativa de desenvolver imunoproteção falharam no desenvolvimento de um método profilático contra infecções por esse patógeno (COGEN; NIZET; GALLO, 2008; GIERSING et al., 2016; KANE; CAROTHERS; LEE, 2016). Assim, trabalhos que buscam formas complementares de terapias antimicrobianas tradicionais apresentam

resultados promissores. A aPDT tem sido usada para combater infecções estafilocócicas há muito tempo (KASHIWABUCHI et al., 2012; FU; FANG; YAO, 2013; MORIMOTO et al., 2014; ROSA et al., 2015).

No entanto, mesmo ocorrendo poucos relatos de resistência ao aPDT, é possível que ocorra o desenvolvimento de cepas resistentes. Por se tratar de uma nova terapia, com expansão em seu uso, não se pode garantir que a resistência não ocorrerá (PARK et al., 2013), a aPDT tem um tempo de meia-vida curto e atua com base no ataque de vários alvos celulares por EROs gerada por fótons ou fotossensibilizadores, então, um conceito prévio é que o desenvolvimento de resistência ao aPDT é improvável (AL-MUTAIRI et al., 2018). Porém, existem relatos na literatura indicando que algumas cepas de *S. aureus* são mais tolerantes a aPDT do que outras, e alguns isolados clínicos demonstraram menor suscetibilidade ao aPDT após exposição fotodinâmica. Somados, estes dados apontam para a necessidade de investigações detalhadas sobre bactérias e as respostas adaptativas ao tratamento fotodinâmico (GRINHOLC et al., 2007; CASSIDY; DONNELLY; TUNNEY, 2010; KASHEF; HAMBLIN, 2017; AL-MUTAIRI et al., 2018). Além do que é preciso aumentar a disponibilidade de fotosensibilizadores, para eventuais resistências desenvolvidas.

Até o momento, estudos têm procurado determinar parâmetros adequados para a aplicação clínica da aPDT, envolvendo a avaliação de diferentes fotossensibilizadores, fontes e doses de luz e concentrações de fármacos (SELMAN et al., 1987; GARCIA et al., 2014). Nesse sentido, pesquisas objetivam identificar fotossensibilizadores com atividade antimicrobiana quando associados à luz LED por ser uma fonte de luz de menor custo e tecnologia mais simples em comparação aos dispositivos a laser, facilitando a aplicação clínica dessa terapia (HEMPSTEAD et al., 2015).

Além disso, quando comparado a outras terapias antimicrobianas, a aPDT tem muitas vantagens, como maior especificidade (o fotosensibilizador pode ser entregue à célula e pode ser altamente focado no local da lesão em casos de tratamento cutâneo) e menos efeitos adversos indesejáveis (MUSSER; OSEROFF, 2001; HAYASHI et al., 2015). Diante desses fatos, optamos por usar o Resveratrol ativado pela luz azul devido à sua atividade antimicrobiana, além de sua similaridade estrutural com outros fotosensibilizadores com grupos fenólicos, como a curcumina. Em nosso laboratório, foram testados diversos componentes fenólicos, porém, apenas o Resveratrol demonstrou atividade fotodinâmica.

Além disso, o Resveratrol na varredura do espectro UV/Visível tem como banda característica a absorção a 306 nm. Então, a emissão de luz está próxima da faixa máxima de absorção da molécula. No entanto, isso não é uma regra, pois existem estudos que estimularam fotossensibilizadores em várias faixas de comprimento de onda e não apenas na faixa máxima de absorção (LEITE et al., 2014; MANOIL et al., 2014; NIU et al., 2015; MAMALIS; KOO; JAGDEO, 2016; PANHOCA et al., 2016). Neste trabalho, fotoativamos o propilenoglicol e confirmamos que este composto não exibiu inibição do crescimento bacteriano *in vitro* (dados não mostrados). Levando isso em consideração, usamos propilenoglicol como solvente para o Resveratrol nos experimentos.

4.2.3. Após a fotoativação o Resveratrol exibe alteração no tempo de retenção na análise por HPLC

No intuito de analisar se a fotoativação causa algum tipo de alteração estrutural no Resveratrol, a análise por HPLC (Figura 8) foi realizada. Os testes foram realizados para a forma fotoativada e não fotoativada. A alteração no tempo de retenção da molécula pressupõe que o resveratrol interagiu por mais tempo com a fase estacionária. Inicialmente, é possível que tenham ocorrido alterações na estrutura do resveratrol que geraram um componente secundário que interagiu com a fase estacionária por um período maior. Na literatura, estão presentes trabalhos que fotoativaram o resveratrol e foi observado uma alteração no tempo de retenção tal como o nosso. Autores relatam a ocorrencia de uma isomerização que resultou na abertura do anel presente na estrutura deste polifenol (Fig. 7B), (YANG et al., 2012; RODRIGUEZ-CABO et al., 2015).

Porém, devido às limitações técnicas e financeiras, não podemos afirmar com veemência de que seja esse composto porque seriam necessárias mais técnicas para corroborar essa informação, mas é provável que seja o mesmo composto.

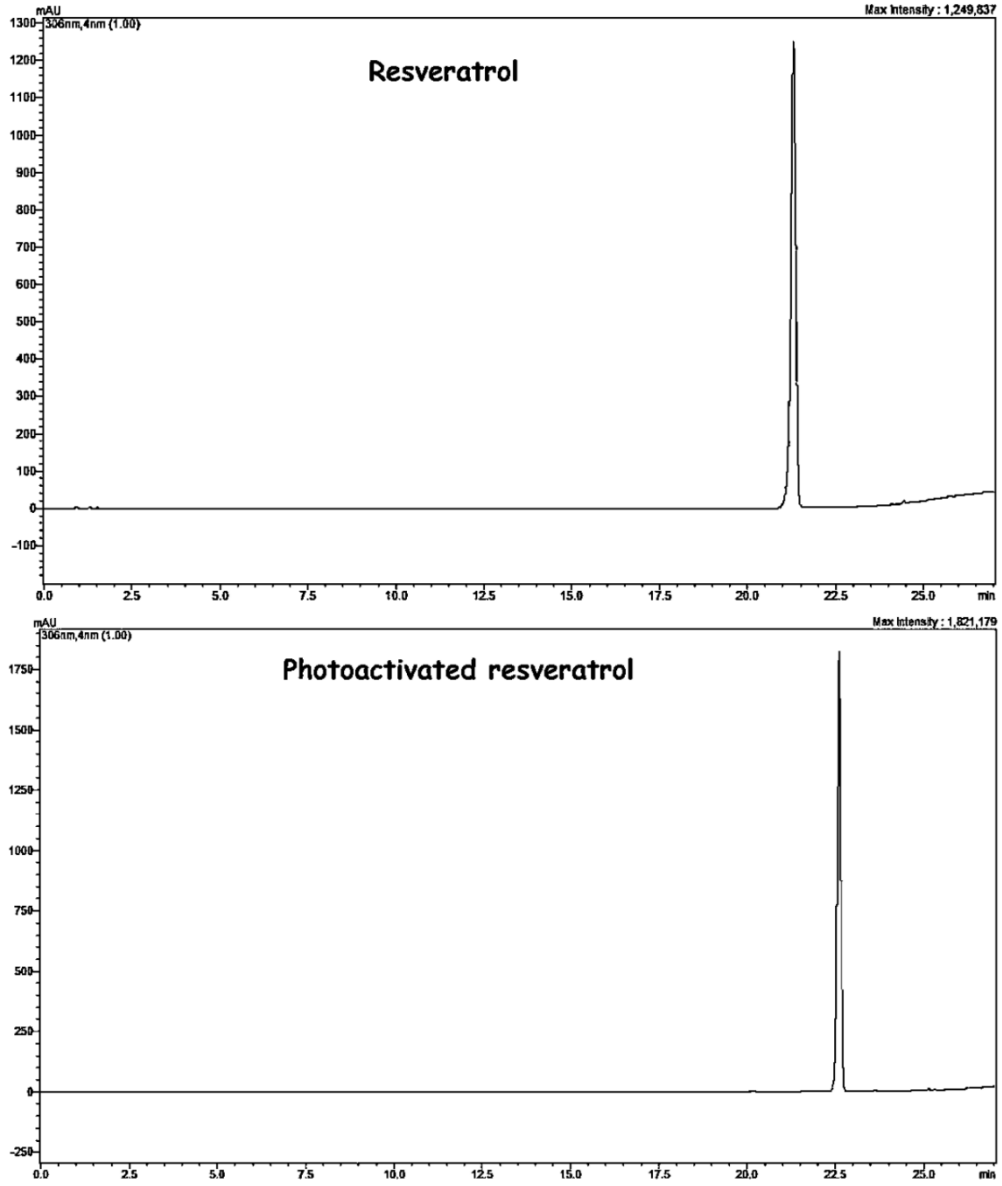


Figura 8: Cromatogramas do Resveratrol - Uma solução de 0,001 mg/mL de Resveratrol foi preparada para análise. Análises de compostos fotoativados e não fotoativados foram realizadas. As separações cromatográficas foram monitorizadas a 306 nm.

4.2.4. Terapia usando Resveratrol fotoativado reduz o número de células inflamatórias no bolsão de ar

Modelos animais podem ser utilizados, para desenvolver protocolos que permitam uma observação do perfil de infiltrado celular. Dentre estes modelos de observação, temos o bolsão de ar. O modelo de bolsão trata-se de um espaço de fácil acesso que pode ser

produzido no tecido subcutâneo dorsal do camundongo, com uma injeção de um pequeno volume de ar. Este modelo tem se mostrado eficaz em estudos de observação da quimioatração celular para o sítio inflamatório induzido por infecções (EDWARDS; SEDGWICK; WILLOUGHBY, 1981). Logo, por ser um modelo de avaliação de inflamação, pode contribuir para o entendimento das respostas iniciais geradas na infecção por *S. aureus* em camundongos.

Então, para avaliar se havia diferença quantitativa nas células inflamatórias no bolsão de ar, o lavado do ambiente inflamatório foi analisado para contagem total e diferencial de células inflamatórias. Como visto na Figura 9, os dados obtidos demonstram que houve uma redução nas células inflamatórias totais nos animais tratados com o Resveratrol fotoativado. Houve também redução do número de células na pele do bolsão de ar, como pode ser visto na análise das imagens.

A fotoativação das soluções foi realizada fora do ambiente corporal dos animais C57Bl/6, uma vez que a melanina poderia ser um agente interferente na aPDT (HUANG et al., 2013). Portanto, aqui estamos propondo uma adaptação do aPDT para o tratamento de infecções em pacientes negros, uma vez que, na literatura, existem poucos estudos avaliando essa técnica nesses pacientes (RIBEIRO, J. B. et al., 2016). Após a fotoativação, as soluções foram aplicadas imediatamente aos animais. O modelo de bolsão de ar é um modelo conveniente *in vivo* para estudar a inflamação localizada com efeitos sistêmicos reduzidos (DUARTE; VASKO; FEHRENBACHER, 2016). Nesse contexto, foi possível observar os efeitos do Resveratrol fotoativado contra uma infecção por MRSA em modelo vivo.

O que se pode inferir dos resultados obtidos neste estudo é que há interação do Resveratrol fotoativado com o sistema imune do hospedeiro (Fig. 9).

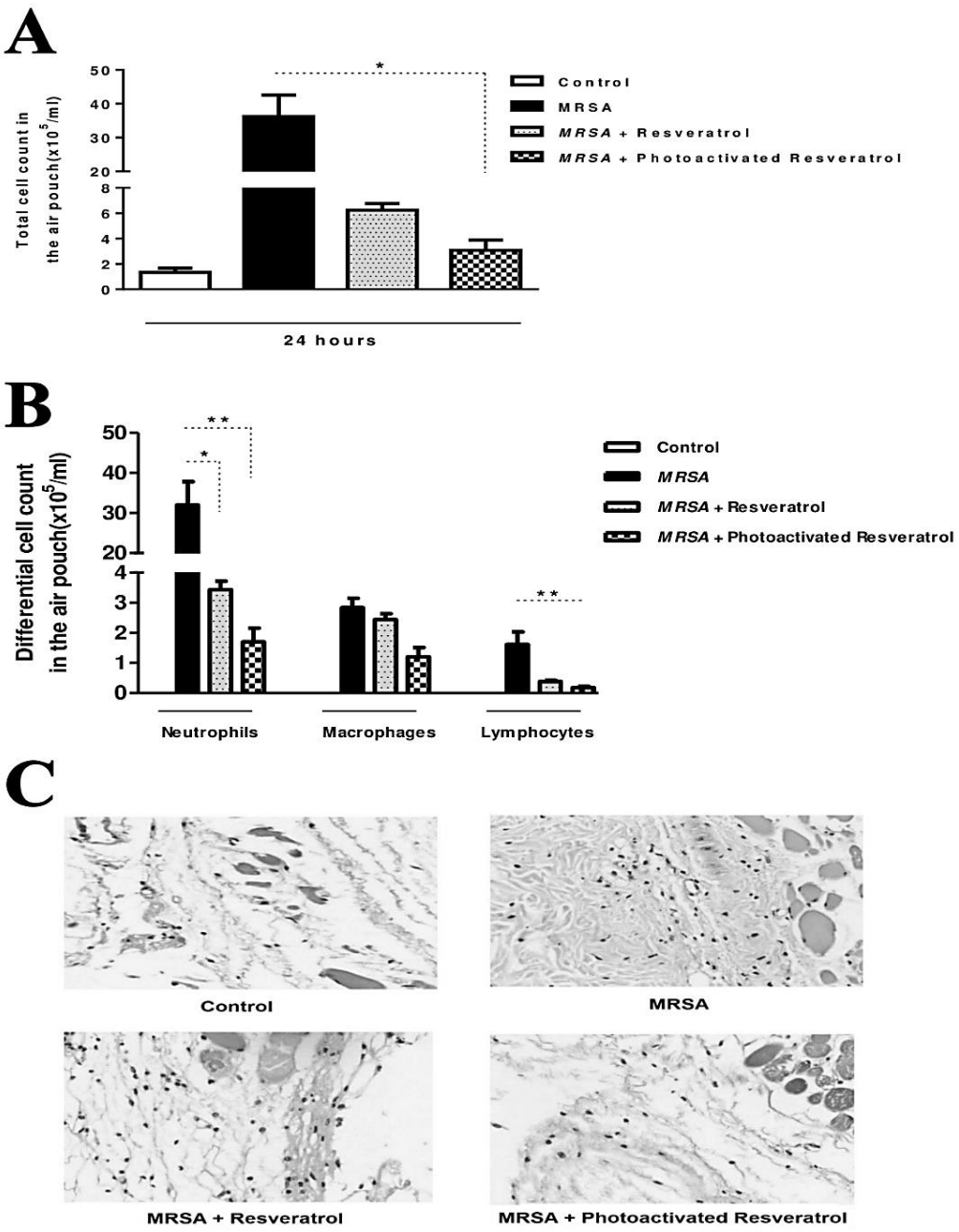


Figura 9: Contagem total e diferencial de leucócitos no lavado do bolsão de ar e visualização da inflamação na pele que recobre o bolsão (x10^5/mL) - Amostras de pele do bolsão de ar foram coletadas, fixadas em formalina a 10% e coradas pela hematoxilina e eosina. Após a obtenção de foto das lâminas, as contagens total (**A**) e diferencial (**B**) foram realizadas, bem como as imagens representativas das lâminas histológicas (**C**). (n = 6); (MRSA: Methicillin-resistant *Staphylococcus aureus*); (* p <0.05, ** p<0.01).

4.2.5. O Resveratrol fotoativado aumenta a produção de TNF-α e IL-17 no bolsão de ar

Com o objetivo de analisar se os animais tratados com Resveratrol fotoativado possuem uma produção de citocinas quantitativamente diferentes dos não tratados, as citocinas IL-1β, IL-10, TNF-α e IL-17A foram analisadas. Animais tratados com o polifenol fotoativado apresentaram uma produção maior de TNF-α e IL-17A (Fig. 10B e 10C). Tal fato é fundamental para a depuração de *S. aureus*, relatado em trabalhos presentes na literatura (KAPETANOVIC et al., 2011; WU et al., 2012). A estimulação da liberação destas citocinas é um fator determinante para o tratamento, principalmente em infecções por cepas resistentes.

Foi demonstrado anteriormente que camundongos *knockout* para IL-17 são incapazes de eliminar efetivamente *S. aureus*. Modelos experimentais usando imunógenos associados à resposta Th17 contra patógenos como *S. aureus* e *Candida albicans* demonstraram um aumento no recrutamento e na ativação de fagócitos nos locais de infecção, além da liberação mais eficaz desses patógenos nos tecidos (LIN et al., 2009). Nesse contexto, a produção de IL-17 na indução de mecanismos efetores contra *S. aureus* e a presença de células que produzem essa citocina são fatores fundamentais para o controle da infecção.

O aumento da liberação de TNF-α é um achado interessante, uma vez que existem dados conflitantes na literatura sobre a capacidade do Resveratrol de alterar a produção dessa citocina. Aqui, mostramos que esse polifenol quando não fotoativado, foi associado com uma redução na produção de TNF-α. Diferentes estudos demonstraram a importância da liberação de TNF-α, uma citocina pró-inflamatória, na redução da carga bacteriana na infecção por MRSA associada à terapia com laser (EVANS et al., 1990; BELLNIER, 1991; ZIOLKOWSKI et al., 1997; SZLISZKA et al., 2011). Além disso, tem sido relatado que a aPDT estimula macrófagos a produzir TNF-α em grandes quantidades, levando a um aumento dessa citocina no ambiente inflamatório (STEUBING et al., 1991; HUANG et al., 2012; LI et al., 2016; PANSA et al., 2016). Os leucócitos ativados pelo TNF-α produzem espécies reativas de oxigênio e nitrogênio, essenciais para a depuração de microrganismos. Dessa forma, a ativação das células do sistema imunológico pelo TNF-α é essencial para o controle de infecções (DAS, U. N. et al., 1990; LARYSZ-BRYSZ et al., 2012).

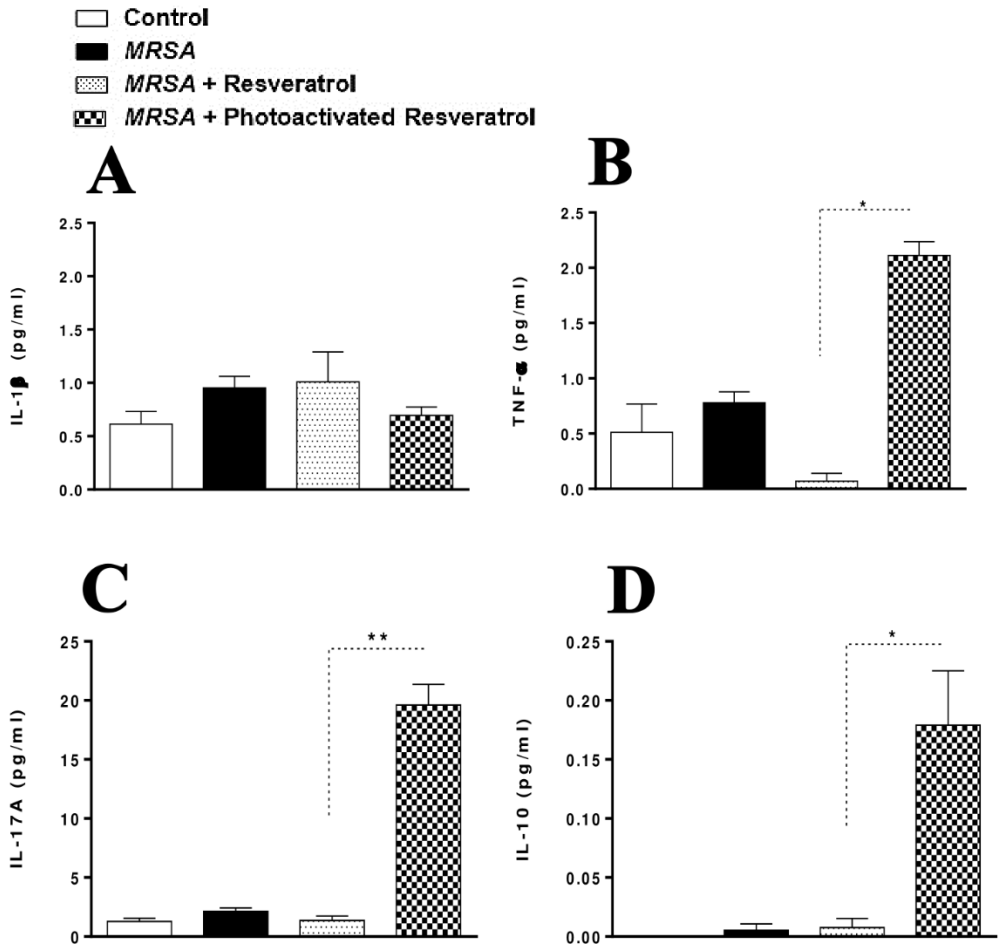


Figura 10: Quantificação de citocinas no ambiente inflamatório (pg/mL) - As lavagens da bolsa de ar foram coletadas posteriormente e avaliadas por ELISA para a presença de IL-17A, IL-10, IL-1β e TNF-α. (n = 6); (BHI = brain heart infusion; MRSA: Methicillin-resistant *Staphylococcus aureus*);(* p <0.05, ** p<0.01).

Quantitativamente, o número de células inflamatórias nos grupos tratados com Resveratrol foi menor do que nas não tratadas (Fig. 9A). Esses dados corroboram com vários trabalhos mostrando que, após o tratamento de modelos animais com esse polifenol, houve redução da inflamação local (JEONG et al., 2016). No entanto, em nossos dados, mesmo com a redução do número de células no local, a inibição do crescimento bacteriano foi maior (Fig. 11), fato importante, pois a exacerbação da inflamação local pode ser prejudicial ao organismo. Além disso, é possível que, mesmo em menor quantidade, as células inflamatórias sejam direcionadas pelo sinergismo de IL-17A e TNF-α (LIU, Y. et al., 2011; LAURIDSEN et al., 2017), resultando em uma maior redução da carga bacteriana, mesmo com um número menor de células.

Por outro lado, é possível que 24 horas após o tratamento com Resveratrol fotoativado, tempo analisado por nosso trabalho, a diminuição do número de células inflamatórias já tenha ocorrido devido à depuração bacteriana. Esse fato também explicaria os níveis mais altos de produção de IL-10 (Fig.10D), uma citocina anti-inflamatória, secretada para modular as respostas inflamatórias (IYER; CHENG, 2012). Não foram observadas diferenças entre os grupos em relação à produção de IL-1β. Além desses dados, o modelo do bolsão de ar foi um dos fatores que auxiliou na visualização eficaz da inflamação, pois é de fácil manipulação e obtenção de amostras clínicas que auxiliam na compreensão da resposta inflamatória local.

4.2.6. Animais tratados com Resveratrol fotoativado demonstraram maiores níveis de depuração bacteriana no bolsão inflamatório

A figura 11 apresenta o comportamento do crescimento bacteriano após 24 horas de cultivo. Observou-se que camundongos C57Bl/6 tratados com Resveratrol fotoativado apresentaram menor formação de UFC que os grupos não tratados.

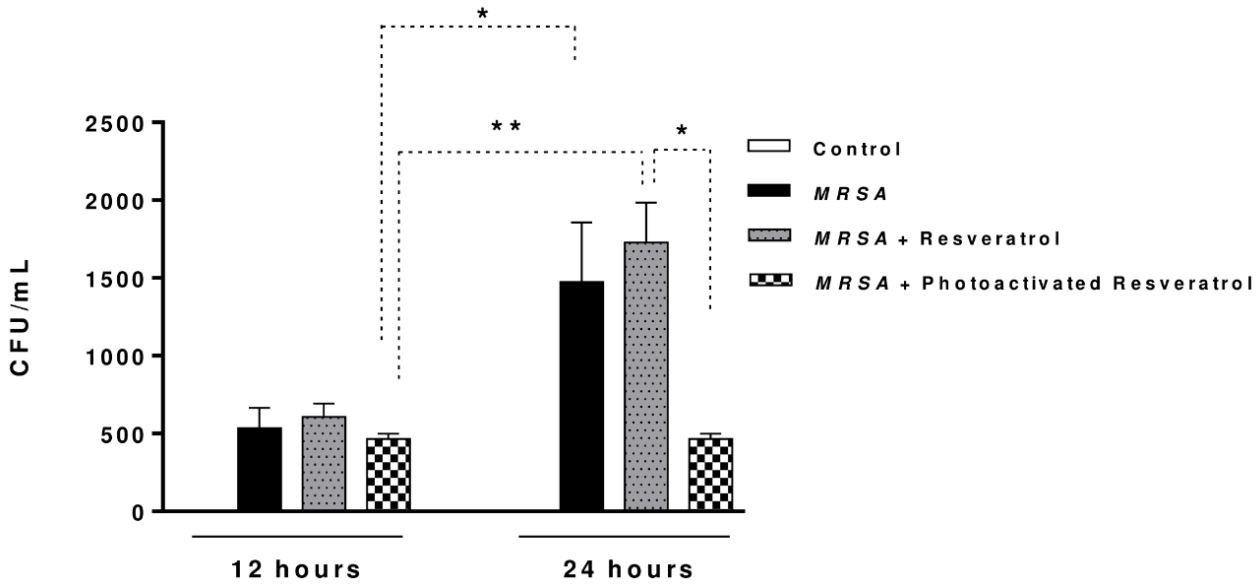


Figure 11: Carga bacteriana de *Staphylococcus aureus* no bolsão de ar (CFUx10^5 /mL) – O lavado do bolsão de ar foi coletado e posteriormente cultivadas no BHI. As UFCs foram quantificadas após 24 horas. (n = 6); (BHI = *brain heart infusion*; MRSA: Methicillin-resistant *Staphylococcus aureus*);(* p <0.05, ** p<0.01).

4.3. Experimento III – Resveratrol fotoativado controla a infecção intradérmica por *Staphylococcus aureus* em camundongos

4.3.1. Resveratrol fotoativado promove aumento da expressão de mieloperoxidase na derme da orelha

A figura 12 mostra que após a fotoativação, o Resveratrol foi capaz de aumentar as expressões de MPO e E-caderina nas orelhas de camundongo tratados com o composto ativado. Pode-se verificar também que não há expressão elevada quando o estímulo luminoso não ocorre.

Na literatura, os trabalhos já relatam que após a fotoativação, o Resveratrol produz oxigênio *singlete* (DOS SANTOS et al., 2019b), um potente agente antimicrobiano. Aqui, estamos relatando outro efeito importante do Resveratrol fotoativado: produção de MPO. Sendo assim, acreditamos que a depuração bacteriana (Fig.13) ocorreu de duas maneiras. Primeiro, o próprio Resveratrol fotoativado, gerando espécies reativas de oxigênio (DOS SANTOS et al., 2019b), e o segundo, aumentando a expressão de MPO (fig. 12A) em animais tratados com o composto fotoativado (discussão no tópico 4.1.5. sobre a importância da MPO).

Além disso, ao analisar a expressão da E-caderina (Fig. 12B), notamos que os animais tratados com Resveratrol fotoativado apresentaram níveis mais altos dessa glicoproteína. Estudos demonstraram que *S. aureus* é capaz de reduzir a expressão da E-caderina liberando α-hemolisina, que por sua vez intensifica a atividade das metaloproteases, resultando na clivagem da E-caderina (VANDENESCH; LINA; HENRY, 2012). A clivagem está associada à interrupção da função da barreira epitelial, contribuindo para a patogênese, facilitando a penetração bacteriana no tecido infectado. Aqui observamos que o Resveratrol fotoativado evitou esse possível mecanismo de virulência bacteriana. No entanto, não temos informações suficientes para garantir tais propostas, de modo que incentivamos outros autores a realizar pesquisas sobre esse assunto para colaboração e elucidação de dados.

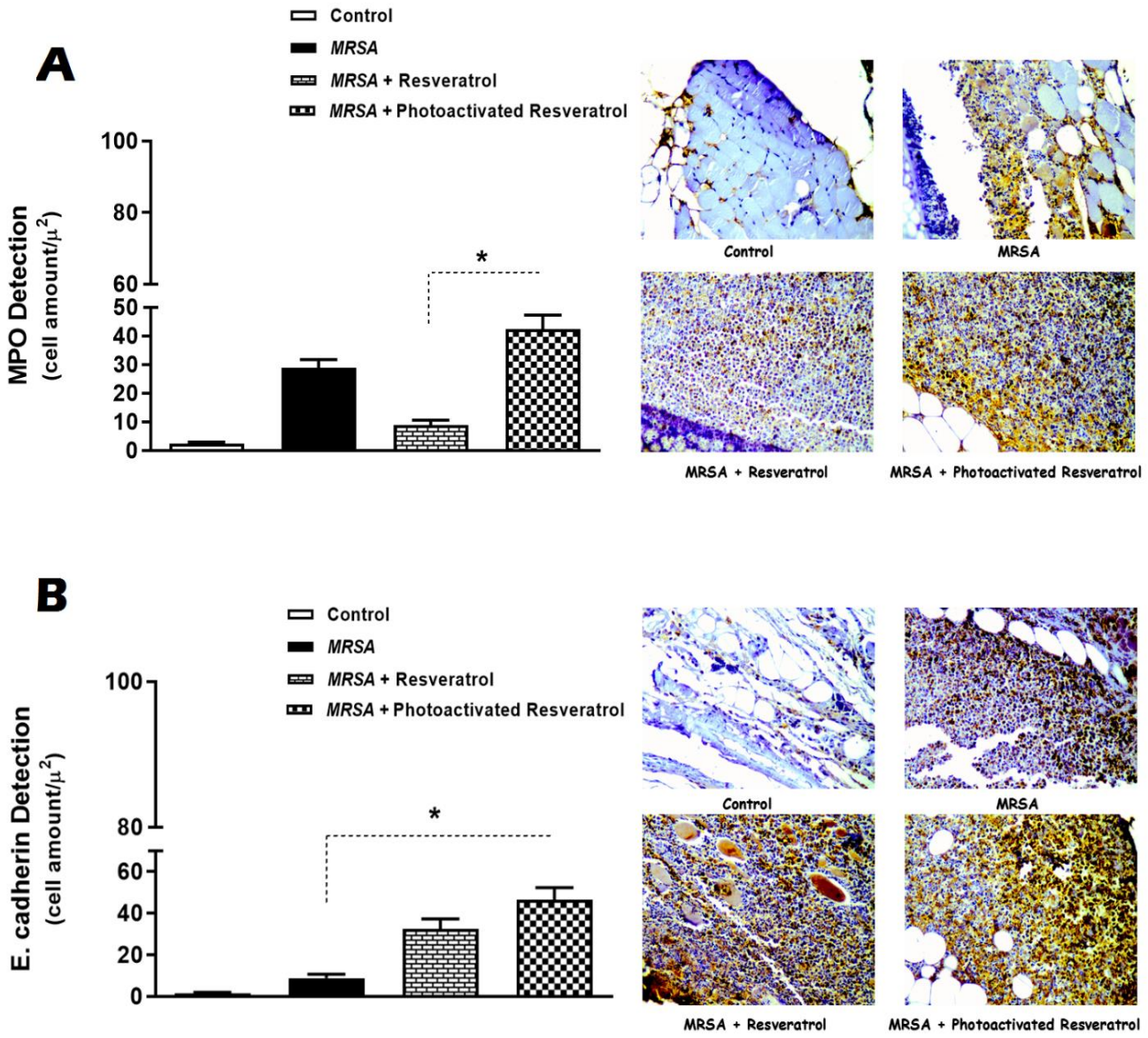


Figura 12: Determinação da expressão de mieloperoxidase e E-caderina - As orelhas dos animais foram cortadas, embebidas em parafina, foram feitos cortes histológicos, a técnica imuno-histoquímica para a expressão de mieloperoxidase (A) e E-caderina (B) foram prosseguidos. (n = 6); (* p <0,05).

O que se pode afirmar é que menos células migraram para o linfonodo drenante nos animais tratados com o composto fotoativado, o que resultou em uma menor hiperplasia desse órgão. Assim, é possível dizer que a resolução da inflamação ocorreu principalmente na orelha do animal, onde ocorreu a maior proporção de depuração bacteriana. Devido à baixa espessura do tecido de camundongos na área de inoculação, supõe-se que as camadas superficiais da pele não tenham atuado como uma tela, de modo que quase toda a luz irradiada atingiu o Resveratrol. Esses dados são reforçados quando analisamos a quantidade bacteriana no linfonodo drenante dos grupos estudados neste

trabalho, nos quais os animais não tratados com o composto fotoativado apresentaram maior carga bacteriana. Além disso, nesses animais não tratados, houve menor expressão de E-caderina, portanto, houve um maior descolamento e migração celular para os linfonodos drenantes.

4.3.2. Camundongos tratados com Resveratrol fotoativado apresentam carga bacteriana reduzida no linfonodo drenante

O tráfego de bactérias da orelha para o linfonodo foi menor nos animais que foram tratados com RSV fotoativado (Figura 13) nos tempos de cultura avaliados do sobrenadante do macerado do linfonodo drenante.

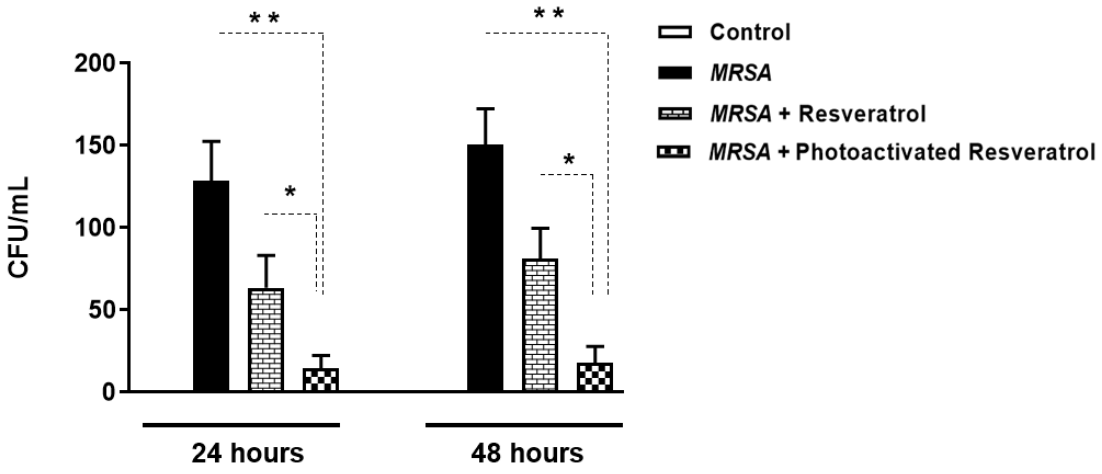


Figura 13: Determinação da carga bacteriana no linfonodo drenante - Os animais foram eutanasiados 24 horas após o desafio. Em ambiente estéril, o nódulo linfático drenante foi removido, macerado em 1 mL de solução salina estéril e 5 µL foram cultivados em meio BHI. As UFCs foram quantificadas após 24 e 48 horas de cultura. (n = 6); (BHI = *Brain Heart Infusion*); (CFU: *Colony Forming Unit*); (*$p<0.05$); (**$p<0.01$).

4.3.3. O linfonodo drenante dos animais tratados com Resveratrol fotoativado controla a inflamação através da produção de IL-10

Vinte queatro horas após o tratamento, o padrão de citocinas nos animais tratados com Resveratrol fotoativado foi diferente (figura 14). Neste contexto, foi possível observar uma redução na produção de citocinas pró-inflamatórias, como o TNF-α (Fig. 14A) e a citocina quimioatrativas neutrofílicas, a IL-17A (fig. 14E). No entanto, foi observado um nível mais alto de produção de IL-10 (fig. 14F), uma citocina moduladora

da inflamação (as discussões sobre a importância dessas citocinas foram realizadas anteriormente neste texto). Assim, através da quantificação de IL-10 em camundongos tratados com o polifenol fotoativado, podemos verificar uma resolução da inflamação nesses animais**.** Não foram detectadas diferenças significativas para as outras citocinas avaliadas.

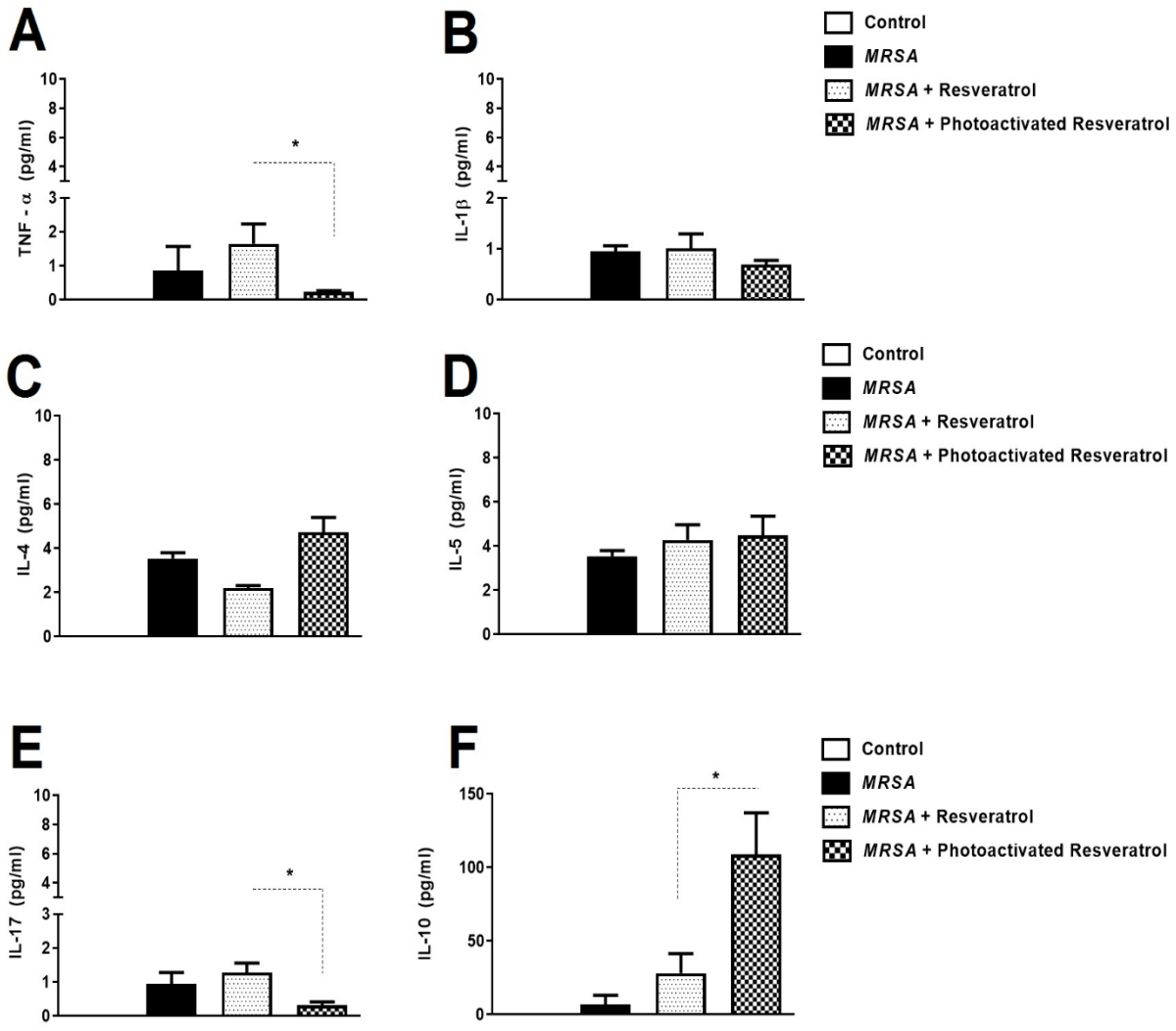


Figura 14: Quantificação de citocinas no ambiente inflamatório (pg/mL) - Em ambiente estéril, o linfonodo drenante foi removido, macerado em 1 mL de solução salina estéril e avaliado por ELISA para a presença de TNF-α (A), IL-1β (B), IL-4 (C), IL-5 (D), IL-17A (E) e IL-10 (F). (n = 6); (MRSA: Methicillin-resistant *Staphylococcus aureus*);(* p <0.05).

5. CONCLUSÕES

Aqui, foi demonstrado que o extrato de *M. cauliflora* possui atividade fotossensibilizante, promovendo a produção de oxigênio *singlete*, TNF-α e MPO provendo depuração bacteriana. Diante disso, foi depositada uma patente com o número de processo BR1020170246930 (Anexo II). Somado a essa validação de fotossensibilizador, o Resveratrol ativado pela luz LED azul também demonstrou ser um fotossensibilizador promissor para ser usado na terapia fotodinâmica antimicrobiana. Este composto, após o estímulo luminoso, produziu também oxigênio *singlete*, como a *M. cauliflora*, além disso, exerceu efeitos no sistema imune com a produção de TNF-α e IL-17A, que são citocinas pró-inflamatórias, com indução da expressão de MPO e controlando a infecção pela produção de IL-10. Por fim, este polifenol apresentou atividade antimicrobiana em diferentes sítios corpóreos. Por se tratar de um trabalho pioneiro, são necessários mais estudos para permitir uma clara elucidação do mecanismo de ação dos novos fotossensibilizadores aqui validados.

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7. ANEXO I – Parecer da Comissão de Ética Em Uso Animal da UFBA - IMS/CAT

UNIVERSIDADE FEDERAL DA BAHIA
Instituto Multidisciplinar em Saúde
Campus Anísio Teixeira
Comissão de Ética Em Uso de Animais
(CEUA - IMS/CAT - UFBA)

PROJETO DE PESQUISA – Reapreciação em 20/03/2016 - Aprovado sem restrições

Título: Avaliação de novos fotossensibilizadores para a terapia fotodinâmica no tratamento de infecção intradérmica por *Staphylococcus aureus* em modelo murino.

Protocolo: 042/2017

Pesquisador: Robson Amaro Augusto da Silva.

Instituição: Instituto Multidisciplinar em Saúde - Campus Anísio Teixeira – UFBA

PARECER DO RELATOR

Data da Relatoria: 20 de março de 2017

Este projeto teve seu primeiro parecer apreciado na Comissão de Ética no Uso de Animais no dia 17 de março de 2017. O pesquisador Robson Amaro Augusto da Silva reapresentou-o atendendo à solicitação de preenchimento do item sobre o grau de invasividade. Sendo assim, o projeto assume parecer final de **aprovado sem restrições**.

Parecer do Relator: Aprovado sem restrições

PARECER CONSUBSTANCIADO CEUA E CONSIDERAÇÕES FINAIS:

Conforme parecer do relator transcrito acima as adequações solicitadas foram plenamente atendidas, mantendo a aprovação do referido projeto apreciado na 15ª Reunião Ordinária do dia 17 de março de 2017.

Status Final deste Parecer: Aprovado sem restrições

Vitória da Conquista, 20 de março de 2017.

Ricardo Evangelista Fraga
Coordenador CEUA - IMS/CAT - UFBA

CEUA - IMS/CAT – UFBA
Prédio Administrativo 1º andar - Rua Rio de Contas, 58 - Quadra 17 - Lote 58 - Bairro Candeias - Vitória da Conquista - Ba - CEP 45.029-094
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8. ANEXO II – Depósito de patente

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

17/11/2017 870170088838 14:28

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 024693 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DA BAHIA
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 15180714000104
Nacionalidade: Brasileira
Qualificação Jurídica: Instituição de Ensino e Pesquisa
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PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 17/11/2017 às 14:28, Petição 870170088838

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): COMPOSIÇÃO FOTOSSENSIBILIZADORA CONTENDO EXTRATO DE Myrciaria cauliflora CONTRA Staphylococcus aureus

Resumo: A presente invenção refere-se a utilização do extrato de Myrciaria cauliflora ativado por luz LED azul contra Staphylococcus aureus. Neste contexto, a Terapia Fotodinâmica (TFD) surge como uma tecnologia promissora através da inativação microbiana com o uso da luz. A TFD envolve a utilização de corantes não tóxicos, denominados fotossensibilizadores, ou substancias sensíveis a luz que são ativadas quando expostas a determinado comprimento de onda de luz visível específico para sua ativação. Diante disso, com a intensa utilização de antimicrobianos e a possibilidade de resistência bacteriana, produtos naturais são estudados como alternativas, como o uso de extrato de plantas. A M. cauliflora possui potencial antimicrobiano devido às antocianinas, compostos fenólicos presentes nas suas cascas e sementes. Assim sendo, este trabalho trouxe uma nova abordagem da utilização do extrato de M. cauliflora, com associação com TFD, ativado por luz LED azul (LED Bluephase, Ivoclar Vivadent, Liechtenstein, comprimento de onda de 380-515 nm, intensidade de 1200 mW/cm2 durante 5 min) no tratamento de infecção bacteriana causada por S. aureus. Foi observada a inibição do crescimento bacteriano in vitro bem como in vivo. Além disso, foi observado que animais tratados com extrato de M. cauliflora fotoativado reduz a produção de IL-17, IL-10, IL-5, IL-4 e IL-1ß, e aumenta a produção de TNF-a.

Figura a publicar: 1

Dados do Procurador

Procurador:

Nome ou Razão Social: Marlos André Pereira de Jesus

Numero OAB:

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Documentos anexados

Tipo Anexo	Nome
Resumo	Resumo.pdf
Relatório Descritivo	Relatório descritivo.pdf
Reivindicação	Reivindicações.pdf
Desenho	Figuras.pdf
Comprovante de pagamento de GRU 200	Comprovante de PGTO - GRU 200.PDF
Procuração	PROCURAÇÃO.pdf

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 17/11/2017 às 14:28, Petição 870170088838

9. ANEXO III – Modelo de Tese no formato de compilação de artigos

Parágrafo único: A critério do Colegiado Geral, a tese de doutorado poderá ser substituída por uma compilação de no mínimo 2(dois) artigos científicos publicados ou aceitos em periódico Qualis B1 ou superior e relacionados a tese, em que o estudante seja o primeiro autor. O formato da tese nesse caso deverá conter:

I- Resumo em Português e Inglês;

II- Introdução geral e justificativa do estudo;

III-Cópia dos trabalhos publicados;

IV- Resumo dos resultados e discussão geral;

V- Conclusões;

VI- Referências Bibliográficas.

Art. 67º. O orientador deverá requerer ao Coordenador as providências necessárias à defesa de dissertação ou tese.

Parágrafo único. A dissertação ou tese, em formato preliminar, deverá ser encaminhada ao Colegiado Administrativo Local que nomeará e encaminhará para a Banca Examinadora para análise e sugestões.

Art. 68º. A defesa da tese será pública e se fará perante a Banca Examinadora indicada pelo Colegiado Administrativo Local, integrada pelo orientador e pelo menos 04 (quatro) membros portadores do grau de Doutor, sendo, no mínimo, 3(três) examinadores externos ao quadro de orientadores da Instituição Associada, dos quais, 2(dois) externos à Instituição associada. Um dos examinadores externos deve pertencer a uma das Instituições Nucleadoras, exceto em casos excepcionais aprovados pelo Colegiado Geral.

Parágrafo Único: A critério das normas das IES Associadas o número mínimo de componentes da banca poderá ser modificado.

Art. 69º. A defesa de dissertação será pública e se fará perante Comissão Examinadora indicada pelo Colegiado Administrativo Local e constituída pelo orientador e pelo menos mais 2(dois) membros portadores do grau de doutor, sendo um deles externo ao quadro de orientadores da Instituição Associada e, preferencialmente, pertencente a uma das Instituições Nucleadoras.

Parágrafo único. Na hipótese de co-orientadores virem a participar de comissão examinadora de tese ou dissertação, estes não serão considerados para efeito de integralização do número mínimo de componentes previstos respectivamente nos artigos 68 e 69.

Art. 70º. Será considerado aprovado na defesa de dissertação/tese o candidato que obtiver aprovação unânime da Comissão Examinadora.

Parágrafo único. No caso de tese de doutorado, os membros da banca examinadora deverão elaborar parecer único, por escrito, indicando a avaliação do candidato e de sua tese, que será divulgado publicamente juntamente com o resultado da defesa.

Art. 71º. A dissertação e tese, na forma em que for aprovada pela Banca Examinadora e visto do orientador, deverá ser impressa e encaminhada à Secretaria Local do Programa, no prazo estipulado pela IES associada.