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# POTENCIAL ANTIMICROBIANO DA LECTINA DA SARCOTESTA DE *Punica granatum* (PgTeL) CONTRA PATÓGENOS HUMANOS

**Recife**
**2019**

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como parte dos requisitos para obtenção do título de Doutora em Bioquímica e Fisiologia.

**Orientador:** Prof. Dr. Thiago Henrique Napoleão

**Coorientadoras:** Profa. Dra. Patrícia Maria Guedes Paiva e Profa. Dra. Hanne Ingmer

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Aprovado em: 19/02/2019

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Universidade Federal de Alagoas

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## RESUMO

O aumento no número de microrganismos resistentes e a elevada toxicidade das drogas atualmente utilizadas torna necessária a busca por novos agentes com ação antimicrobiana. Lectinas são proteínas que se ligam de forma seletiva e reversível a carboidratos, apresentando diversas atividades biológicas, incluindo atividade antimicrobiana. A sarcotesta das sementes de romã (*Punica granatum*) contém uma lectina ligadora de quitina denominada PgTeL, já descrita como agente antibacteriano. A presente tese investigou a atividade antimicrobiana de PgTeL contra fungos do gênero *Candida* e isolados clínicos bacterianos de *Staphylococcus aureus* (não-resistente e resistente à meticilina, MRSA) e *Escherichia coli* (produtores de β-lactamases das famílias CTX-M, CMY e metalo-β-lactamases). Adicionalmente, foi investigada a toxicidade para células mononucleares de sangue periférico (PBMCs) humano. Atividade antifúngica de PgTeL foi detectada contra *Candida albicans* (concentração mínima inibitória, CMI: 25 µg/mL; concentração mínima fungicida, CMF: 50 µg/mL) e *Candida krusei* (CMI e CMF de 12,5 µg/mL). O tratamento das leveduras com PgTeL, mesmo em concentrações sub-inibitórias (½CMI e ¼CMI), resultou na diminuição do conteúdo de ATP intracelular e induziu peroxidação lipídica. Além disso, PgTeL danificou a integridade da parede celular de ambas as espécies, com efeitos mais pronunciados em *C. krusei*. Por fim, a lectina mostrou atividade antibiofilme contra *C. albicans*. Com relação aos efeitos sobre *S. aureus*, PgTeL apresentou atividade antibacteriana contra os isolados 8325-4 (não-resistente) e LAC USA300 (MRSA), interferindo tanto no crescimento (CMI de 6,25 e 12,5 µg/mL, respectivamente) quanto na sobrevivência (concentração mínima bactericida, CMB, de 25,0 e 50,0 µg/mL, respectivamente). O crescimento das culturas começou apenas na nona (8325-4) e na décima (LAC USA300) horas na presença de PgTeL na CMI, enquanto foi detectado desde a primeira hora no controle. A lectina causou alterações na morfologia celular como alongamento, enrugamento, perfurações, aumento no tamaho e lise celular, apresentou efeito anti-agregação e exibiu atividade antibiofilme contra ambos os isolados de *S. aureus*. Por outro lado, a lectina não interferiu com a atividade hemolítica de LAC USA300 e na expressão dos genes *hla* e *rnaIII* que codificam hemolisina e RNAIII (efetor do sistema), respectivamente. PgTeL também não ativou a expressão de *spa* que codifica a proteína A. Os genes *hla*, *rnaIII* e *spa* estão ligados ao sistema de virulência Agr. Para os isolados de *E. coli* PgTeL apresentou tanto ação bacteriostática (CMI: 12,5 a 50 µg/mL) quanto bactericida (CMB: 25 a 100 µg/mL). A incubação com PgTeL atrasou o início da replicação das culturas

em no mínimo 10 horas. Na CMI, a lectina causou alterações no tamanho, forma e estrutura das células de *E. coli*. A combinação PgTeL-ceftazidima apresentou efeito sinérgico com todos os isolados; efeito sinérgico também foi detectado com carbenicilina, cefotaxima, cefalexina e cefuroxima para um ou mais isolados de *E. coli*. Ainda, PgTeL apresentou ação antibiofilme contra todos os isolados. Quanto à citotoxicidade para células humanas PgTeL não interferiu na viabilidade das PBMCs quando testada nas concentrações de 1, 10 e 100 µg/mL. Em conclusão, PgTeL é uma molécula com potencial para tratamento de infecções fúngicas e bacterianas, inclusive contra isolados multirresistentes.

**Palavras-chave:** Romã. Atividade antibacteriana. Atividade antifúngica. Sinergismo. *Candida*. *Staphylococcus aureus*. *Escherichia coli*.

## ABSTRACT

The increase in the number of resistant microorganisms and the high toxicity of the currently used drugs makes necessary the search for new agents with antimicrobial action. Lectins are proteins that bind selectively and reversibly to carbohydrates, presenting several biological activities, including antimicrobial activity. The sarcotesta of pomegranate (*Punica granatum* L.) seeds contains a chitin-binding lectin called PgTeL, already described as antibacterial agent. The present thesis investigated the antimicrobial activity of PgTeL against fungi of *Candida* genus and bacterial clinical isolates of *Staphylococcus aureus* (non-resistant and methicillin resistant, MRSA) and *Escherichia coli* (producers of β-lactamases from the CTX-M, CMY and metallo-β-lactamases families). Additionally, toxicity to human peripheral blood mononuclear cells (PBMCs) was investigated. Antifungal activity of PgTeL was detected against *Candida albicans* (minimum inhibitory concentration, MIC: 25 μg/mL, minimal fungicidal concentration, MFC: 50 μg/mL) and *Candida krusei* (MIC and MFC of 12.5 μg/mL). Treatment of yeasts with PgTeL, even at subinhibitory concentrations (½MIC and ¼MIC), resulted in a decrease in intracellular ATP content and induced lipid peroxidation. In addition, PgTeL damaged the cell wall integrity of both species, with more pronounced effects on *C. krusei*. Finally, the lectin showed antibiofilm activity against *C. albicans*. In respect to the effects on *S. aureus*, PgTeL presented antibacterial activity against the isolates 8325-4 (non-resistant) and LAC USA300 (MRSA), interfering in both growth (MIC of 6.25 and 12.5 μg/mL, respectively) and survival (minimum bactericidal concentration, MBC, 25.0 and 50.0 μg/mL, respectively). Growth of the cultures started only at the ninth (8325-4) and tenth (LAC USA300) hours in the presence of PgTeL at MIC, while it was detected since the first hour in the control. The lectin caused changes in cell morphology such as stretching, wrinkling, perforations, increase in size and cellular lysis, showed anti-aggregation effect and exhibited antibiofilm activity against both *S. aureus* isolates. On the other hand, the lectin did not interfere with the hemolytic activity of LAC USA300 and the expression of *hla* and *rnaIII* genes encoding hemolysin and RNAIII. PgTeL also did not activate expression of *spa* encoding protein A. The *hla*, *rnaIII* and *spa* genes are linked to the Agr virulence system. For *E. coli* isolates, PgTeL presented both bacteriostatic (MIC: 12.5 to 50 μg/mL) and bactericidal (MBC: 25 to 100 μg/mL) activities. Incubation with PgTeL delayed the start of culture replication in at least 10 hours. At the CMI, the lectin caused changes in the size, shape, and structure of *E. coli* cells. The PgTeL-ceftazidime combination showed synergistic effect with all isolates; synergistic effect was also detected

with carbenicillin, cefotaxime, cephalexin and cefuroxime for one or more *E. coli* isolates. In addition, PgTeL presented antibiofilm action against all the isolates. In regard to the cytotoxicity to human cells, PgTeL did not interfere with the viability of PBMCs when tested at concentrations of 1, 10 and 100 μg/mL. In conclusion, PgTeL is a molecule with potential for the treatment of fungal and bacterial infections, including against multiresistant isolates.

**Keywords:** Pomegranate. Antibacterial activity. Antifungal activity. Synergism. *Candida*. *Staphylococcus aureus*. *Escherichia coli*.

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# 1 INTRODUÇÃO

As plantas têm sido utilizadas pelos seres humanos para fins medicinais desde a Pré-História e é notável o crescente interesse em estudos que avaliam propriedades medicinais de compostos de origem vegetal (MARTINS et al. 2015; JESUTHASAN; ULUWADUGE, 2017; JAMSHIDI-KIA; LORIGOOINI; AMINI-KHOEI, 2018). Segundo a Política Nacional de Plantas Medicinais e Fitoterápicos (2006), plantas com valor medicinal são aquelas que possuem, em um ou vários órgãos, substâncias usadas com finalidade terapêutica ou que sejam ponto de partida para a síntese de produtos químicos e farmacêuticos. Essas substâncias são chamadas de "princípios ativos".

O uso de plantas na medicina popular tem grande relevância principalmente nos países em desenvolvimento, onde a disponibilidade e a eficiência dos serviços de saúde são limitadas. Cerca de 80% da população nesses países utilizam plantas no tratamento de doenças (MAROYI, 2013; VAN WYK; PRINSLOO, 2018). No entanto, alguns compostos de origem vegetal podem gerar efeitos adversos, apresentando efeito tóxico para o organismo humano a curto, médio ou longo prazo. Por isso, faz-se necessária uma análise das atividades biológicas e da toxicidade de preparações vegetais utilizadas pela população, a fim de confirmar cientificamente os efeitos relatados, bem como definir a segurança do uso (AZIZ et al. 2016; KHARCHOUFA et al., 2018).

Doenças infecciosas são responsáveis por uma alta taxa de morbidade e mortalidade em todo o mundo. Ao longo dos anos, houve grandes avanços no campo da Microbiologia Médica visando o controle de microrganismos; porém, o uso excessivo e/ou indiscriminado dos antibióticos disponíveis tem criado uma pressão de seleção que culmina no crescimento da resistência microbiana, de forma que vários patógenos permanecem como graves ameaças à saúde pública. Adicionalmente, são descritos vários efeitos colaterais das drogas atualmente utilizadas. Nesse cenário, novos agentes antimicrobianos têm sido pesquisados e os compostos de origem vegetal têm se mostrado boas alternativas (ABDOLLAHZADEH et al., 2011; CHOI et al., 2011; HE et al., 2018).

As lectinas são proteínas que se ligam especificamente a carboidratos de forma reversível, sendo capazes de induzir alterações celulares ao inteagirem com glicoconjugados de membrana. Podem assumir diferentes papeis biológicos, todavia, não existe uma função universal para todas elas. As lectinas apresentam diversas atividades biológicas como, por

exemplo, ação antimicrobiana, sendo apontadas como possíveis alternativas aos antibióticos existentes (MOURA et al., 2017; COELHO et al., 2018).

*Punica granatum* L. (romãzeira), pertencente à família Lythraceae, é uma planta bastante utilizada na medicina popular contra diversos tipos de doenças. Vários estudos relatam que os diversos tecidos da planta apresentaram atividades antimicrobiana, antioxidante, hepatoprotetora, anticâncer, anti-inflamatória, antiproliferativa, antiangiogênica, cardioprotetora, neuroprotetora, antidiabética e anti-aterosclerose (JURENKA, 2008; WAFA et al., 2017; WANG et al., 2018; KHWAIRAKPAM et al., 2018; LOIZZO et al., 2019). Na medicina popular, extratos de folha, casca, sementes e o suco da romã são usados para fins protetores e preventivos de obesidade, diabetes, hipertensão, hipercolesterolemia e doenças cardiovasculares (AL-MUAMMAR; KHAN, 2012).

A sarcotesta das sementes da romã contém uma lectina (PgTeL) ligadora de quitina que apresentou atividades bacteriostática (*Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus mutans*, *Aeromonas* sp., *Escherichia coli*, *Klebsiella* sp., *Salmonella enterica* serovar. Enteritidis, *Serratia marcescens*) e bactericida (*M. luteus*, *S. mutans*, *S. marcescens*). Adicionalmente, as capacidades de adesão e invasão de *Aeromonas* sp., *S. aureus*, *S. marcescens* e *S. enterica* foram reduzidas quando as células dessas bactérias foram previamente tratadas com PgTeL (SILVA et al., 2016).

Esses resultados prévios estimularam a continuidade da avaliação do potencial antimicrobiano de PgTeL. Na presente tese, foi investigada a atividade anti-*Candida* de PgTeL, bem como sua ação antibacteriana contra isolados de *S. aureus* resistentes ou não à meticilina e isolados de *E. coli* resistentes a antibióticos β-lactâmicos. Ainda, foi avaliada a toxicidade de PgTeL para células mononucleares de sangue periférico humano.

## 1.1 OBJETIVOS

### 1.1.1 GERAL

Avaliar o potencial antimicrobiano da lectina da sarcotesta de *Punica granatum* (PgTeL) contra patógenos humanos e a toxicidade para células humanas.

### 1.1.2 ESPECÍFICOS

- Isolar PgTeL seguindo protocolo previamente estabelecido.
- Avaliar os efeitos de agentes desnaturantes (ureia, pH e temperatura) na conformação de PgTeL.
- Investigar a citotoxicidade de PgTeL sobre células mononucleares de sangue periférico (PBMCs) humano.
- Determinar o efeito de PgTeL no crescimento e sobrevivência de *Candida albicans* e *Candida krusei.*
- Inferir mecanismos envolvidos na ação anti-*Candida* de PgTeL por meio da determinação dos níveis intracelulares de ATP, ocorrência de peroxidação lipídica e alterações na integridade da parede celular.
- Avaliar a ação antibiofilme de PgTeL sobre *C. albicans*.
- Analisar os efeitos de PgTeL no crescimento, viabilidade e nas capacidades de agregação, hemolítica e formadora de biofilme de isolados de *Staphylococcus aureus* resistentes (MRSA) ou não à meticilina.
- Avaliar a expressão de genes de virulência do sistema *agr* em células de *S. aureus* resistente à meticilina tratadas ou não com PgTeL.
- Determinar os efeitos de PgTeL no crescimento, viabilidade e capacidade de formação de biofilme de isolados de *Escherichia coli* produtores de β-lactamases.
- Analisar o potencial de sinergismo entre PgTeL e antibióticos frente os isolados de *E. coli*.
- Avaliar alterações ultraestruturais nas células de *C. albicans*, *C. krusei*, *S. aureus* e *E. coli* tratadas com PgTeL.

# 2 FUNDAMENTAÇÃO TEÓRICA

## 2.1 MICRORGANISMOS PATOGÊNICOS

Os microrganismos são seres que vivem em colônias ou como célula isolada, sendo representados por bactérias, vírus, fungos, pequenos protozoários e microalgas. São encontrados em diversos lugares, incluindo a pele, superfície dos objetos, microbiota humana e animal, nas plantas, no solo e no ar (JIA et al., 2018). Compreendem a maior biodiversidade da Terra e possuem papel funcional na base da cadeia alimentar em todos os ecossistemas da biosfera. Vivem em simbiose com organismos multicelulares, inclusive o homem e apresentam potencial biotecnológico para serem usados na indústria como biocatalisadores capazes de produzir compostos naturais, tais como: aromas, biossurfactantes, polissacarídeos e óleos microbianos (AMSELLEM et al., 2016; PESSÔA et al., 2019). Por outro lado, diversos microrganismos são causa de várias doenças em humanos, animais e plantas (JIA et al., 2018). Doenças infecciosas causam altas taxas de morbidade e mortalidade em todo mundo; cerca de 5,7 milhões de pessoas morrem anualmente (KALIA et al., 2018).

A descoberta de drogas antimicrobianas foi um passo revolucionário na luta contra as doenças infecciosas. A penicilina-G tem sido usada em terapia desde 1940; porém, o fenômeno da resistência microbiana sempre esteve atrelado ao combate a infecções, devido à forte pressão seletiva exercida pelos agentes antimicrobianos. Por exemplo, Alexander Fleming encontrou uma linhagem de *Staphylococcus aureus* resistente à penicilina-G já em 1944. A meticilina foi aprovada em 1958 e a primeira cepa resistente à meticilina foi isolada em 1961(DURAND et al., 2018). Existem dois tipos de resistência: a natural ou intrínseca, que ocorre quando uma droga não tem ação sobre um microrganismo devido a características inerentes a esse; e a adquirida, que se desenvolve quando uma cepa se torna resistente a uma droga para a qual era sensível antes (MOREHEAD; SCARBROUGH, 2018) .

Cepas multirresistentes têm se espalhado rapidamente em hospitais em todo o mundo devido à pressão seletiva exercida pelo uso não-racional de agentes antimicrobianos (HORVÁTH et al., 2016). Na década de 80, foram introduzidos na clínica as cefalosporinas de 3ª geração que possuíam atividade contra Enterobacteriaceae que eram resistentes aos antibióticos β-lactâmicos disponíveis; porém, o uso generalizado foi associado ao surgimento e disseminação de bacilos Gram-negativos, como da espécie *Klebsiella pneumoniae*, que

passaram a expressar enzimas chamadas β-lactamases de espectro estendido (ESBLs), capazes de clivar todos os β-lactâmicos. Atualmente, a resistência antimicrobiana é causa de 700.000 mortes por ano, e pesquisas indicam que nos próximos 30 anos esse número pode superar as mortes por câncer, que é considerada a maior causa de morte em todo o mundo (RICE, 2018; SEMRET; HARAOUI, 2019).

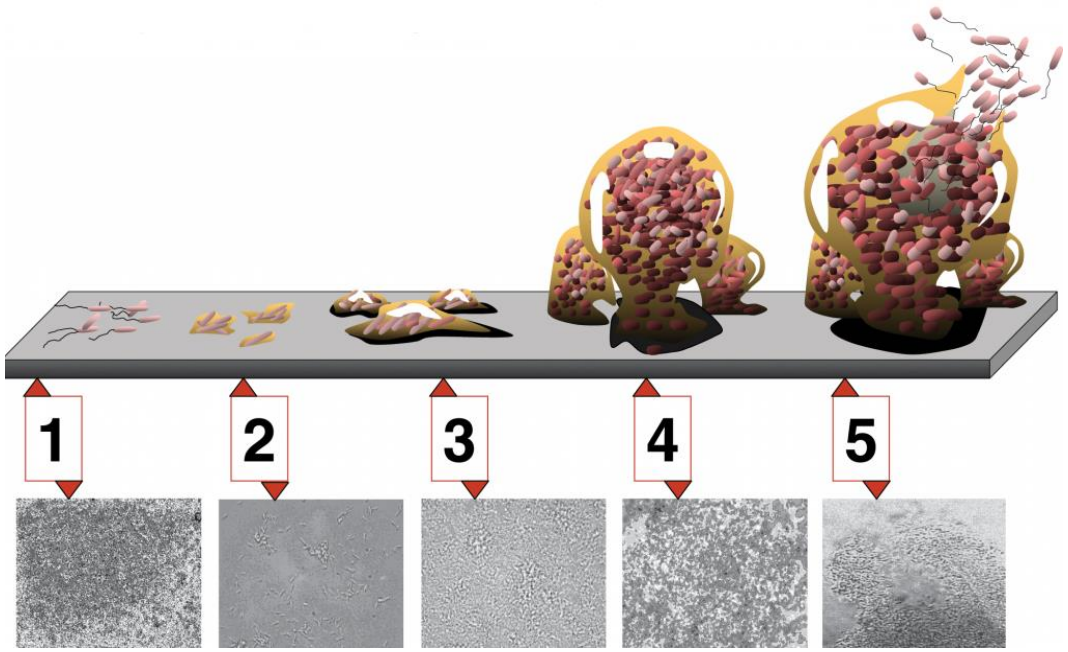
A patogenicidade é a capacidade de um microrganismo causar doença e, para isso ocorrer, é necessário que o microrganismo produza agentes de virulência, sendo capaz de vencer as defesas do hospedeiro e se multiplicar dentro desse organismo. Há várias etapas envolvidas no processo de infecção, tais como transmissão do agente infeccioso, seguida de colonização, aderência e invasão. As estratégias de adesão mais comum são as fímbrias, que geralmente são constituídas por resíduos de carboidratos, glicoproteínas ou glicolipídios, e adesinas. O processo de invasão se dá pelas invasinas que se ligam a receptores das células do hospedeiro. Curiosamente há algumas bactérias Gram-positivas como: *Clostridium tetani* e *Corynebacterium diphtheria* que não necessitam se ligar às superfícies das células hospedeiras para exercerem virulência e patogenicidade, atuando por toxinas mediadas por genes que causam danos ao tecido hospedeiro (ORJI et al., 2018).

Os patógenos bacterianos sintetizam diferentes compostos e estruturas que lhes permitem colonizar e danificar seus hospedeiros, denominados fatores de virulência. Dentre esses, podemos citar as toxinas, modulinas, agressinas, cápsulas, pigmentos, enzimas e formação de biofilme. A regulação de genes ligados a virulência é feita pelo sistema *quorum sensing* (QS) em resposta à presença de pequenas moléculas sinalizadoras (DEFOIRDT, 2018). Cerca de 65% a 80% destas infecções são causadas por bactérias formadoras de biofilme, cuja produção é regulada pelo sistema QS (KALIA et al., 2018).

Os microrganismos existem na forma planctônica (flutuação livre) ou séssil (aderidos a uma superfície biótica ou abiótica; nesse último caso formam os biofilmes. Os biofilmes são comunidades biológicas heterogêneas com um elevado grau de organização. Em seu inteior, as células crescem em agregados multicelulares que se encontram envolvidos em matrizes de substâncias poliméricas extracelulares (EPS) autoproduzidas, que fornecem proteção contra agressões ambientais, favorecem a difusão de água e nutrientes e a troca de material genético entre as células. A associação dos organismos em biofilmes confere maior proteção à ação de agentes antimicrobianos: microrganismos dentro de biofilmes toleram mil vezes maiores concentrações de antibióticos que as células planctônicas não só pela maior dificuldade de

penetração da droga como também pela maior produção de bombas de efluxo induzida pelo sistema QS. A formação de biofilmes consiste em várias etapas: adesão, desenvolvimento, maturação e dispersão (Figura 1). Os microrganimos de um biofilme se comunicam uns com os outros através do sistema QS para realizar funções coletivamente e atuam em resposta a alta densidade célular (LIN et al., 2017; MOURA et al., 2017; KALIA et al., 2018; GUO et al., 2019).

**Figura 1.** Representação esquemática da formação de biofilme em superfícies abióticas (1) fixação microbiana, (2) e (3) replicação e crescimento celular, secreção de substância polimérica extracelular (EPS), formação de colônias, (4) maturação e (5) desprendimento de microrganismo.




**Fonte:** LIN et al. (2017).

### 2.1.1 *Candida*

Os fungos são organismos não fotossintéticos que crescem como uma massa de filamentos entrelaçados e ramificados, conhecida como micélio (fungos filamentosos), ou na forma de leveduras, que são organismos unicelulares (fungos leveduriformes). Os fungos, em sua maioria, têm sua parede celular constituída por celulose ou quitina (JAWETZ et al., 1991). Esses microrganismos são ubíquos, encontrados no solo, água, vegetais, animais e detritos em geral (TRABULSI, 2000).

Os fungos do gênero *Candida*, pertencente à família Saccharomycetaceae, são polimorfos, podendo ser oportunistas (que causam doença em indivíduos que se encontram com atividade imunológica comprometida) ou patogênicos (quando estão sempre associados doenças) (NOBRE *et al.*, 2002). As infecções fúngicas tornaram-se uma das mais importante das infecções nosocomiais (causadas por bactérias adquiridas no hospital) e são comuns entre pacientes em estado grave e em terapia intensiva (SULEYMAN; ALANGADEN, 2016).

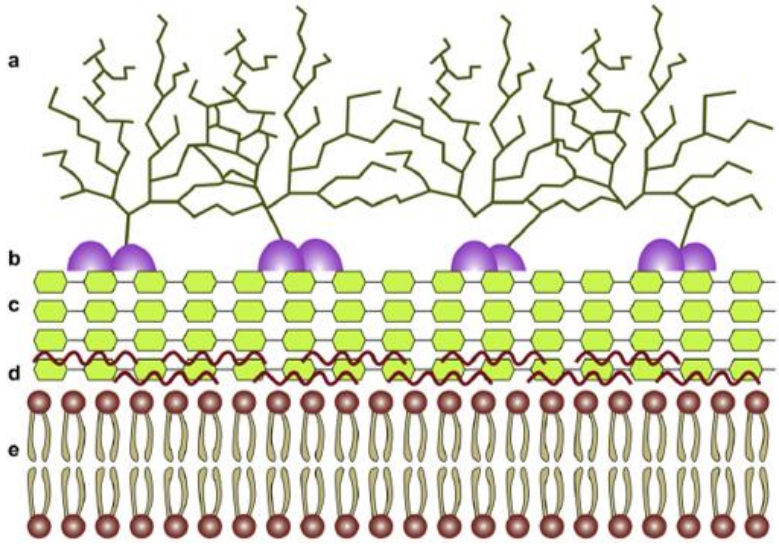
A espécie *C. albicans* faz parte da microbiota no trato gastrointestinal, cavidade oral, pele e vagina em humanos (TONG; TANG, 2017). É uma espécie de fungo polimórfico que cresce como leveduras unicelulares, hifas filamentosas ou pseudo-hifas e forma clamidósporos. A formação de hifas é importante no processo de invasão de tecidos e na expressão de fatores de virulência como adesinas, invasinas, hidrolases e uma toxina citolítica, a candidalisina (WILSON, 2019). *C. albicans* é o principal agente causador da candidíase, infecções superficiais na pele e mucosas ou infecções invasivas, principalmente em pacientes imunocomprometidos (MUSTHAFA et al., 2016; ABDELRAHMMAN et al., 2018). A forma sistêmica pode alcançar diversos órgãos, causando candidíase pulmonar e endocardite dentre outros, podendo levar os pacientes a óbito (MENEZES et al., 2008; TONG; TANG, 2017). Para candidíase invasiva existem estimativas de 250.000 casos e pelo menos 50 000 mortes por ano (WILSON, 2019).

Infecções fúngicas invasivas podem ser provocadas também por outras espécies de *Candida* tais como: *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* e *Candida krusei*. Foi relatado que essas espécies possuem alta capacidade de formar biofilme onde as hifas são incorporadas a uma matriz extracelular dificultando a ação de agentes antifúngicos (ABOUALIGALEHDARI et al., 2016; RODRÍGUEZ-CERDEIRA et al., 2018). *Candida krusei* é um dos agentes causadores de úlceras persistentes nas pernas de pacientes portadores de doença vascular crônica (JUD et al., 2017). Petrocheilou-Paschou et al. (2002) relataram caso de pneumonia nosocomial causada por *Candida krusei* em paciente transplantado.

A patogenicidade de espécies de *Candida* está associada a vários fatores como a adesão tecidual do hospedeiro, estresse ambiental, secreção de hidrolases e produção de biofilme. As hidrolases desempenham um papel crucial na adesão, penetração tecidual e invasão, que facilita o dano dos tecidos do hospedeiro pelas fosfolipases e hemolisinas produzidas (EL-HOUSSAINI et al., 2019). A parede celular (Figura 2) também é componente

essencial para a patogênese, é importante no crescimento, confere rigidez e a proteção à célula, além disso serve como ponto de contato entre a candida e o meio ambiente.

**Figura 2:** Estrutura da parede celular de *Candida* spp. (a) camada de fibrila; (b) manoproteínas; (c) glucanos; (d) complexo glucano-quitina e (e) membrana citoplasmática.




**Fonte:** RODRÍGUEZ-CERDEIRA et al.(2018)

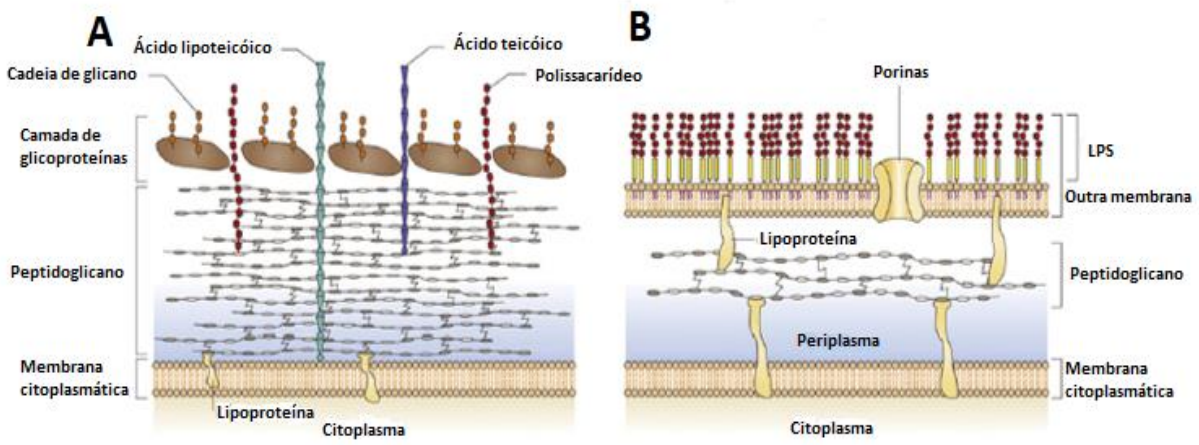
### 2.1.2. *Staphylococcus aureus*

As bactérias são organismos unicelulares e procariontes. Sua classificação é feita de acordo com a constituição da parede celular em dois grupos: Gram-positivas (+) e Gram-negativas (-) (Figura 3). As bactérias Gram-positivas apresentam em sua parede celular polissacarídeos, ácidos teicoicos e peptidoglicanos, enquanto as Gram-negativas apresentam na sua parede celular peptideoglicanos, lipídeos, proteínas e uma membrana adicional rica em lipopolissacarídeos (TRABULSI, 2000; HANKINS et al., 2012; MOLE, 2015).

*Staphylococcus* é um gênero de bactérias Gram-positivas que fazem parte da microbiota normal da pele humana (KOOKEN et al., 2012). No entanto, são os mais comuns microrganismos causadores de infecções superfíciais na pele produzindo erupções pruriginosas acompanhadas de coceira intensa; podem causar também infecções invasivas em regiões mais profundas como músculos, vísceras e tecidos ósseos; e ocasionalmente provocam problemas graves, tais como: choque séptico, endocardite, infecção pulmonar e meningite, além de promover intoxicações alimentares ao homem por intermédio da produção de enterotoxinas (KOOKEN et al., 2012; FOSTER et al., 2014; LEE et al., 2016). Das mais

de 30 espécies do gênero *Staphylococcus*, *S.aureus, S. epidermidis* e *S. saprophyticus* são as mais estudadas por se apresentarem com maior incidência em infecções humanas. A espécie *S. aureus* tem uma alta capacidade de formar biofilme (PERIASAMY *et al.*, 2012; FOSTER *et al.*, 2014).

**Figura 3:** Esquema de estruturas que compõem a parede celular de bactérias: (A) Gram-positivas e (B) Gram-negativas.



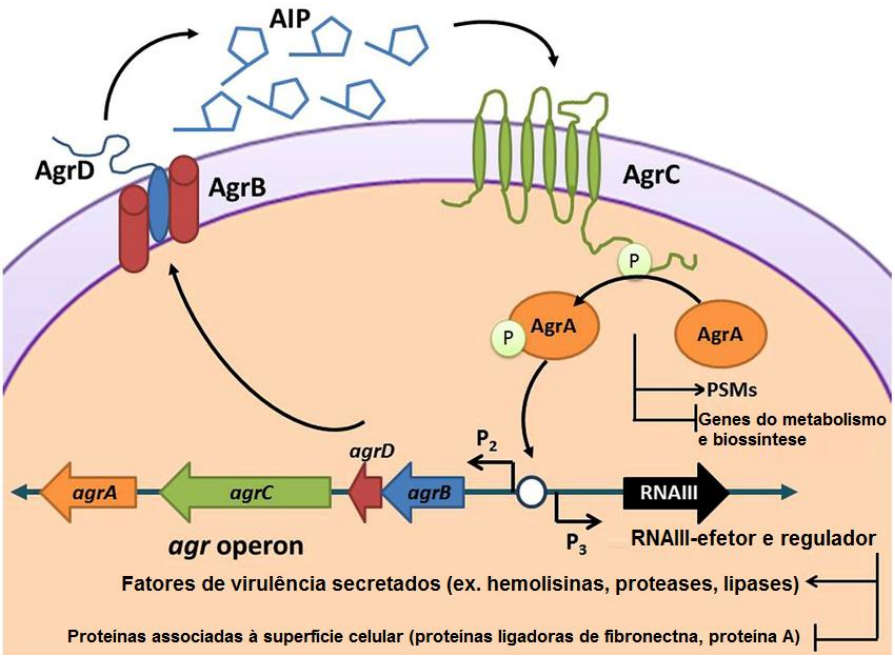

Fonte: DEPELTEAU; BRENZINGER; BRIEGEL (2019).

As infecções por *S. aureus* podem causar sérios danos ao organismo hospedeiro devido a vários fatores de virulência, como formação de biofilme, agregação, ação hemolítica e secreção de proteases (TANG et al., 2017; KONG et al., 2018). Hoerr et al. (2018) relataram que o aumento da idade, a resistência microbiana, as condições de tratamento intensivo (hemodiálise renal, imunossupressão e cateter venoso de longa duração) e aplicação de implantes cardíacos e próteses valvares levaram a incidências emergentes de endocardite infecciosa por *S. aureus* nos serviços de saúde. Infecção sanguínea por *S. aureus* é uma das principais causas de morbidade e mortalidade de pacientes em hemodiálise, particularmente aqueles dependentes de cateteres venosos centrais para acesso vascular (CONNOLLY et al., 2019)

*Staphylococcus aureus* tem alta capacidade de desenvolver resistência a uma gama de antibióticos, tais como meticilina, e tem causado infecções nosocomiais em todo mundo. Isolados de *S. aureus* resistentes à meticilina (MRSA) são continuamente relatados e demonstram resistência a múltiplos antibióticos. Linhagens MRSA têm se estabelecido como agentes patogênicos endêmicos em ambientes hospitalares e isso tem se tornado uma

preocupação devida a poucas opções de tratamento (NIELSEN et al., 2014; HASAN et al., 2016). *S. aureus* apresenta um sistema QS que regula os processos de colonização e a expressão de fatores de virulência (Figura 4). Esse sistema atua por meio do gene regulador acessório *agr*, o qual tem sua transição aumentada da fase exponencial para a fase estacionária e gera dois transcritos primários, RNA II e RNA III, que são regulados pelos promotores P2 e P3, respectivamente. O RNA II contém os genes *agrA*, *agrB*, *agrC* e *agrD*, que dão origem às chamadas proteínas reguladoras de genes acessórios (Agr). As proteínas AgrB e AgrD se unem para formar um peptídeo autoindutor (AIP) e a proteína AgrC é um receptor transmembrana para o AIP (YARWOOD; SCHLIEVERT, 2003; NOVICK, 2003 *apud* RODRIGUES, 2013). O AIP é secretado e se ligará à proteína AgrC, presente na membrana da própria célula que o produziu ou de outras células, o que resultará na ativação de AgrA, que é uma proteína capaz de se ligar ao DNA e promover: 1) feedback positivo nessa via descrita por meio da ativação de P2; 2) ativação de P3, que regula a transcrição da δ-hemolisina e do RNA III. Esse último é um RNA efetor que ativa expressão de várias proteínas de virulência e diminui a expressão de proteínas de superfície (BENNETT et al., 2015).

**Figura 4.** Representação esquemática do sistema do gene regulador acessório (Agr) de *Staphylococcus aureus*.




O locus *agr* é conhecido por conter dois transcritos divergentes denominados RNAII e RNAIII. O transcrito de RNAII é um operon de quatro genes, *agrBDCA*, que codificam os fatores necessários para sintetizar o peptído autoindutor (AIP) e ativar a cascata reguladora. AgrD é o peptídeo precursor do AIP, AgrB é uma protease de membrana envolvida na geração do AIP, AgrC é uma histidina quinase que é ativada pela ligação de AIP e AgrA é um regulador de resposta que induz a transcrição de RNAII e RNAIII através dos promotores P2 e P3,

respectivamente. A AgrA também promove diretamente a produção de PSMs (modulinas solúveis em fenol). O transcrito de RNAIII produz uma molécula reguladora de RNA que atua como efetor primário do sistema *agr*, regulando positivamente os fatores de virulência extracelulares e regulando negativamente as proteínas da superfície celular. Fonte: QUAVE; HORSWILL (2014).

### 2.1.3 *Escherichia coli*

Enterobacteriaceae é uma família de bactérias Gram-negativas que fazem parte da flora intestinal humana, mas são facilmente transmitidas por mãos, água e comida. Membros desse grupo como *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* spp. e *Acinetobacter* spp., podem causar sérias infecções com risco de vida tanto na comunidade como nos hospitais (TZOUVELEKIS *et al.*, 2012).

*Escherichia coli* forma colônias lisas, convexas e circulares e, apesar de fazer parte da microbiota intestinal, pode causar infecção do trato urinário, diarreia, meningite e septicemia (ABDELHAMID et al., 2018). As infecções nosocomiais causadas por essa bactéria são tratadas com antibióticos β-lactâmicos. Porém, nos últimos anos, tem surgido cepas produtoras de enzimas chamadas "β-lactamases de espectro estendido (ESBLs)", as quais são capazes de inativar estes antibióticos, o que tem interferido seriamente no sucesso do tratamento e na sobrevivência dos pacientes (HORVÁTH *et al.*, 2016).

As β-lactamases são classificadas de acordo com suas propriedades moleculares e/ou sequência de aminoácidos em diferentes grupos. As enzimas pertencentes às classes A, C e D utilizam um resíduo de serina no sítio ativo para hidrolisar a molécula β-lactâmica e alguns exemplos são as ESBLs, as β-lactamases AmpC e a oxacilinase, respectivamente. A classe B inclui as metalo-β-lactamases, cujo mecanismo de catálise envolve íons zinco presentes no sítio ativo (BUSH; JACOBY 2010; BUSH et al., 2013; JU et al., 2018).

Várias infecções causadas por bactérias resistentes têm sido associadas às enzimas CTX-M (família das ESBLs), que conferem resistência às oximino-cefalosporinas; CMY (família das β-lactamases AmpC), capazes de inativar as cefalosporinas de terceira geração; e metalo-β-lactamases que são capazes de hidrolisar todos os antibióticos β-lactâmicos (D'ANDREA et al., 2013; ESPINOSA et al., 2018; JU et al., 2018). A disseminação de cepas de Enterobacteriaceae produzindo estas enzimas é de grande preocupação, pois elas podem hidrolisar a maioria dos β-lactâmicos, incluindo cefalosporinas, cefamicinas, penicilinas, monobactamas, cefamicinas e carbapenêmicos. Neste cenário, há uma concentração de esforços na descoberta de novos antibióticos e inibidores de β-lactamases para serem usados

sozinhos ou em combinação com medicamentos padrão, a fim de minimizar o impacto da resistência (HORTON et al., 2016; ROTONDO; WRIGHT, 2017; SARAVANAN; RAMACHANDRAN; BARABADI, et al., 2018; TEPELI et al., 2018). ,

## 2.2 LECTINAS

### 2.2.1. Características gerais

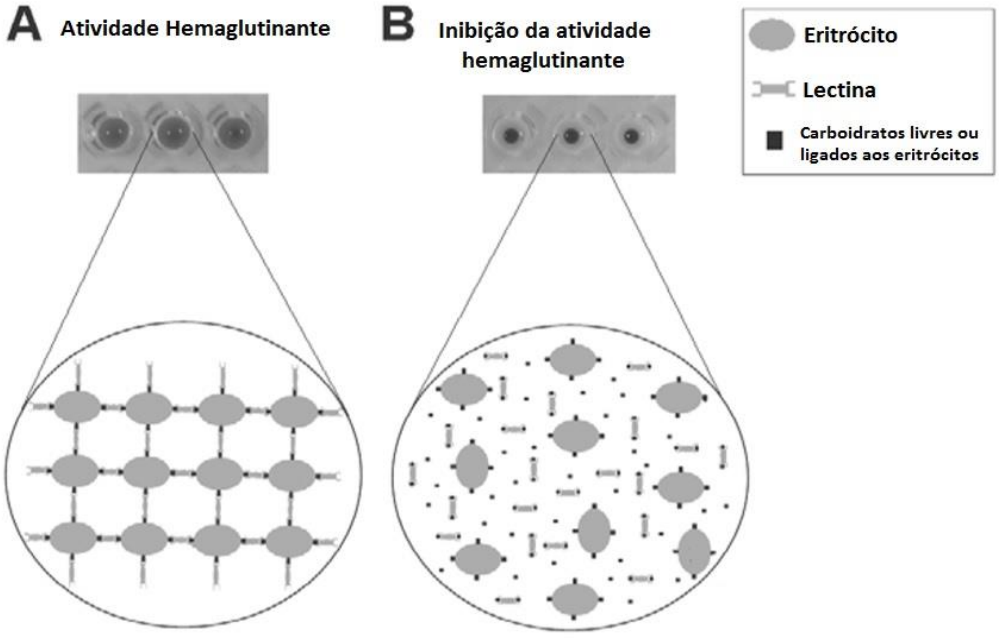
Lectinas constituem um grupo diversificado de proteínas que são caracterizadas por conter pelo menos um domínio não catalítico capaz de se ligar seletiva e reversivelmente a carboidratos (SILVA et al., 2014; COELHO et al., 2017). O primeiro relato sobre lectinas foi feito em 1888 quando Stillmark, ao estudar a toxicidade de extratos de *Ricinus communis* (mamona), observou a capacidade de aglutinar eritrócitos e a atribuiu à presença de uma proteína, a ricina (KENNEDY et al., 1995). Pouco tempo depois, outra hemaglutinina, chamada abrina, foi encontrada em sementes de *Abrus precatorius* (jequiriti). Entretanto, o estudo sobre estas proteínas só começou a ganhar ímpeto com os trabalhos de Sharon e Lis em 1972, abrindo uma vasta área de aplicação para essas proteínas (GABOR et al., 2004; BIES; LEHR; WOODLEY, 2004; JUAN et al., 2017).

O termo lectina (originado do latim *lectus*, que significa selecionado) refere-se à habilidade dessas proteínas de ligarem-se seletivamente aos carboidratos (PAIVA et al., 2011). Não são produtos de uma resposta imune, o que as difere de anticorpos anticarboidratos que também aglutinam células. Ainda, os anticorpos são estruturalmente similares, enquanto as lectinas diferem entre si quanto à composição aminoacídica, requerimentos de metais, massa molecular e estrutura tridimensional (PAIVA et al., 2013).

As lectinas possuem ampla distribuição na natureza, sendo encontradas em microrganismos (SINGH; WALIA; KENNEDY, 2018), veneno de serpentes (CASTANHEIRA et al., 2017), esponjas (MARQUES et al., 2018) e algas (ABREU et al., 2018). Em plantas já foram isoladas de diversos tecidos, tais como: cerne (SÁ et al., 2008), casca (COSTA et al., 2018), sementes (SILVA et al., 2012; CARVALHO et al., 2015), folhas (GOMES et al., 2013; PROCÓPIO et al., 2017b), flores (CHOLAK et al., 2016), raízes (AGRAWAL et al., 2011; SOUZA et al., 2011) e rizomas (BISWAS; CHATTOPADHYAYA, 2015).

As lectinas são, em sua maioria, di ou polivalentes e são capazes de interagir por ligações não covalentes (ligação de hidrogênio, forças de van der Waals e interações hidrofóbicas, com carboidratos ou glicoproteínas que se apresentam em solução ou ligados à membrana celular (COELHO et al., 2017). A presença de lectinas em uma amostra pode ser facilmente detectada a partir de ensaios de aglutinação, nos quais elas interagem com carboidratos da superfície celular através de seus sítios, formando diversas ligações reversíveis entre as células. O ensaio mais comumente utilizado é o de atividade hemaglutinante (AH) (Figura 5A). A rede formada entre os eritrócitos constitui o fenômeno de hemaglutinação. Os eritrócitos utilizados podem ser de humanos ou de animais, os quais podem ser tratados enzimaticamente (com tripsina, papaína, entre outras), aumentando a sensibilidade das células à lectina, ou quimicamente (com glutaraldeído ou formaldeído), para fixação dos eritrócitos e possibilidade de armazenamento a maior prazo (PAIVA et al., 2010; NAPOLEÃO et al., 2013).

**Figura 5.** Aspectos do ensaio de hemaglutinação (A) e de inibição da hemaglutinação (B) em placas de microtitulação. Os círculos mostram representações esquemáticas da rede de eritrócitos formada pela lectina (A) e inibição da atividade hemaglutinante por carboidratos livres (B).




Fonte: PAIVA *et al*. (2013)

Alguns compostos, tais como taninos, lipídios ou íons bivalentes podem dispersar os eritrócitos dando um falso resultado, visualmente similar ao da hemaglutinação. Para assegurar que houve aglutinação e que o agente aglutinante é uma lectina, são necessários ensaios de inibição da AH (Figura 5B). Nesse ensaio, a amostra é incubada em uma solução contendo carboidratos ou glicoproteínas livres previamente à incubação com eritrócitos. Os sítios de reconhecimento de carboidratos das lectinas serão ocupados pelos carboidratos ou glicoproteínas livres em solução e não poderão interagir com os açúcares das superfícies celulares, ocorrendo a precipitação dos eritrócitos. Este ensaio pode ser utilizado para determinar a especificidade da lectina a diferentes carboidratos (NAPOLEÃO et al., 2011; PAIVA et al., 2013). A grande maioria das lectinas de plantas apresenta dois ou mais sítios de ligação a carboidratos simples (monossacarídeos) ou complexos (oligossacarídeos e glicanas), tais como manose, N-acetilglicosamina e ácidos N-glucurônico, galacturônico, xilurônico, L-idurônico, siálico e N-acetilmurâmico (AMBROSI et al., 2005; MITCHELL; RAMESSAR; O'KEEFE, 2017).

Métodos comuns utilizados na purificação de proteínas são aplicados para purificar as lectinas. O primeiro passo para a purificação consiste na extração de proteínas. Extratos podem ser feitos utilizando solução salina, como no caso do isolamento das lectina de rizoma de *Microgramma vaccinifolia* (SANTANA et al., 2012), de folhas de *Calliandra surinamensis* (PROCÓPIO et al., 2017b) e da raiz de *Portulaca elatior* (SILVA et al., 2019); soluções-tampão, como na obtenção das lectinas de sementes de *Salvia bogotensis* (VEGA; PÉREZ, 2006), do feijão preto (*Phaseolus vulgaris*) (HE et al., 2013) e da casca de *Genipa americana* (COSTA et al., 2018); ou água destilada, como na obtenção da lectina de sementes de *Moringa oleifera* (SANTOS et al., 2005).

Para a preparação do extrato, o material pode ser lisado ou triturado e mantido sob agitação constante em tempo e temperatura estabelecidos. Este processo resulta no aumento da solubilidade das proteínas do material. A partir do extrato bruto, as proteínas podem ser parcialmente purificadas por precipitação frente a diferentes concentrações de sais, solventes orgânicos e polímeros não-iônicos. A precipitação salina é mais comumente utilizada por permitir que a conformação nativa da proteína seja mantida após o processo. O sulfato de amônio, altamente hidrofílico, remove a camada de solvatação das proteínas fazendo com que as mesmas precipitem (NELSON; LEHNINGER; COX, 2008; CAGLIARI; KREMER; PINTO, 2018). As lectinas parcialmente purificadas pelo fracionamento salino são então

submetidas ao processo de diálise em membranas semipermeáveis e podem ser isoladas através de métodos cromatográficos que utilizam matrizes cuja escolha dependerá da especificidade a carboidratos (cromatografia de afinidade), carga líquida (cromatografia de troca iônica) ou tamanho (cromatografia de gel filtração) da proteína (LAM; NG, 2011; QU et al., 2015). O isolamento de lectinas é estimulado pela sua potencial utilização em diversas áreas da Medicina Clínica, Agricultura e na indústria farmacêutica, bem como é fundamental para o estudo estrutural e funcional (VARROT; BASHEER; IMBERTY, 2013; JUAN et al., 2017).

As lectinas apresentam uma grande variedade estrutural, mas uma característica comum a todas é a presença de ao menos um sítio específico de ligação a carboidratos, que corresponde ao chamado domínio de reconhecimento de carboidrato (GABIUS 1994; ZANETTI, 2007; LAM; NG, 2011; CARVALHO et al., 2018). As lectinas de plantas têm sido subdivididas em merolectinas, hololectinas, quimerolectinas e superlectinas (PEUMANS; VAN DAMME, 1998). Merolectinas são aquelas que possuem apenas um domínio para ligação a carboidratos e, por isso, não podem precipitar glicoconjugados ou aglutinar células. Hololectinas contêm dois ou mais domínios idênticos de ligação a açúcares, aglutinam células e/ou precipitam glicoconjugados. A maioria das lectinas de plantas pertence a esse grupo. Quimerolectinas são proteínas com um ou mais domínios de ligação a carboidratos e um domínio não-relacionado, o qual pode ter uma atividade enzimática bem definida ou outra atividade biológica, agindo independentemente dos domínios de ligação a carboidratos. Superlectinas consistem de pelo menos dois domínios de ligação a açúcares diferentes.

Algumas lectinas requerem a presença de íons bivalentes para exercerem sua função biológica. Lectinas que não requerem íons metálicos já possuem a conformação estrutural necessária para o reconhecimento de carboidratos (QU et al., 2015). As lectinas, assim como outras proteínas, podem apresentar uma porção glicídica associada. Essa porção aumenta a estabilidade da lectina, reduzindo a susceptibilidade à degradação proteolítica e à desnaturação por ureia e alterações de pH e temperatura, bem como influencia na associação com outras moléculas, na solubilidade e viscosidade em solução aquosa (KILPATRICK, 1986; ALBUQUERQUE et al., 2012; ARAÚJO et al., 2012).

### 2.2.2 Propriedades biológicas e potencial biotecnológico de lectinas

As lectinas, por terem a habilidade de se ligar a mono e oligossacarídeos, apresentam uma variedade de efeitos biológicos. As lectinas podem, por exemplo, induzir apoptose em células tumorais, bloquear infecções causadas por microrganismos, regular o processo de adesão e migração de células bacterianas e desempenhar um importante papel no sistema imune por reconhecer carboidratos que são exclusivos de patógenos (DIAS et al., 2015).

Lectinas isoladas de *Rana catesbeiana* e *Abelmoschus esulentus* são proteínas que possuem atividade antitumoral por induzirem morte por apoptose em células mesoteliais malignamente diferenciadas e linhagens de células malignas mamárias (MCF-7), respectivamente (TATSUTA et al., 2014; MONTE et al., 2014). Uma lectina isolada do rizoma de *Microgramma vacciniifolia* apresentou atividade antitumoral sobre células de carcinoma mucoepidermóide de pulmão e nenhuma citotoxidade para células mononucleares de sangue periférico humano (ALBUQUERQUE et al., 2014b). Já a lectina isolada das folhas de *Schinus terebinthifolius* apresentou atividade antitumoral contra sarcoma 180 em camundongos, sendo capaz de induzir apoptose das células tumorais (RAMOS et al., 2019).

Várias lectinas apresentam atividade antiviral, sendo sugerido que a forma de atuação seja através de ligação com glicoproteinas virais que participam no processo de invasão celular (KOHARUDIN; FUREY; GRONENBORN, 2011; MITCHELL; RAMESSAR; O'KEEFE, 2017). São exemplos de lectinas com atividade antiviral a jacalina (isolada das sementes de *Artocarpus heterophyllus*), a concanavalina A (isolada de *Canavalia ensiformis*), a lectina de *Musa acuminata* (banana) e a lectina da raiz de *Myrianthus holstii*, que possuem atividade contra vírus envelopados como o HIV (AKKOUH et al., 2015). A lectina isolada da cianobactéria *Scytonema varium* apresentou potente atividade *in vitro* e *in vivo* contra o vírus do Ebola Zaire (GARRISON et al., 2014) e uma lectina ligadora de quitina isolada de tamarindo diminuiu a infecção viral causada pelo vírus CHIKV, que causa a chikungunya (KAUR et al., 2019).

Devido ao fato de algumas lectinas possuírem habilidade para mediar mucoadesão, citoadesão e citoinvasão de drogas, essas moléculas têm sido exploradas em sistemas de liberação de drogas (GABOR *et al.*, 2004). As lectinas de folhas de *Bauhinia monandra* (pata-de-vaca) e de *Lens culinaris* (lentilha) foram incorporadas e também adsorvidas na

superfície de nanopartículas, mostrando serem ferramentas potenciais em medicamentos de administração oral com liberação controlada (RODRIGUES et al., 2003).

Lectinas ligadoras de manose (MBL) desempenham um importante papel na resposta imune como um receptor padrão de reconhecimento. Vários estudos têm mostrado que as MBLs de humanos e aves participam na proteção do hospedeiro contra infecções virais como, por exemplo, o vírus da bronquite infecciosa (IBV), que possui grande importância econômica no setor avícola. A MBL desempenha um papel importante contra a infecção causada por IBV por induzir a produção de anticorpos específicos, o que pode ser explorado como estratégia para otimizar uma vacina contra IBV (KJÆRUP et al., 2014). Também tem sido descrito na literatura que as colectinas, uma família de lectinas encontradas no pulmão de mamíferos, participam na proteção contra alérgenos e patógenos respiratórios, sendo importantes ferramentas no estudo de doenças alérgicas (SALAZAR *et al*., 2013).

Interações de lectinas com porções glicídicas da superfície celular podem desencadear eventos de morte, mas, também, induzir a proliferação celular ou a expressão de proteínas específicas. Isto tem sido demonstrado em testes *in vitro* para avaliar a proliferação de linfócitos e esplenócitos e promover imunomodulação. Vários estudos têm mostrado lectinas com atividade mitogênica e/ou imunomoduladora, dentre elas podemos citar: a isolada da babosa, *Aloe arborescens*, das sementes de *Cratylia mollis*, do cogumelo *Pleurotus ferulae*, de fronde de *Microgramma vacciniifolia*, do fungo *Penicillium duclauxii*, das sementes de *Phaseolus vulgaris*, do micélio de *Rhizoctonia bataticola* e dos corpos de frutificação de *Hygrophorus russula* (KOIKE et al., 1995; MACIEL et al., 2004; XU et al., 2014; PATRIOTA et al., 2017; SINGH; WALIA; KENNEDY, 2018; CARVALHO et al., 2018). Lectinas têm sido reportadas como agentes imunomoduladores atuando na modulação da secreção de citocinas, óxido nítrico e na diferenciação de linfócitos podendo ser ferramentas para auxiliar no combate de doenças como infecções microbianas e câncer (PATRIOTA et al., 2017; BRITO et al., 2017; PROCÓPIO et al., 2018). Aranda-Souza et al. (2018) mostraram que a lectina isolada do veneno de *Bothrops leucurus* apresentou efeitos leishmanicida e imunomodulatório sobre macrófagos infectados com *L. braziliensis* e *L. amazonenses*. Já a lectina isolada do fruto da jaqueira (*Artocarpus integrifolia)* mostrou efeito imunoestimulante na imunização de camundongos contra neosporose induzindo um efeito protetor por estimular a liberação de citocinas pró-inflamatórias, revelando seu potencial para formulações de vacina contra *Neospora caninum* (CARDOSO et al., 2011).

A atividade antinociceptiva de lectinas também tem sido descrita. Lectina ligadora de galactose isolada de folhas *Bauhinia monandra* apresentou propriedades anti-inflamatória e antinociceptiva (CAMPOS et al., 2016) e a lectina ligadora de manose isolada de sementes de *Lonchocarpus campestris* apresentou efeito inibitório na nocicepção inflamatória via mecanismos periféricos associados a eventos celulares de inflamação (PIRES et al., 2019).

Lectinas têm sido utilizadas na detecção e separação de glicoconjugados. A lectina de *Cratylia mollis* (feijão camaratu), quando imobilizada em suporte Sepharose CL-4B, foi utilizada para purificar a enzima lecitina colesterol aciltransferase, importante no metabolismo do colesterol (LIMA et al., 1997) e inibidor de tripsina presente em sementes *Echinodorus paniculatos* (PAIVA et al., 2003). Matrizes contendo lectinas de *Cratylia mollis* (Cramoll 1,2,3-Sepharose e Cramoll 3-Sepharose) foram empregadas no isolamento de glicoproteínas de soro fetal bovino, colostro humano, clara de ovo e plasma sanguíneo (NAPOLEÃO et al., 2013). A lectina da casca de *Crataeva tapia*, imobilizada em Sepharose CL–4B, foi capaz de ligar glicoproteínas de interesse comercial (ARAÚJO et al., 2012).

Lectinas também podem ser usadas na determinação de tipos sanguíneos como um método simples e de baixo custo (KHAN et al., 2002; COLLINS et al., 2017), no diagnóstico de processos de desenvolvimento, diferenciação e transformação neoplásica (LI et al., 2008; RAMBARUTH; DWEK, 2011) e no tratamento de condições pré-cancerosas como a colite ulcerativa, através de conjugação com drogas (WROBLEWSKI et al., 2001). Devido a sua capacidade de reconhecer carboidratos especificamente, lectinas têm sido usadas como marcadores histoquímicos para processos inflamatórios e em interações parasita-hospedeiro (MELO-JUNIOR et al., 2006; MELO-JUNIOR et al., 2008).

Lectinas conjugadas como nanossondas (por exemplo, pontos quânticos) podem ser usadas para análise de expressão de carboidratos, podendo ser úteis em pesquisas relacionadas ao câncer e patogenicidade de microrganismos por fornecer informações sobre mudanças na glicosilação celular (FONTES et al., 2012; KARAKOTI et al., 2015). Hovhannisyan et al. (2017) conjugaram pontos quânticos de CdSe com uma lectina isolada da casca do ovo de galinha para detecção de *Staphylococcus aureus*. Pontos quânticos de CdTe foram conjugados às lectinas concanavalina A e Cramoll para potencial uso como sondas moleculares fluorescentes na detecção de sacarídeos na superfície celular de *Candida albicans* (TENÓRIO et al., 2015; CUNHA et al., 2018).

2.2.2.1 Atividade antimicrobiana de lectinas

O uso excessivo e indiscriminado de agentes antimicrobianos tem levado à seleção de microrganismos cada vez mais resistentes, tornando necessária a busca por novas substâncias com propriedades antimicrobianas (YIM et al., 2013; RAMOS et al., 2014; RICE, 2018). Peptideos e proteínas com ação antimicrobiana são candidatos promissores para uso como novos agentes antibióticos. As lectinas merecem destaque, uma vez que são capazes de afetar a fisiologia dos microrganismos de diferentes formas ao interagirem com carboidratos da parede celular de bactérias e fungos, promovendo inibição do crescimento e morte, dentre outros efeitos (GOMES et al., 2014; PROCÓPIO et al. 2017a; COELHO et al., 2018).

As lectinas possuem a capacidade de se ligar especificamente a hifas fúngicas, impedindo o consumo de nutrientes e a incorporação de precursores necessários para o crescimento do fungo e perturbando a síntese e/ou a deposição de quitina na parede celular (YAO et al., 2010). A superfície bacteriana é revestida com glicoconjugados tais como glicoproteínas, glicolipideos, glicosaminoglicanos e proteoglicanos e esses carboidratos constituem alvos de ligação de lectinas que podem causar inibição do crescimento (PAIVA et al., 2010; CARVALHO et al., 2015). Além disso, lectinas podem afetar a permeabilidade da célula por danificar a integridade da membrana, causando a morte do microrganismo pelo extravasamento do conteúdo celular (MOURA et al., 2015), bem como alterar o metabolismo energético por interferir no ciclo dos ácidos tricarboxílicos e na via da hexose monofosfato afetando a cadeia respiratória e produção de energia pelas células bacterianas (QU et al., 2015).

A quitina é um polissacarídeo de ocorrência natural composto por monômeros de *N*-acetilglicosamina. Lectinas ligadoras de quitina têm sido isoladas de diversas fontes, incluindo bactérias, insetos, plantas e mamíferos. Muitas delas apresentam atividade antifúngica, uma vez que a quitina é o componente-chave da parede celular de fungos (SITOHY; DOHEIM; BADR, 2007; ALBUQUERQUE et al., 2014a). A lectina ligadora de quitina do rizoma de *Setcreasea purpurea* foi capaz de inibir a germinação de *Rhizoctonia solani, Penicillium italicum, Sclerotinia sclerotiorum* e *Helminthosporiun maydis* (YAO *et al.*, 2010). Gomes et al. (2013) isolaram uma lectina ligadora de quitina a partir de folhas de *Schinus terebinthifolius* com ação fungicida contra *Candida albicans* e bactericida *contra Staphylococcus aureus*, Hasan et al. (2014) purificaram uma lectina ligadora de quitina de

*Solanum tuberosum* com atividade antifúngica contra *Rhizopus* spp., *Penicillium* spp. e *Aspergillus niger*. Silva *et al.* (2019) isolaram uma lectina ligadora de quitina a partir da raiz de *Portulaca elatior* com ação fungicida contra *Candida albicans, Candida parapsilosis, Candida krusei* e *Candida tropicalis*.

Lectinas com outras especificidades a carboidratos também mostraram atividade antifúngica. Lectina ligadora de galactose isolada de raízes secundárias de *Bauhinia monandra* apresentou atividade contra espécies de *Fusarium* (SOUZA et al., 2011). A lectina ligadora de N-acetilglicosamina isolada do feijão *Phaseolus vulgaris* inibiu o crescimento micelial de *Valsa mali*, fungo causador de tumores em macieras e pereiras (ANG et al., 2014) e lectina isolada do líquen *Cladonia verticillaris* inibiu o crescimento do fungo *Trichophyton rubrum* (RAMOS et al., 2014). Lectina ligadora de manose isolada da cianobactéria *Scytonema varium* mostrou ação antifúngica contra *Cryptococcus neoformans* var. *neoformans* e *Cryptococcus gattii* inibindo o crescimento por ligação à parede celular (JONES et al., 2017).

Lectinas antibacterianas já foram isoladas de animais como *Bothrops leucurus e Crassostrea hongkongensis*, líquens como *C. verticillaris* e plantas tais como: *Microgramma vaccinifolia, S. terebinthifolius* e *P. elatior* (NUNES et al., 2011; HE et al., 2011; RAMOS et al., 2014; SANTANA et al., 2012; GOMES et al., 2013; SILVA et al., 2019). Lectina de sementes de *Lablab purpureus* foi ativa contra *Salmonella typhi* e sementes de *Apuleia leiocarpa* contêm lectina com ação contra bactérias Gram-positivas e Gram-negativas causadoras de doenças em humanos e plantas (SAHA et al., 2014; CARVALHO et al., 2015). Ferreira et al. (2011) e Moura et al. (2015) demonstraram o efeito da lectina solúvel em água isolada de sementes de *Moringa oleifera* (WSMoL) no crescimento e sobrevivência *Staphylococcus aureus* e *Escherichia coli* e no crescimento, sobrevivência e permeabilidade celular de *Bacillus sp., Bacillus cereus, Bacillus pumillus, Bacillus megaterium, Micrococcus sp., Pseudomonas sp., Pseudomonas fluorescens Pseudomonas stutzeri e Serratia marcescens*. A lectina isolada de *Archidendron jiringa* foi capaz de inibir o crescimento de bactérias Gram-positivas possivelmente pela interação da lectina com ácido murâmico, N-acetilmurâmico e carboidratos presentes na parede celular bacteriana (CHARUNGCHITRAK et al., 2011).

As lectinas isoladas do veneno de *Bothrops jararacuçu* (KLEIN et al., 2015), de folhas de *Calliandra surinamensis* (PROCÓPIO et al., 2017b), de sementes de *Moringa oleífera*

(MOURA, et al., 2017) e da inflorescência de *Alpinia purpurata* (FERREIRA et al., 2018) mostraram ação antibiofilme contra *Staphylococcus aureus*.

Paralelamente aos estudos de atividade antimicrobiana, alguns trabalhos demonstram que a habilidade de lectinas em reconhecer especificamente carboidratos permite o emprego dessas proteínas como sondas-diagnóstico para identificação de bactérias patogênicas (GAO *et al.*, 2010). Athamna et al. (2006) analisaram os diferentes padrões de aglutinação de bactérias promovidas por 23 lectinas e mostraram que a interação lectina-bactéria é uma boa ferramenta para identificar rapidamente espécies de *Mycobacterium*. Campuzano et al. (2011) desenvolveram uma sonda contendo a lectina concavalina A para detecção e isolamento de *Escherichia coli* com aplicação em diversos setores como: segurança alimentar, diagnóstico médico e avaliação da qualidade de água. Lectina também podem ser usadas como sondas celulares que podem servir como carreadores de agentes antifúngicos utilizando, como alvos específicos, os carboidratos existentes na superfície da célula do microrganismo (LEAL et al., 2007; SHIOKAWA; YAMASAKI; SAIJO, 2017).

### 2.3 *Punica granatum* L.

A família Lythraceae pertence à ordem Myrtales, segundo a APG III (2009), e possui distribuição pantropical, com algumas espécies nativas de regiões temperadas. Compreende aproximadamente 30 gêneros e 600 espécies. Diversas espécies são cultivadas no Brasil, como a romãzeira (*Punica granatum* L.) (MOSTAFA et al., 2018). Essa planta é nativa da região norte da Índia, mas é encontrada, além do Brasil, em países como Argentina, Chile, Estados Unidos e África do Sul (HAGHAYEGHI et al., 2013; SILVA et al., 2013). É uma planta caducifólia, sendo uma excelente árvore para o cultivo em zona árida por sua resistência a condições de seca. Tolera ainda solos alcalinos, o calor do verão ou temperaturas mínimas de inverno de até -12 ºC (MENEZES et al., 2008; SHAYGANNIA et al., 2016; ERKAN; DOGAN, 2018). As plantas maduras (Figura 6A) têm geralmente de 1,5 a 8 metros de altura. Tem folhas simples e inflorescências constituídas por flores de corola vermelha alaranjada e cálices esverdeados, duros e coriáceos (Figura 6B) (APG II, 2003; ZAGO et al., 2009; ERKAN; DOGAN, 2018).

A romã é uma infrutescência (Figura 6C) conhecida em alguns países como fruto do Éden por possuir sabor agradável e proporcionar vários benefícios à saúde (AKHTAR et al.,

2015). É um fruto do tipo baga, redondo, de casca coriácea, amarela ou avermelhada (Figura 6D), contendo inúmeras sementes, com sabor doce e levemente acidulado (MENEZES et al., 2008; ERKAN; DOGAN, 2018). A romã apresenta um pericarpo carnoso-coriáceo e é dividida internamente em muitas lojas (Figura 6E), contendo diversas sementes (Figura 6F). A testa (tecido originado do óvulo) é formada por células polposas e é dividida em uma mesotesta esclerótica e uma sarcotesta translúcida (parte mais externa), a qual envolve as sementes (Figura 6G). A sarcotesta apresenta coloração róseo, é comestível e pode ser removida física, química ou mecanicamente (LOPES et al., 2001), sendo utilizada na obtenção do chamado "suco de romã" (Figura 6H).

Pesquisas têm demonstrado que diferentes partes de *P. granatum* constituem um reservatório de compostos bioativos o que justifica seu uso tão amplo na medicina popular. Estudos comprovam efeitos terapêuticos encontrados em extratos, bem como em moléculas isoladas de praticamente todos os tecidos da planta (BEKIR et al., 2013; PANDE; AKOH, 2016). Nos últimos anos, tem sido mostrada a importância dos alimentos funcionais de origem vegetal por seus vários benefícios à saúde, estando o fruto da romãzeira incluído na lista desses alimentos devido às propriedades terapêuticas (VIUDA-MARTOS et al., 2010; SAEED et al., 2018).

**Figura 6.** *Punica granatum* L. (A) Hábito da planta; (B) inflorescência; (C) infrutescência em desenvolvimento; (D) fruto maduro; (E) fruto aberto, evidenciando as lojas onde as sementes estão alojadas; (F) e (G) sementes envoltas pela sarcotesta e (H) suco.


### 2.3.1 Propriedades biológicas de *P. granatum*

Extratos de casca e sementes e o suco de *P. granatum* são ricos em polifenois que têm sido relacionados à sua alta atividade antioxidante (DERAKHSHAN et al., 2018). Extrato metanólico de casca apresentou ação antiplasmodial, estrogênica, melhorou o desenvolvimento cognitivos em camundongos, reduziu proliferação e induziu apoptose em células de câncer mamário (MCF-7) e apresentou propriedade antioxidante (DELL'AGLI *et al.*, 2009; ADIGA *et al.*, 2010; SATPATHY; PATRA; PUROHIT, 2013; AKHTAR et al., 2015). Extrato etanólico de sementes apresentou atividade antioxidante e antiproliferativa em células LNCaP encontradas em câncer de próstata (AL-HUQAIL; ELGAALY; IBRAHIM, 2015; LUCCI et al., 2015) e nanoemulsões de óleo extraído das sementes apresentaram ação antioxidante, fotoprotetora e citotóxica para células tumorais da coluna cervical, colorretal e pulmonar (HeLa, LS174 e A549) (BACCARIN et al., 2015; ĐURĐEVIĆ et al., 2018). Um

polissacarídeo isolado da casca apresentou efeitos hepatoprotetor e antioxidante (ZHAI et al., 2018).

Extrato metanólico de folhas foi eficiente no combate à dislipidemia decorrente da obesidade e apresentaram ação protetora na nefropatia diabética (LEI et al., 2007; PATEL; BANDAWANE; MHETRE, 2014; MESTRY et al., 2017). Extratos metanólico e etanólico de folhas possuem compostos com atividade antioxidante, anti-inflamatória, anticolinesterase e antiproliferativa em células MCF-7 (BEKIR et al., 2013; MESTRY et al., 2017). Fração etanólica de folhas apresentou ação anti-hiperglicêmica, anti-hiperlipidêmica e antioxidante (PATEL; BANDAWANE; MHETRE, 2014). Extratos aquoso e etanólico de flores mostraram ação contra a demência associada à doença de Alzheimer (BHASKAR; KUMAR, 2012; BEKIR et al., 2016).

São diversos os relatos as propriedades antimicrobianas de *P. granatum*. O extrato acetônico de casca apresentou atividade antifúngica contra *Aspergillus flavus, Candida albicans Candida krusei, Candida guilliermondii, Epidermophyton floccosum, Epidermophyton floccosum, Trichophyton verrucosum* e *Trichophyton mentagrophytes.* (AKROUM, 2017). Extratos metanólico e aquoso da casca mostraram ação antibacteriana contra *Staphylococcus aureus, Escherichia coli, Salmonella enteritidis, Streptococcus mutans* e antifúngica contra *Aspergillus parasiticus* (AL-ZOREKY et al., 2009; ORAK *et al.*, 2011) e contra patógenos orais causadores da cárie, estomatites e doenças periodontais (ABDOLLAHZADEH et al., 2011; GULUBE; PATEL, 2016). A infusão da casca é empregada no tratamento de aftas, diarreia, estomatite, infecções da garganta e processos inflamatórios da mucosa oral, sendo utilizada como adstringente, germicida e antisséptico bucal (ORAK et al., 2011; DELL'AGLI et al., 2013; GULUBE; PATEL, 2016). O pó da casca mostrou-se um componente eficaz na produção de filmes antimicrobianos (ALI et al., 2018). Outros metabólitos secundários, como punicalina, punicalagina e corilagina 3, extraídos da casca, apresentaram atividade antimicrobiana contra as bactérias *Staphylococcus aureus* e *Pseudomonas aeruginosa* e fungos dermatófitos e atividade nematicida (ZHOU et al., 2010; FOSS et al., 2014; GUO et al., 2016).

2.3.1.1 Propriedades biológicas do suco (sarcotesta) de *P. granatum*

Nos últimos anos, houve um aumento significativo no consumo mundial do suco da romã devido aos potenciais benefícios à saúde que lhe são atribuídos. Ele é rico em polifenóis

(antocianinas e taninos) e ácido ascórbico (SÁNCHEZ-RUBIO et al., 2016) e apresenta atividade antioxidante maior que o vinho tinto e o chá verde (KALAYCIOĞLU; ERIM, 2017; DERAKHSHAN et al., 2018). Ainda, o suco apresenta atividades anti-inflamatória, antidiabética, antiateroesclerótica, antimicrobiana, antitumoral, antiviral, antitrombocitopênica, cardioprotetora, musculoprotetora e vasodilatadora; ainda, é descrita ação neuroprotetora em doenças de Alzheimer e Parkinson (TAPIAS; CANNON; GREENAMYRE, 2014; LANTZOURAKI et al., 2015; BRUNO, 2016; ACHRAF et al., 2018; LOIZZO et al., 2019). O suco da romã também foi eficiente em diminuir as pressões sistólica e diastólica em pacientes diabéticos (SOHRAB et al., 2019).

Uma fração acetonitrila obtida do suco induziu parada na fase S da divisão celular de vários tipos de células tumorais (KHWAIRAKPAM et al., 2018) e o suco foi descrito como agente preventivo do câncer de mama através da modulação dos níveis de hormônios sexuais (KAPOOR et al., 2015). Luteína, ácido gálico e ácido púnico extraídos do suco exibiram propriedade antimetástica no tratamento de câncer de próstata, interferindo em vários processos ligados ao desenvolvimento de metástase, tais como crescimento celular, adesão, migração e quimiotaxia (WANG et al., 2012). Ácido elágico, ácido gálico e punicalagina A e B apresentaram atividade anti-inflamatória (BENSAAD et al., 2017).

Extrato hidroalcoólico do suco apresentou atividade antibacteriana contra bactérias causadoras da placa dentária e cárie (JURENKA, 2008; GULUBE; PATEL, 2016) e ação antibacteriana contra espécies Gram-positivas e Gram-negativas transmitidas por alimentos e contra *Helicobacter pylori*, causadora de gastrite crônica e úlceras (HAGHAYEGHI et al., 2013). Extrato metanólico do suco e seus compostos ácido gálico, quercetina e miricetina apresentaram atividade contra corinebactérias, estafilococos, estreptococos, *Shigella*, *Salmonella*, *Bacillus subtilis*, *Vibrio cholerae* e *Escherichia coli* (NAZ et al., 2007). Ácido caféico, ácido elágico, galato de epigalocatequina e quercetina isolados do suco mostraram ação contra *Klebsiella pneumoniae* produtora de β-lactamase (DEY; RAY; HAZRA, 2015). Compostos fenólicos purificados do suco inibiram a replicação do vírus influenza A responsável por gripe sazonal, preveniram a infecção causada pelo enterovírus 71 (responsável por doenças neurológicas em crianças) e apresentaram atividade antibacteriana contra *Staphylococcus aureus* e *Escherichia coli* (HAIDARI et al., 2009; YANG et al., 2012; PAGLIARULO et al., 2016).

### 2.3.2 Lectina da sarcotesta de *P. granatum* (PgTeL)

A sarcotesta da romã contém uma lectina (denominada PgTeL, do inglês *P. granatum testa lectin*), cujo procedimento de purificação compreende a solubilização das proteínas da sarcotesta em solução salina, tratamento com sulfato de amônio (30% de saturação) para remoção de proteínas contaminantes e etapa cromatográfica realizada em coluna de quitina. A estrutura primária de PgTeL apresentou homologia (37%) com a de uma quitinase isolada das sementes de *P. granatum*. PgTeL apresentou atividades bacteriostática (*Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus mutans*, *Aeromonas* sp., *Escherichia coli*, *Klebsiella* sp., *Salmonella enterica* serovar. Enteritidis, *Serratia marcescens*) e bactericida (*M. luteus*, *S. mutans*, *S. marcescens*). Adicionalmente, as capacidades adesiva e invasiva de *Aeromonas* sp., *S. aureus*, *S. marcescens* e *S. enterica* foram reduzidas quando as células dessas bactérias foram previamente tratadas com PgTeL (SILVA et al., 2016).

A necessidade de novos agentes com ação antimicrobiana é crescente devido ao aumento no número de cepeas de microrganismos resistentes. Os resultados descritos acima estimulam novas investigações acerca do potencial de PgTeL como alternativa aos antibióticos atualmente utilizados.

# 3 RESULTADOS

Os resultados dessa pesquisa são apresentados na forma de artigos

## 3.1 ARTIGO 1 - PgTeL, THE LECTIN FOUND IN *Punica granatum* JUICE, IS AN ANTIFUNGAL AGENT AGAINST *Candida albicans* AND *Candida krusei*

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# PgTeL, the lectin found in *Punica granatum* juice, is an antifungal agent against *Candida albicans* and *Candida krusei*

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ABSTRACT

The pomegranate (*Punica granatum*) sarcotesta contains a chitin-binding lectin (PgTeL) with antibacterial activity against human pathogenic species. In this work, the structural stability of PgTeL was evaluated by fluorimetric analysis and the lectin was evaluated for cytotoxicity to human peripheral blood mononuclear cells (PBMCs) and antifungal activity against *Candida albicans* and *Candida krusei*. PgTeL folding was impaired when lectin was incubated at pH ≥ 6.0. On the other hand, the lectin did not undergo unfolding even when heated at 100 °C. PgTeL (1, 10, and 100 μg/mL) was not cytotoxic to PBMCs. Antifungal activity was detected for *C. albicans* (MIC: 25 μg/mL; MFC: 50 μg/mL) and *C. krusei* (MIC and MFC of 12.5 μg/mL). Treatment of yeast cells with PgTeL resulted in decrease of intracellular ATP content even at sub-inhibitory concentrations (½MIC and ¼MIC) and induced lipid peroxidation. In addition, PgTeL damaged the integrity of fungal cell wall of both species, with more pronounced effects in *C. krusei*. The lectin showed significant antibiofilm activity on *C. albicans* at sub-inhibitory concentrations (0.195 and 0.39 μg/mL). In conclusion, PgTeL is an anti-*Candida* agent whose action mechanism involves oxidative stress, energetic collapse, damage to the cell wall and rupture of yeast cells.



## 1. Introduction

Lectins, proteins widely distributed in nature, have the ability to bind specifically and reversibly to carbohydrates [1,2]. They have also been reported as antifungal agents, interacting with carbohydrates present in fungal cell wall (including mannoproteins, glucans, cellulose and chitin) and then causing growth inhibition and cell death [3–5]. Lectins were also reported to inhibit the formation of microbial biofilms (aggregates of cells distributed along an exopolymeric matrix) by interfering on cell adhesion to surfaces as well as cell–cell interactions [6,7].

*Punica granatum* L., popularly known as pomegranate, is a plant native to India but currently cultivated worldwide [8,9]. This plant possesses a wide use in folk medicine, being considered as a reservoir of bioactive compounds [10,11]. For example, studies have demonstrated the antimicrobial potential of extracts and juice from pomegranate fruit against bacteria and fungi [12–14]. Silva et al. [15] reported the isolation of a chitin-binding lectin (PgTeL) from

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*P. granatum* sarcotesta. PgTeL is a 26-kDa protein, with sequence similarities with a chitinase found in seeds of pomegranate, showing bacteriostatic and bactericidal effects on human pathogenic species. The authors also showed that this lectin interferes with bacteria adhesion and invasion process in human cells.

Species from *Candida* genus are widely distributed in nature and are usually found as saprophytic constituents of the normal human microbiota; however, they can also become opportunistic pathogens [16]. *Candida albicans* is the most common causative agent of candidiasis and it has also been associated with nosocomial infections around the world, together with other species of the genus [17]. *Candida krusei* is a fungal pathogen that affects mainly hospitalized patients [18]. The treatment of infections caused by these fungi has been challenging because of resistance to drugs currently used. In addition, biofilm formation is considered as an important factor for virulence and resistance of *C. albicans* [19]. This scenario has increased the search for new compounds with anti-*Candida* activity. In this regard, medicinal plants used to treat infections are considered as potential sources of antifungal agents [20,21].

In this work, we evaluated PgTeL for antifungal activity against *C. albicans* and *C. krusei* and investigated the putative mechanisms of action using different approaches. PgTeL was also characterized for its stability towards variation of urea concentration, temperature and pH.

## 2. Materials and methods

### 2.1. Isolation of PgTeL

*Punica granatum* fruits were collected in Glória do Goitá (Pernambuco, Brazil), with authorization (36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of Environment. The sarcotesta was obtained after sieving (mesh of 1 mm) of the fresh fruit content. PgTeL was isolated as described by Silva et al. [15]. First, the sarcotesta was mixed with 0.15 M NaCl (in a proportion of 9:1, v/v) during 6 h using a magnetic stirrer. Next, the mixture was filtered through gauze, centrifuged (3000g, 15 min, 4 °C) and the supernatant was treated with ammonium sulphate at 30% saturation [22] during 4 h under magnetic stirring. After this period, the material was centrifuged (3000g, 15 min, 4 °C) and the supernatant fraction was dialyzed against distilled water (4 h) followed by 0.15 M NaCl (2 h) and loaded (4.0 mg of protein) onto a chitin (Sigma-Aldrich, USA) column (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl. The adsorbed PgTeL was recovered by elution with 1.0 M acetic acid and dialyzed against distilled water (4 h) for eluent removal. The extract, dialyzed fraction and PgTeL were evaluated for protein concentration and hemagglutinating activity as described in the next section.

### 2.2. Protein concentration and hemagglutinating activity (HA)

The samples were evaluated for protein concentration according to Lowry et al. [23] using bovine serum albumin (31.25–500 μg/mL) as standard. The hemagglutinating activity (HA) was determined as described by Silva et al. [15] using a suspension of rabbit erythrocytes (2.5% v/v) previously treated with glutaraldehyde [24]. Collection of erythrocytes was approved by Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* (process 23076.033782/2015-70). In the HA assay, the sample (50 μL) was serially two-fold diluted in 0.15 M NaCl in a 96-well V-bottomed microplate row and then 50 μL of the erythrocyte suspension were added to each well. In negative control, erythrocytes were incubated with 0.15 M NaCl. After incubation for 45 min at 28 °C, the number of HA units (HAU) was determined as the reciprocal of the highest dilution of the sample that promoted full agglutination of rabbit erythrocytes. The specific HA was defined as the ratio between the HAU and the protein concentration (mg/mL).

### 2.3. PgTeL characterization

#### 2.3.1. Effects of pH and temperature on PgTeL HA

For determination of pH effect on PgTeL HA, the agglutination assay was performed as described above but replacing the 0.15 M NaCl solution by the following buffer solutions: 10 mM citrate phosphate buffer, pH 3.0–6.0, 10 mM sodium phosphate pH 7.0, and 10 mM Tris–HCl, pH 8.0–11.0. The effect of temperature on PgTeL HA was evaluated using lectin samples previously heated for 30 min at 30, 40, 50, 60, 70, 80, 90 or 100 °C.

#### 2.3.2. Fluorimetric analysis

The effects of urea, pH and temperature on PgTeL conformation were evaluated by fluorimetric analysis using the extrinsic probe bis-ANS [(bis(8-anilinonaphthalene-1-sulfonate))], which emits fluorescence when it binds to hydrophobic regions surrounded by positively charged residues. Fluorescence measurements used the following PgTeL samples (2.0 μM): PgTeL in water, PgTeL previously incubated for 24 h at 25 °C with urea (0–8.0 M), PgTeL incubated at pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 with the same buffers described in section 2.3.1, and PgTeL previously incubated for 30 min at different temperatures (30, 50 and 100 °C). The samples were transferred to a quartz cuvette (10 × 10 mm light path; Hellma GmbH & Co. KG, Germany) and then incubated with 50-fold molar excess of bis-ANS in the dark. The bis-ANS fluorescence spectra were recorded on a Jasco spectrofluorometer FP-6300 (Jasco Corporation, Japan) at 25 °C by setting the excitation wavelength at 360 nm and emission in the range 400–600 nm. Bis-ANS binding was evaluated by area of fluorescence intensity in arbitrary units. The spectra displayed in the figures are the averages of five scans and the signals were corrected by subtracting the solution spectrum.

### 2.4. Evaluation of PgTeL toxicity to human peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from blood of healthy volunteer donors (n = 5) using tubes containing EDTA and isolated by density-gradient centrifugation over Ficoll-Hypaque (GE Healthcare Life Sciences, Sweden). The viability of the cells was evaluated by the trypan blue exclusion method and they were only used when viability was higher than 98%. All donors signed a document of free consent and the study was approved by the Human Research Ethics Committee of the *Universidade Federal de Pernambuco* (CEP/CCS/UFPE N° 145/09).

PBMCs (100 μL, $10^6$ cells/mL) were plated in 96-well microtiter plates (TPP-Techno Plastic Products, Trasadigen, Switzerland) in RPMI 1640 medium supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin (all purchased from Sigma-Aldrich, USA). PBMCs were placed at 25 °C at 5% $CO_2$ atmosphere. After 24 h, PgTeL (100 μL, from dilutions of a 400 μg/mL stock solution in water) was added to each well in order to obtain the concentrations 1, 10, and 100 μg/mL. After incubation for 72 h, 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5.0 mg/mL) was added to each well [25,26]. After 3 h, the formazan product, derived from the ability of the living cells to reduce the yellow tetrazolium, was dissolved in dimethyl sulphoxide and the absorbance at 540 nm was measured using an Epoch microplate reader (BioTek Instruments, USA). Negative control corresponded to cells not treated with PgTeL. The assays were performed in triplicate in three independent experiments.

### 2.5. Antifungal assays

#### 2.5.1. Determination of minimal inhibitory (MIC) and fungicidal (MFC) concentrations

Stored cultures of *Candida albicans* (URM 5901) and *Candida krusei* (URM 6391) were obtained from the culture collection University Recife Mycologia (URM), *Departamento de Micologia, Universidade Federal de Pernambuco*, Brazil. The fungi were cultured in Sabouraud Dextrose Broth (SDB), pH 5.6, at 28 °C overnight under gentle shaking. For use in the assays, the density of the cultures was adjusted turbidimetrically at a wavelength of 600 nm to $3 \times 10^6$ colony forming units (CFU) per mL. Each antifungal assay corresponded to a row of a flat-bottomed 96-well microplate (TPP Techno Plastic Products AG, Switzerland). First, 100 μL of SDB were added to each well of the row and then PgTeL (100 μL; 0.24 mg/mL in water) was added to the third well and subjected to a two-fold serial dilution in the culture medium until a final ratio of 1:1024. Next, all wells (except the first) were inoculated with 20 μL of the fungal culture. The first well contained only the SDB medium and corresponded to the sterility control. The second well corresponded to the 100% growth control (negative control) and contained a 1:1 dilution of SDB in distilled water as well as the fungal culture. The optical density at 600 nm ($OD_{600}$) was measured (time zero) and then the assay was incubated at 28 °C for 24 h. After this period, the $OD_{600}$ was recorded again. The increase in $OD_{600}$ in comparison with time zero was considered as fungal growth. Fluconazole (Sigma-Aldrich, USA) was used as positive control. The minimal inhibitory concentration ($MIC_{50}$) was determined as the lowest PgTeL concentration able to promote a reduction of $OD_{600}$ higher or equal to 50% in comparison with the 100% growth control [27]. Each assay was achieved in triplicate and three independent experiments were performed.

To determine the minimal fungicidal concentration (MFC), inoculations (10 μL) from the wells containing PgTeL at concentrations higher or equal to the $MIC_{50}$ were transferred to Sabouraud Dextrose Agar plates, which were incubated at 28 °C for 24 h. The MFC corresponded to the lowest PgTeL concentration able to reduce the number of CFU in 99.9% in comparison with the initial inoculum. Each assay was achieved in triplicate and three independent experiments were performed.

#### 2.5.2. Growth curves

Growth curves for *C. albicans* and *C. krusei* in the first six hours of incubation with PgTeL were established. The assay was performed in 96-well microplates as described in Section 2.5.1 using six wells of each row: the first well corresponded to the sterility control; the second well corresponded to the 100% growth control; the third to sixth wells corresponded to treatments with PgTeL at concentrations corresponding to 2 × $MIC_{50}$, $MIC_{50}$, ½$MIC_{50}$, and ¼$MIC_{50}$. The microplate was incubated at 28 °C and the $OD_{600}$ was determined every hour. Three experiments were performed in triplicate.

#### 2.5.3. ATP quantification assay

The viability of *Candida* cells in culture was evaluated by the quantification of the intracellular ATP concentration using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, USA). *C. albicans* and *C. krusei* were treated for 24 h at 28 °C with different concentrations of PgTeL (2 × $MIC_{50}$, $MIC_{50}$, ½$MIC_{50}$, ¼$MIC_{50}$) or with 0.15 M NaCl (negative control) as described in Section 2.5.1 in a 96-well microplate (TPP Techno Plastic Products AG, Switzerland). After this period, the microplate was incubated for 25 min at 25 °C and then 100 μL of control and test wells were transferred to an opaque-walled 96-well microplate (Corning Inc., Corning, USA). Then, the CellTiter-Glo® Reagent (containing the luciferase from *Photuris pennsylvanica*) was added to each well and the plate was placed for 2 min on a shaker to induce cells lysis. After incubation at 25 °C for 10 min, the luminescence was recorded in a GloMax® 96 microplate luminometer (Promega Corporation, USA) and ATP level was calculated according to manufacturer's instructions. Three independent experiments were performed in triplicate.

#### 2.5.4. Fungal cell wall analysis

Yeast cells treated with PgTeL (2 × $MIC_{50}$, $MIC_{50}$) or 0.15 M NaCl (control) were stained with Calcofluor White M2R (LIVE/DEAD® Yeast Viability Kit, Molecular Probes, USA), a fluorochrome that binds cellulose and chitin, in order to investigate possible damages caused on fungal cell wall. The yeast culture (50 μL) was added to 1 mL of 10 mM Na-HEPES pH 7.2 prepared in sterile 0.2 μm-filtered water. After centrifugation (10,000g, 5 min), the pellet was resuspended in 1 mL of this same buffer. The cells were then stained with 25 mM Calcofluor White M2R according to manufacturer's instructions and observed using a confocal microscope (Leica SPII AOBS, Leica Microsystems, Wetzaler, Germany) at excitation and emission wavelengths of 355 and 440 nm, respectively. The images were collected and analyzed using the LITE 2.0 software (Chem-Table Software, Moscow, Russia).

#### 2.5.5. Ultrastructural analysis

In order to investigate the effects of PgTeL on the ultrastructure of *C. krusei* and *C. albicans*, a Scanning Electron Microscopy (SEM) assay was performed. Yeast cells treated with different concentrations of PgTeL ($MIC_{50}$, ½$MIC_{50}$) were harvested by centrifugation at 1500g, washed in 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde/4% paraformaldehyde/5 mM $CaCl_2$ in 0.1 M cacodylate buffer pH 7.2 for 30 min at room temperature. After washing with the same buffer the cells were allowed to adhere to polylysine-coated coverslips and post-fixed for 1 h with 1% osmium tetroxide/0.8% potassium ferricyanide/5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The cells were dehydrated in graded ethanol, critical-point-dried with $CO_2$, coated with a 20 nm-thick gold layer, and observed with a JEOL T-200 scanning electron microscope. Yeast cells incubated with distilled water instead of lectin were used as a control.

#### 2.5.6. Lipid peroxidation assay

The occurrence of lipid peroxidation was investigated by determining the concentration of malondialdehyde (end product of lipid peroxidation) in non-treated and lectin-treated cells of *C. albicans* and *C. krusei*, using the Lipid Peroxidation (MDA) Assay Kit (MAK085) from Sigma–Aldrich (USA). The *C. albicans* and *C. krusei* cells were treated with PgTeL (2 × $MIC_{50}$, $MIC_{50}$, ½$MIC_{50}$, ¼$MIC_{50}$) or 0.15 M NaCl (control) as reported in Section 2.5.1. After incubation at 28 °C for 24 h, the cells were incubated with a lysis buffer (300 μL) containing 3 μL of butylated hydroxytoluene (100×) and then centrifuged (13,000g, 10 min) to remove insoluble material. The supernatants were collected (200 μL) and incubated with thiobarbituric acid (600 μL) at 95 °C for 60 min. Next, the samples were transferred to a microplate and the absorbance at 532 nm was recorded. Reactions performed in absence of cell lysates were used as blank. The concentration of malondialdehyde (MDA) was determined using a standard curve (0–20 nmol). To correct the data regarding the cell density present at the final of the experiment, the results were expressed as relative MDA content according to the ratio between MDA content and the $OD_{600}$. Three independent experiments were performed in triplicate.

### 2.6. Antibiofilm assay

*C. albicans* culture was grown in SDB medium overnight at 28 °C and then suspended in sterile saline solution (0.9%, w/v, NaCl) to obtain a fungal suspension equivalent to $10^8$ CFU/mL. To each well of a 96-well polystyrene microplate, it was added 40 μL of SDB

medium and 80 μL of the fungal suspension as well as 80 μL of ultrapure Milli-Q water (control) or 80 μL of PgTeL (to final concentrations of 0.195–100 μg/mL, in Milli-Q water). The $OD_{600}$ was recorded at this time and the microplate was incubated at 28 °C for 24 h. After this period, the $OD_{600}$ was read again, the culture medium was removed and the plate wells were washed three times with saline solution. The remaining attached fungal cells were heat-fixed at 60 °C for 40 min and the adherent biofilm layer was stained with 0.4% (w/v) crystal violet for 15 min at 25 °C. After washing with water, the stain bound to the biofilm was solubilized with ethanol (15-min incubation) and the absorbance was measured at 570 nm [28]. Three independent experiments were performed in triplicate.

### 2.7. Statistical analysis

Data are expressed as the mean of replicates ± standard deviation (SD). One-way ANOVA (significance at $p < 0.05$) was conducted using Action 2.8.29.357.515 software (Estatcamp, Brazil). Significant differences between the treatment groups were analyzed using Tukey's test (significance at $p < 0.05$).

## 3. Results and discussion

*Punica granatum* fruits have been called as "superfood" due to their range of biological activities described, being effective for treatment of dental caries and prevention of oral candidiasis [8,29]. In a previous study, we demonstrated that a lectin from *P. granatum* sarcotesta (PgTeL) showed to be active against the cariogenic bacterium *Streptococcus mutans* [15]. In the present work, we investigated the stability of PgTeL folding towards denaturing agents and evaluated its effects on two of the main yeast species that cause candidiasis.

PgTeL was successfully isolated following the previous established protocol. The sarcotesta extract showed specific HA of 18 and was submitted to treatment with ammonium sulphate. Chromatography of the dialyzed supernatant fraction (specific HA of 850) on chitin column yielded isolated PgTeL with specific HA of 17,225 (purification fold: 957). These data are in according with the previous results reported by Silva et al. [15].

Fluorimetric analysis employed the bis-ANS probe, which is an indicator of protein folding. Fig. 1A and B show that, in absence of urea, the bis-ANS bound to the hydrophobic cavities of PgTeL and then it was possible to detect the characteristic fluorescence of this probe in nonpolar environments. An increase in fluorescence intensity was observed when the lectin was treated with 1 M urea, suggesting a conformational change that favored the bis-ANS binding. On the other hand, the fluorescence decreased when PgTeL was incubated with 3 M urea being almost abolished at 8 M urea, indicating the denaturation of the protein and consequent exposure of the bis-ANS to water. These results showed that the use of bis-ANS probe is a good strategy to follow PgTeL denaturation. This probe was then employed to monitor proper folding of this lectin at different pH and temperature values in order to determine the best conditions for maintenance of the carbohydrate-binding function.

The intensity of bis-ANS fluorescence was higher when the lectin was incubated at pH 3.0, had some decrease at pH 4.0 and 5.0, and was reduced at pH ≥ 6.0, suggesting an impairment of PgTeL folding (Fig. 1C). Alterations in carbohydrate-binding ability of the lectin with variation of pH were evaluated by hemagglutinating activity assay. The results show that PgTeL was more active at acidic pH (3.0–5.0), showed lower HA at pH 6.0 and 7.0 and had its HA lost at pH 8.0 (Fig. 1D). The comparison between these results reveals a correlation between the folding level and hemagglutinating ability of PgTeL. However, the lectin maintained the ability to agglutinate the erythrocytes at pH 6.0 and 7.0, in spite of the alteration in conformation indicated by fluorimetric analysis. This result suggests that the folding status of this protein at these pH values is still enough to allow the binding to carbohydrates at erythrocyte surface.

The *Microgramma vacciniifolia* frond lectin (MvFL) showed a behavior partially similar to PgTeL in regard to the ability to bind erythrocytes at different pH values [30]: highest activity was also detected at acidic pH, and was intermediate at pH 6.0 and 7.0; on the other hand, MvFL maintained its HA at alkaline pH, unlike PgTeL. These differences are also reflected in the fluorescence spectra of bis-ANS when incubated with these lectins: MvFL showed to be folded at all tested pH values [30], while the results for PgTeL indicated a considerable unfolding level at pH 8.0.

The fluorimetric analysis revealed an increase of bis-ANS fluorescence in samples that were heated (Fig. 1E) and PgTeL remained active after heating at temperatures from 30 until 100 °C (Fig. 1F). This result suggests possible aggregation of the lectin molecules, resulting in binding of more bis-ANS molecules and shows that PgTeL did not suffer unfolding, which explains the persistence of hemagglutinating activity in heated samples. Similar results (increase of bis-ANS fluorescence after heating) were found for other thermo-stable lectins [30,31].

The search for new chemotherapeutic agents to treat fungal infections has increased every year due to reports of toxicity of antifungal drugs and resistance of pathogenic microorganisms [32]. Plants have been broadly studied as sources of alternative drugs having a diversity of bioactive compounds that act by different mechanisms than conventional antibiotics [33]. In addition, the plant compounds usually promote fewer side effects than synthetic chemicals [34]. In this regard, we evaluated the effects of PgTeL on viability of human PBMCs and of the fungi *C. albicans* and *C. krusei*.

PgTeL was investigated for cytotoxicity on normal PBMCs before the evaluation of antifungal activity, since compounds with unselective cytotoxicity are not viable for use in chemotherapy. PgTeL did not have cytotoxic effects on PBMCs. The cell viability rate was higher than 90% at all tested concentrations and did not show significant difference as compared with control cells ($p > 0.05$). Silva et al. [15] reported that PgTeL affected growth, survival, adherence and invasiveness of pathogenic bacteria at concentrations between 3.0 and 100 μg/mL, i.e. within the concentration range nontoxic to human PBMCs.

The results from antifungal assays revealed that PgTeL was able to inhibit the growth of *C. albicans* and *C. krusei* with $MIC_{50}$ of 25.0 and 12.5 μg/mL, respectively. The lectin also induced cell death of both species, with MFC values of 50.0 μg/mL (*C. albicans*) and 12.5 μg/mL (*C. krusei*). The $MIC_{50}$ and MFC values found for PgTeL are smaller or greater than the values reported for other plant lectins against *Candida* species. For example, the lectin from *Schinus terebinthifolius* leaves inhibited the growth of *C. albicans* with lower $MIC_{50}$ and MFC of 6.5 and 26.0 μg/mL, respectively [35], and a lectin from *Cicer arietinum* seeds was active against *C. krusei*, *Candida tropicalis*, and *Candida parapsilosis* with MIC values between 1.56 and 12.5 μg/mL [36]. Lectins isolated from *Dioclea violacea*, *Dioclea rostrata* and *Canavalia brasiliensis* seeds showed antifungal activity against *Candida guilliermondii*, *Candida shehatae*, and *Candida membranaefaciens* with MIC values ranging from 2.0 to 128 μg/mL; among these lectins, only that from *D. violacea* was active against *C. albicans*, with MIC of 32 μg/mL [37], value higher than that found for PgTeL. Klafke et al. [38] evaluated the effect of lectins from *Abelmoschus esculentus* leaves, *Mucuna pruriens* seeds and *Clitoria fairchildiana* seeds against medically important fungi from different genera but found antifungal activity only against *C. parapsilosis* (MIC from 0.97 to 3.9 μg/mL and MFC from 3.9 to 31.25 μg/mL). PgTeL was more active on *C. krusei* than the lectin from *Calliandra surinamensis* leaf pinnulae, which showed $MIC_{50}$ and MFC of 125 and 250 μg/mL, respectively [31], both values higher than PgTeL.

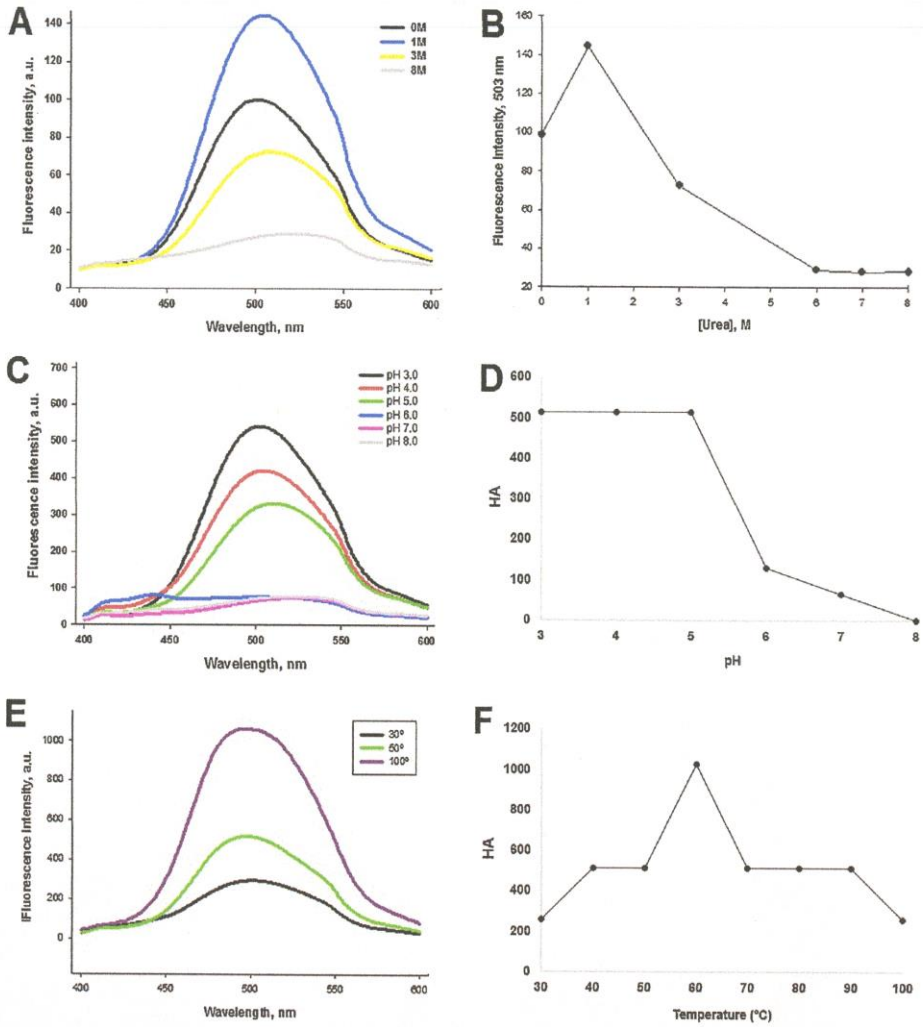


**Fig. 1.** The effects of urea, pH variation and heating on PgTeL stability. (A) Denaturation of PgTeL induced by urea and monitored by bis-ANS fluorescence. Measurements were performed after 24 h-incubation of PgTeL with urea at 25 °C. (B) Change in bis-ANS spectral area as a function of increased urea concentrations. (C) Fluorescence of bis-ANS incubated with PgTeL at different pH values. (D) Hemagglutinating activity (HA) of PgTeL at different pH values. (E) Fluorescence of bis-ANS incubated with PgTeL previously heated at 30, 50 or 100 °C. (F) HA of PgTeL after heating at different temperatures.

PgTeL also showed antifungal efficiency similar or higher than other natural products such as pogostone isolated from *Pogostemon cablin* aerial parts, which showed MIC ranging from 3.1 to 50 μg/mL and MFC from 50 to 400 μg/mL against isolates of *C. albicans* [39], and the proteinaceous trypsin inhibitor from *Tecoma stans* leaves, which presented antifungal activity on *C. albicans* and *C. krusei* with $MIC_{50}$ and MFC values of 100 and 200 μg/mL, respectively, for both species [40].

The detection of antifungal activity stimulated us to investigate the mode of action of PgTeL. First, we evaluated the kinetic of fungal growth inhibition by monitoring the $OD_{600}$ of cultures incubated during 6 h with the lectin at concentrations equivalent to 2 × $MIC_{50}$, $MIC_{50}$, ½$MIC_{50}$, and ¼$MIC_{50}$. The growth of *C. albicans* (Fig. 2A) was significantly ($p < 0.05$) inhibited since the first hour of incubation in treatments with PgTeL at concentrations ≥ $MIC_{50}$. In presence of the lectin at ½$MIC_{50}$ and ¼$MIC_{50}$, the growth of this species was significantly ($p < 0.05$) lower than control cells obtained from cultures of fourth hours. For *C. krusei* (Fig. 2B), the growth inhibition started only after 3 h of incubation with PgTeL at ½$MIC_{50}$ and higher concentrations. Thereafter, the values of optical density in treatments with concentration ≥ $MIC_{50}$ decreased to the initial values. These results reveal that PgTeL is already able to exert its fungistatic effect within few hours of incubation with the fungal cells.

The evaluation of intracellular ATP content in *C. albicans* cells (Fig. 2C) revealed a significant ($p < 0.05$) decrease in viability in treatments at sub-inhibitory concentrations (½$MIC_{50}$ and ¼$MIC_{50}$) and that, at the $MIC_{50}$, PgTeL was able to cause a strong reduction in ATP level indicating that the cells present are with their functionality greatly impaired. As expected, ATP was not detected in treatment at 2 × MIC, which corresponds to the MFC for this species. For *C. krusei* (Fig. 2D), significant ($p < 0.05$) reduction of ATP levels was already observed since ¼$MIC_{50}$ and were negligible at concentrations ≥ $MIC_{50}$. This result is in agreement with the fact the $MIC_{50}$ and MFC for *C. krusei* correspond to the same value. In addition, the results of ATP level evaluation are in accordance with the growth curves established for *C. krusei*, since growth inhibition was also

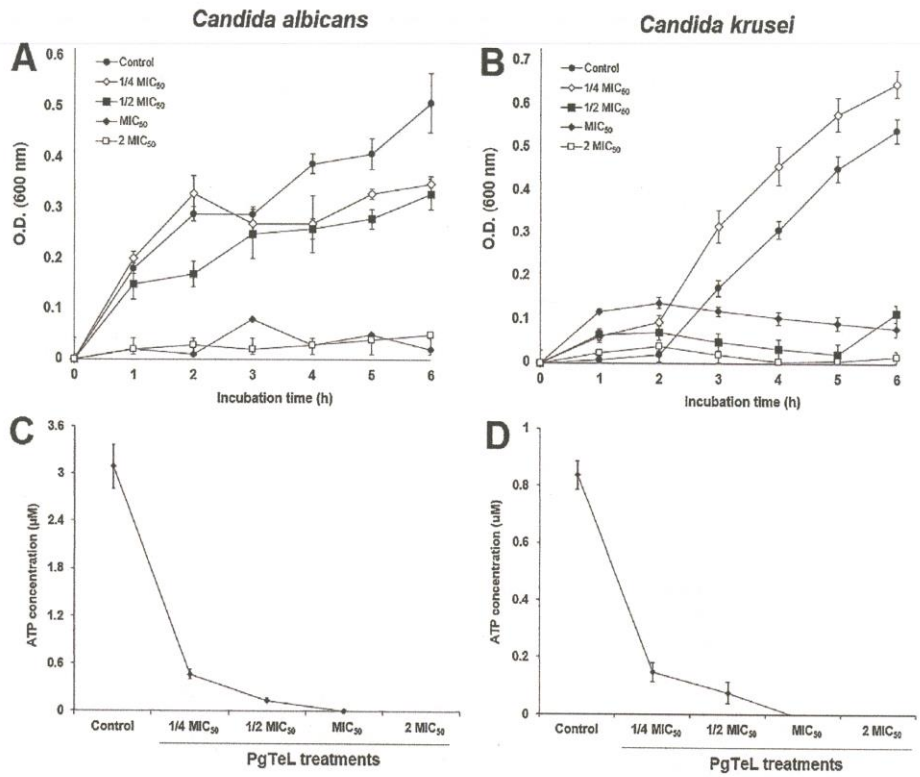


**Fig. 2.** Effects of PgTeL on growth and ATP production in *Candida albicans* (A and B) and *Candida krusei* (C and D). (A and C) Growth curves in absence or presence of PgTeL at different concentrations. The optical density (O.D.) at 600 nm was determined every hour during 6 h. Negative control cells were cultured in a lectin-free medium. Data are expressed as the mean ± standard deviation (SD). (B and D) ATP levels in control and in PgTeL-treated cells at different concentrations. Different letters indicate significant differences ($p < 0.05$) between treatments. $MIC_{50}$: minimal inhibitory concentration.

detected for concentrations lower than the $MIC_{50}$. The ATP depletion is usually linked to a reduction of viability or to cell lysis, among other reasons [41]. Koshlukova et al. [42] showed that salivary histatin 5 (a peptide with antifungal action) promoted the death of *C. albicans* through a mechanism that involves an efflux of cellular ATP without occurrence of cytolysis.

To investigate the possible perturbations induced by lectin on yeast surface we used Calcofluor (CF) fluorescent dye, which binds to chitin emitting a blue fluorescence. Control *C. albicans* presented regular oval shape, as demonstrated by differential interference contrast (DIC) and, in most of cells, the CF staining was concentrated at the budding regions and homogeneously distributed throughout the surface (Fig. 3A). In addition, no intracellular labeling was detected, indicating preserved cell wall integrity. An enhancement in CF fluorescence intensity in yeast wall was observed after treatment at the $MIC_{50}$ (Fig. 3B) and $2 \times MIC_{50}$ (Fig. 3C). Furthermore, the diffusion of fluorescent dye throughout the cytoplasm, suggestive of loss of cell wall integrity, could be observed. Changes in the CF labeling were usually followed by morphological alteration as observed by DIC.

The effects of PgTeL on *C. krusei* morphology were more pronounced than those found in *C. albicans*. The control cells (Fig. 3D) showed a characteristic morphology of this species, being bigger than other *Candida* yeasts, including *C. albicans*. Similarly to *C. albicans*, intense and bright fluorescence was observed at the budding regions. On the other hand, drastic morphological alterations, presence of ruptured cell debris and strong concentration of CF at the cytoplasm was observed in PgTeL-treated cells ($MIC_{50}$) revealing possible impairment of the cell wall (Fig. 3E).

The increase of intensity of CF fluorescence in both species of *Candida* could be due to a rearrangement of wall in response to injuries induced by PgTeL. Consistently with this hypothesis, Rueda et al. [43] demonstrated that the treatment with caspofungin, a fungicidal drug especially efficient towards *Candida* spp., induced chitin accumulation in cell wall as a complex adaptation process, where chitin upregulation and other cell wall rearrangements are necessary to overcome the deleterious effects of caspofungin. According to these authors, the accumulation and cell wall rearrangements causes a decrease in the cell permeability, which can explain the diffusion of CF observed in the present study. These results obtained by us are in accordance with ATP quantification assay, which revealed strong impairment of *C. krusei* viability. Similarly to PgTeL, the lectin from *C. surinamensis* pinnulae was able to promote damage to the cell wall of *C. krusei* cells, being also observed cell rupture and diffusion of CF towards the cytoplasm [31].

The scanning electron microscopy observations of *C. krusei* and *C. albicans* treated with PgTeL showed important morphological damages. Untreated *C. krusei* displayed a well-defined shape, regular size, smooth surface and evident polar bud scars (Fig. 4A). Cells treated with $½MIC_{50}$ assumed elongate shaped body and presented shrinkage of cell surface. Ruptured cells were also commonly observed (Fig. 4B). At higher concentration (Fig. 4C), corresponding to the $MIC_{50}$ value, the number of affected cells increased substantially. Some minor affected cells presenting protuberances at their

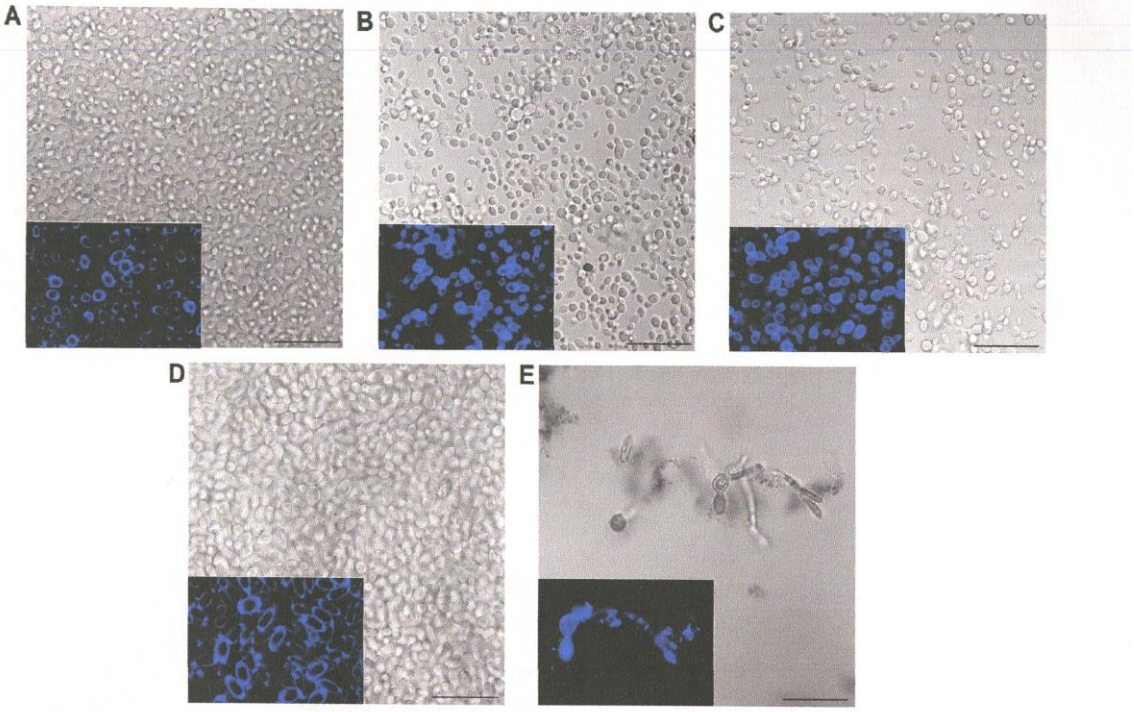


**Fig. 3.** Differential interference contrast and laser confocal microscopy of *Candida albicans* (A–C) and *Candida krusei* (D and E) cells. (A and D) Untreated cells. Cells treated with PgTeL at $MIC_{50}$ (B and E) and 2 × $MIC_{50}$ (C) for 24 h. In the case of *C. krusei*, the $MIC_{50}$ and MFC correspond to the same value. In untreated cells, the Calcofluor staining (blue) was concentrated at the budding regions and homogeneously distributed throughout the surface; in addition, no intracellular labeling was detected, indicating preserved cell wall integrity. In treated cells, it can be observed diffusion of Calcofluor throughout the cytoplasm, suggestive of loss of cell wall integrity. Morphological alterations and, presence of ruptured cell debris were also seen, mainly in *C. krusei*. Scale bars correspond to 23 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surfaces could be observed (Fig. 4C, inset), suggesting that wall disorders preceded the cell shrinkage. *C. albicans* treated with PgTeL (Figs. 4D–F) also presented shrinkage of cell body when compared with control cells. At the ½$MIC_{50}$, it was also already observed the presence of cell membrane perforation (indicated by the arrow), but no ruptured cells, (Fig. 4E). At higher concentration, however, most of cells are shrunken and ruptured cell debris were usually seen (Fig. 4F). These data strongly suggested that PgTeL induced osmotic imbalance and perturbation in cell membranes as also demonstrated by CF labeling, leading to energetic collapse and cell death by lysis.

Imbalance in the production of reactive oxygen species has been associated with structural and functional damages caused by antifungal agents on cell wall and membrane of *Candida* cells [44,45]. The occurrence of lipid peroxidation was then investigated as an indicator of oxidative stress. For both *C. albicans* (Fig. 5A) and *C. krusei* (Fig. 5B) cells treated with PgTeL, there was significant ($p < 0.05$) increase in the relative MDA content at all treatments in comparison with control. The effect was dose-dependent, indicating that PgTeL disturbed the redox state of the fungal cells, resulting in lipid peroxidation. The formation of lipid peroxides has been associated with structural and functional deformities of the membrane of *Candida* cells that, in a higher extent, can lead to cell death [44,46]. The phenylpropanoids eugenol, methyl eugenol and estragole were able to elicit oxidative stress in *C. albicans* and cause membrane damages associated to lipid peroxidation mediated by free radical cascade [47]. The proteinaceous trypsin inhibitor from *T. stans* leaves also caused lipid peroxidation in *C. albicans* and *C. krusei* [40], similarly to PgTeL.

Biofilms are structured communities of microorganisms surrounded by polymeric matrix and adhered to an inert or living surface. The ability of *Candida* species to form biofilms in biotic (e.g. aquatic environments, plant tissues and mammalian tissues) and abiotic (e.g. catheters, prosthetic devices and biomaterials) surfaces has been reported. It has also been also described that cells in the biofilm have decreased sensitivity to drugs than planktonic cells [48]. Thus, plant compounds have been evaluated for antibiofilm activities on *Candida* species. Flavonoids from *Dalea elegans* and *Scutellaria baicalensis* showed antibiofilm activity against *C. albicans* and *C. krusei*, respectively [48,49].

The *C. krusei* isolate used in the present work was not a biofilm producer (data not shown) and thus antibiofilm assay was only performed with *C. albicans*. PgTeL was able to inhibit the biofilm formation by *C. albicans* only at lower concentrations tested (0.195 and 0.39 μg/mL) since biofilm was reduced with no alteration in fungal growth (Fig. 6). The fungal growth was progressively inhibited at concentrations from 6.25 to 50 μg/mL but this was accompanied by an increase of biomass in comparison with control (Fig. 6). This effect of PgTeL on biofilm formation may be due to an agglutinating property of the lectin, which would facilitate cell–cell adhesion, or adsorption of the lectin to the cells. In addition, the presence of an antifungal agent (PgTeL) may have activated the transition of planktonic to biofilm form as a mechanism of protection. This was also observed for amphotericin B, which is an antifungal agent that promoted the formation of *C. parapsilosis* biofilm [48]. It is important to highlight that PgTeL showed antibiofilm activity on *C. albicans* at concentrations that did not promote pronounced fungistatic effects and cell death, which is likely do not favor the selection of resis-

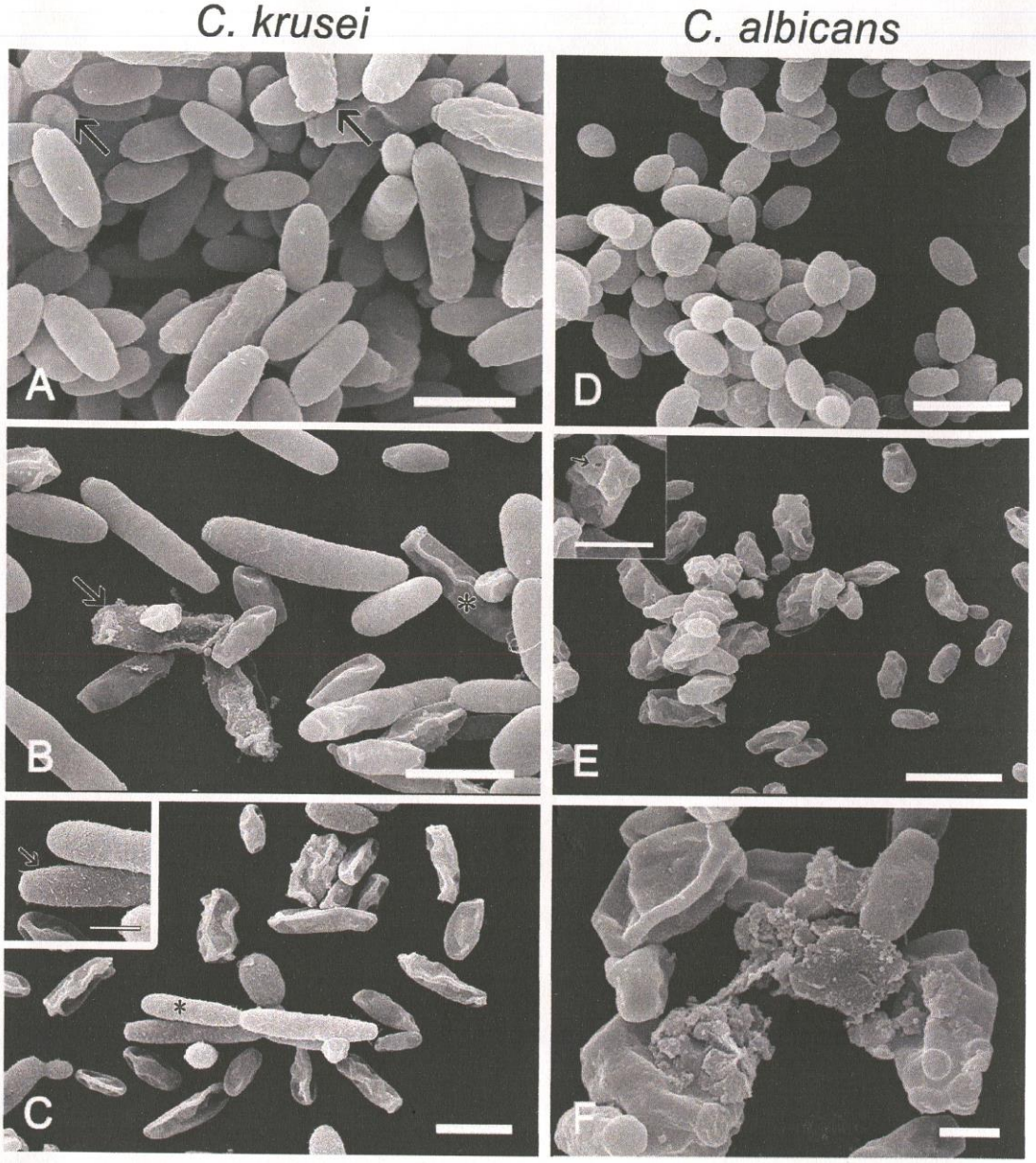


**Fig. 4.** Scanning electron microscopy of *C. krusei* (A–C) and *C. albicans* (D–F) treated or not with PgTeL. (A) Untreated *C. krusei* cells showing oval shape, smooth surface and one or more evident bud scars in the poles of cells (arrow). (B) *C. krusei* treated with ½$MIC_{50}$ PgTeL showing high variability in cell size, with predominance of elongated cells. Note the presence of shrunken (asterisks) and lysed cells (arrow). (C) Cells treated with $MIC_{50}$ of PgTeL with increased number of shrunken cells. Note the presence of protuberances at the surface of an elongated shaped cell (asterisk, inset). (D) Details of *C. albicans* control cells presenting well-preserved shape and smooth surface. (E) *C. albicans* treated with ½$MIC_{50}$, showing severe cell shrinkage. Note the presence of perforation at the cell surface of a shrunken cell (inset, arrow). (F) High magnification of *C. albicans* culture treated with $MIC_{50}$ showing ruptured cell debris. Bars: Figures A, B, C, D and E = 5 μm; F = 1 μm; insets = 2 μm.

tant cells. Thus, the lectin may be used in these concentrations as an antibiofilm agent, preventing about 50% biofilm formation by *C. albicans*, with no antibiotic property.

Antibiofilm activity on *C. albicans* has been reported for other plant-derived agents. A meroterpenoid from *Eucalyptus robusta* called eucarobustol E showed strong inhibitory effect against *Candida albicans* biofilms at a concentration of 16.0 μg/mL [50] and cinnamaldehyde encapsulated in multilamellar liposomes was able to inhibit in 80% biofilm formation by this fungus after 24 h treatment in a concentration of 300 mg/L [51]. In addition, an ethanolic extract from *Peganum harmala* showed weak (6.0 μg/mL) to strong (12.0 μg/mL) antibiofilm activity against *C. albicans* [52]. The concentrations of PgTeL able to inhibit biofilm formation were lower than those reported for all these agents.

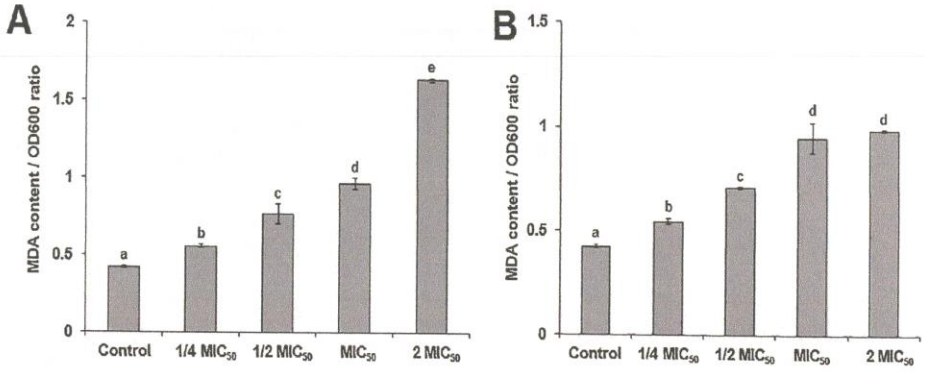


**Fig. 5.** Evaluation of occurrence of lipid peroxidation in *Candida albicans* (A) and *Candida krusei* (B) cells treated or not with PgTeL. Malondialdehyde (MDA) levels (nmol) were determined for cells of control and treated with PgTeL at different concentrations. The bars represent the ratio between MDA content and the $OD_{600}$ recorded after 24-h treatment. Different letters indicate significant differences ($p < 0.05$) between treatments. $MIC_{50}$: minimal inhibitory concentration.

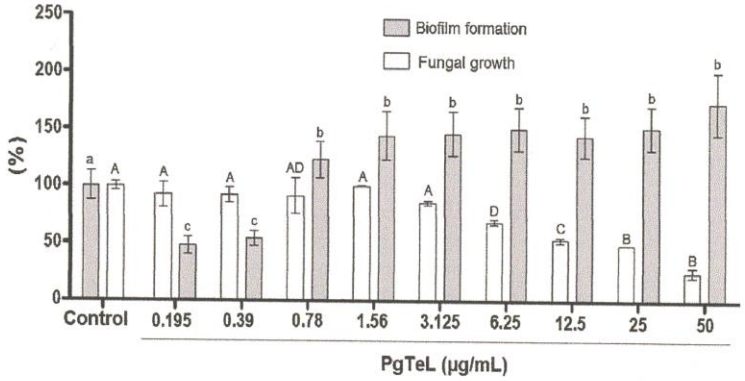


**Fig. 6.** Biofilm formation and growth of *Candida albicans* in absence (control) and presence of PgTeL at different concentrations. Growth was evaluated by optical density at 600 nm ($OD_{600}$). Different letters indicate significant differences ($p < 0.05$) between the treatments. Lowercase letters were used for comparison of biofilm formations and uppercase letters for fungal growth.

## 4. Conclusion

PgTeL, the thermostable lectin from *P. granatum* juice, is an antifungal agent against *C. albicans* and *C. krusei*. The lectin is effective since few hours of incubation, is able to impair the viability of the cells even at sub-inhibitory concentrations, and promotes oxidative stress, damage to the cell wall and rupture of yeast cells. In addition, PgTeL showed antibiofilm effect on *C. albicans*. The present work reinforces PgTeL as a molecule that can be associated with the bioactivities attributed to *P. granatum* fruit.

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## 3.2 ARTIGO 2 - *Punica granatum* SARCOTESTA LECTIN (PgTeL) IMPAIRS GROWTH, STRUCTURE, VIABILITY, AGGREGATION, AND BIOFILM FORMATION ABILITY OF *Staphylococcus aureus* CLINICAL ISOLATES

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# *Punica granatum* sarcotesta lectin (PgTeL) impairs growth, structure, viability, aggregation, and biofilm formation ability of *Staphylococcus aureus* clinical isolates

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ABSTRACT

In this work, we evaluated the ability of *Punica granatum* sarcotesta lectin (PgTeL) to impair the growth and viability of the *Staphylococcus aureus* clinical isolates 8325–4 (non-resistant) and LAC USA300 (MRSA strain). The effects of this lectin on aggregating, hemolytic activity, biofilm-forming ability, and expression of virulence genes (*hla*, *rnaIII*, and *spa*) were also investigated. PgTeL showed antibacterial activity against 8325–4 and LAC USA300 strains by interfering with both the growth ($MIC_{50}$ of 6.25 and 12.5 μg/mL, respectively) and survival (MBC values of 25.0 and 50.0 μg/mL, respectively). Culture growth started only at the ninth (8325–4) and tenth (LAC USA300) hour in the presence of PgTeL at $MIC_{50}$, while growth was detected since the first hour in the control. The lectin caused markedly altered cell morphology in both the strains. Although, at the $MIC_{50}$, PgTeL caused structural alterations, most cells were still viable, while at the MBC it promoted cell injury and death. PgTeL showed anti-aggregation effect and exhibited antibiofilm activity against both the isolates. However, the lectin did not interfere with the hemolytic activity of LAC USA300 and with the expression of *hla*, *rnaIII*, and *spa* genes. In conclusion, PgTeL is a lectin with multiple inhibitory effects on *S. aureus* clinical isolates.



## 1. Introduction

Treating infectious diseases has become an increasingly difficult task due to the spreading of drug resistance, limiting the options of antibiotics; consequently, the mortality and morbidity associated with these diseases have increased worldwide [1]. This framework stimulates the search for new antimicrobial agents, including those of natural origin, which usually have low toxicity to non-target cells and have action mechanisms distinct from that of conventional antibiotics [1,2].

*Staphylococcus aureus* is an opportunistic pathogen that causes infections in humans and animals. Methicillin-resistant *S. aureus* (MRSA) strains are continuously being reported and demonstrate resistance to multiple antibiotics. The MRSA isolates can be classified in the following groups: hospital-acquired (HA-MRSA), community-acquired (CA-MRSA), and livestock-associated (LA-MRSA) [3–5]. The infection by *S. aureus* can lead to severe damage to the host body due to several virulence factors such as biofilm formation, clumping, aggregation, hemolytic action, and secretion of proteases [6]. In addition, *S. aureus* has been found in raw milk, individual cows, surfaces of milkers' hands, and in milking equipments in dairy farms in Brazil [7] and MRSA was detected in goat milk and teat skin samples from Ohio [8]. *S. aureus* isolates from dairy farms were able to produce biofilm, indicating the ability to persist in milking environment [9].

Bacteria use quorum sensing (QS) to regulate several processes including symbiosis, virulence, competence, conjugation, motility, and sporulation [10–12]. The expression of virulence factors in *S. aureus* is regulated by a complex network of many genetic loci, including the QS system associated with the accessory gene regulator (Agr) [13–15]. The QS system allows the regulation of gene expression through cell-cell communication in response to fluctuations in cell-population density, ensuring adaptation to the environment. It comprises some of the most important mechanisms for pathogenesis [15–18].

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Biofilms are communities densely populated of microbial cells that synthesize an exopolymeric matrix that serves to protect them from external adverse conditions and increase resistance to antibiotics [19]. The biofilms can be attached to a tissue or surface and, according to the National Institutes of Health (NIH), bacterial biofilms are responsible for 65–80% of the cases of chronic and nosocomial infections [20–22]. In addition, biofilms have economic impact, such as in the food and petroleum industries, in which large losses can occur due to food spoilage and biocorrosion, respectively [19,23].

Lectins are proteins capable of binding to carbohydrates, which may be present in the free-form or as glycoconjugates [24]. The juicy sarcotesta of *Punica granatum* L. fruit (pomegranate) contains a chitin-binding and thermostable lectin (PgTeL), which is a 26-kDa protein with 37% homology with a chitinase also from pomegranate seeds. PgTeL showed antibacterial, antifungal, and antibiofilm activities against human pathogens. It was also able to inhibit the adhesion and invasion abilities of *Aeromonas* sp., *S. aureus*, *Serratia marcescens*, and *Salmonella enterica* serovar Enteritidis, to HeLa cells. The antifungal activity of PgTeL on *Candida albicans* and *Candida krusei* was associated with lipid peroxidation, energetic collapse, damage to the cell wall, and rupture of yeast cells [25,26].

The objective of the present study was to evaluate the ability of PgTeL in impairing the growth, structure, and viability of *S. aureus* isolates (including a MRSA strain), as well as to investigate its effect on aggregating, hemolytic activity, and biofilm-forming abilities, and the expression of virulence genes (*hla*, *rnaIII*, and *spa*) linked to the Agr system.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The *S. aureus* strains used in this study were as follows: 8325–4 that is a derivative strain of NCTC 8325 (a clinical isolate non-resistant to antibiotics), often used as a background for genetic manipulation and study of *S. aureus* biology [27,28]; LAC USA300, a multidrug resistant CA-MRSA isolate largely associated with infections in the USA, Canada, and Europe, also known as FPR3757, and commonly used as a laboratory MRSA model [28,29]; and the reporter fusion carrying strains PC203 (*spa*::*lacZ*), PC322 (*hla*::*lacZ*), and SH101F7 (*rnaIII*::*lacZ*), all derived from 8325-4 [15,30]. The bacteria are maintained at the Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen (Denmark). They were grown in oxoid tryptone soy broth, TSB (Oxoid Ltd., Basingstoke, Hampshire, UK) in a 1:10 volume/flask ratio, at 37 °C with shaking at 200 rpm.

### 2.2. Lectin isolation

PgTeL was isolated from pomegranate sarcotesta as described by Silva et al. [25]. The fruits were collected in Glória do Goitá (Pernambuco, Brazil), with authorization (36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of Environment. The access was recorded (A9F23D0) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). The sarcotesta was obtained after sieving (mesh of 1-mm) of the fresh fruit content and then mixed with 0.15 M NaCl (in a proportion of 9:1, v/v) for 6 h using a magnetic stirrer. Next, the mixture was filtered through gauze, centrifuged (3000 ×g, 15 min, 4 °C), and the supernatant was treated with ammonium sulphate at 30% saturation [31] for 4 h under magnetic stirring. After this period, the material was centrifuged (3000 ×g, 15 min, 4 °C) and the supernatant fraction was dialyzed against distilled water (4 h) followed by 0.15 M NaCl (2 h) and loaded (4.0 mg of protein) onto a chitin (Sigma-Aldrich, USA) column (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl. The adsorbed PgTeL was recovered by elution with 1.0 M acetic acid and dialyzed against distilled water (4 h) for eluent removal.

### 2.3. Protein concentration and hemagglutinating activity (HA)

Protein concentration was determined according to Lowry et al. [32] using bovine serum albumin (31.25–500 μg/mL) as standard. In order to monitor the carbohydrate-binding ability of PgTeL, the HA was determined as previously described [25] using a suspension of rabbit erythrocytes (2.5% v/v) treated with glutaraldehyde [33]. Collection of erythrocytes was approved by the Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* (process 23076.033782/2015–70). The number of HA units (HAU) was determined as the reciprocal of the highest dilution of the sample that promoted full agglutination of rabbit erythrocytes. The specific HA was defined as the ratio between the HAU and the protein concentration (mg/mL).

### 2.4. Determination of minimal inhibitory (MIC) and bactericidal (MBC) concentrations

The antibacterial activity was performed according to the Clinical and Laboratory Standards Institute [34]. Cultures of 8325–4 and LAC USA300 in TSB had their density adjusted turbidometrically at a wavelength of 600 nm to $3 \times 10^6$ colony forming units (CFU) per mL. Each assay corresponded to a row of a flat-bottomed 96-well microplate (TPP Techno Plastic Products AG, Switzerland). First, 80 μL of distilled water was added to the second upto twelfth well of the row and then PgTeL (80 μL; 0.5 mg/mL) was added to the third well and subjected to a two-fold serial dilution upto the twelfth well. Then, TSB (40 μL) was added from the second upto twelfth well of the row. Next, all wells (except the first) were inoculated with 40 μL of the bacterial culture. The first well contained only the TSB medium and corresponded to the sterility control. The second well corresponded to the 100% growth control (negative control). The optical density at 600 nm ($OD_{600}$) was measured (time zero) and then the assay was incubated at 37 °C for 24 h. After this period, the $OD_{600}$ was recorded again. The increase in $OD_{600}$ in comparison with time zero was considered as bacterial growth. The minimal inhibitory concentration ($MIC_{50}$) was determined as the lowest PgTeL concentration able to promote a reduction in $OD_{600}$, higher or equal to 50% in comparison with the 100% growth control.

To determine the minimal bactericidal concentration (MBC), inoculations (10 μL) from the wells containing PgTeL at concentrations higher or equal to the $MIC_{50}$ were transferred to tryptone soy agar plates, which were incubated at 37 °C for 24 h. The MBC corresponded to the lowest PgTeL concentration able to reduce the number of CFU in 99.9%, in comparison with the initial inoculum. Each assay was conducted in triplicate and three independent experiments were performed.

### 2.5. Growth curves

Twenty-four-hour growth curves of 8325–4 and LAC USA300 in the absence or presence of PgTeL were established. The assay was performed in 96-well microplates as described in Section 2.4: the first well corresponded to the sterility control; the second well corresponded to the 100% growth control; the third to sixth wells corresponded to treatments with PgTeL at $4 \times MIC_{50}$, $2 \times MIC_{50}$, $MIC_{50}$, $1/2 \times MIC_{50}$, and $1/4 \times MIC_{50}$. The microplate was incubated at 37 °C and the $OD_{600}$ was determined every hour. Three experiments were performed in triplicate.

### 2.6. Microscopy analysis

Three-dimensional images of the structure of bacterial cells treated with or without PgTeL were obtained by scanning electron microscopy (SEM). Cells (1.2 mL; $10^6$ CFU/mL) were incubated with MHB or SDB (0.6 mL) and PgTeL (0.4 mL) at the respective $MIC_{50}$ or $2 \times MIC_{50}$ in

Milli-Q water. The negative control was established by adding Milli-Q water instead of lectin. After incubation (24 h at 37 °C), the samples were centrifuged (300 ×g; 10 min, 25 °C) and the precipitated cells were washed three times in 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 28 °C. After washing with the same buffer, the cells were allowed to adhere to stubs (Ø 12.7 mm, 9 mm length; Ted Pella Inc., Redding, CA, USA) and post-fixed for 1 h with 1% osmium tetroxide. The cells were dehydrated in graded acetone, critical-point-dried with $CO_2$, coated with a 20 nm-thick gold layer, and observed with a JEOL JSM-5600 LV microscope (JEOL, Peabody, MA, USA). Cells without treatment were used as controls.

### 2.7. Flow cytometry analysis

The viability of bacterial cells treated with PgTeL was evaluated using the Cell Viability Kit of BD Biosciences (San Jose, CA, USA). The 8325–4 and LAC USA300 strains were incubated as described in Section 2.4 with PgTeL at the $MIC_{50}$ and MBC. The negative control was prepared by adding distilled water instead of PgTeL. For the positive control, cells were treated with 70% (v/v) isopropyl alcohol for 1 h before the analysis. The samples were centrifuged (10,000 ×g, 10 min, 25 °C) and the cell pellets were washed three times with 0.1 M PBS pH 7.0. Next, 42 μM thiazol orange (TO, 5 μL) and 4.3 mM propidium iodide (PI, 5 μL) were added to the assays, which were vortexed and incubated for 5 min at 25 °C. Next, the fluorescent bead suspension (BD Liquid Counting Beads) was added (50 μL) and mixed for 30 s. Data were acquired in a BD Accuri C6 cytometer (BD Biosciences) with an SSC threshold of 200 and stopped after gating 30,000 events for each sample. Analysis was performed using the BD Accuri C6 Software. Three experiments were performed in triplicate.

### 2.8. Bacterial aggregation assay

The bacteria were inoculated in TSB (1 mL per well) in a 24-well plate at a starting $OD_{600} = 0.02$. PgTeL, in sterile distilled water, was added to the wells in order to obtain final concentrations of 4 × $MIC_{50}$ (MBC), 2 × $MIC_{50}$, $MIC_{50}$, 1/2 × $MIC_{50}$, and 1/4 × $MIC_{50}$. Distilled water was used as the control. The plates were incubated for 8.5 h at 37 °C, with gentle shaking. Next, the cultures were allowed to sediment for 30 min and the supernatants were collected for $OD_{600}$ determination. A lower supernatant $OD_{600}$ is indicative of aggregation since mostly single cells remain in the suspension. Three experiments were performed in triplicate.

### 2.9. Hemolysis assay

To verify whether PgTeL would be able of inhibit the secretion of hemolytic toxins, we performed two different experiments on blood agar plates according to Bojer et al. [35] with some modifications. Colonies of LAC USA300 were transferred into 5 mL of TSB in 15-mL test tubes and incubated for 16 h at 37 °C in a shaking incubator (225 rpm). A starter inoculum (400 μL) with $OD_{600} = 0.02$ was used and 400 μL of either distilled water (control) or PgTeL at sub-inhibitory concentrations (1/2 × $MIC_{50}$, 1/4 × $MIC_{50}$, 1/8 × $MIC_{50}$, and 1/16 × $MIC_{50}$), as well as TSB (200 μL), were added to the cultures and incubated for 16 h at 37 °C in shaking incubator (225 rpm). The supernatants (20 μL) were dropped in a blood agar plate and incubated for 24 h at 37 °C. The appearance of a hemolysis halo around the drop was observed. Three experiments were performed in triplicate.

In the other assay, cultures obtained in the same conditions described above were diluted (1:9, v/v) in 0.9% NaCl, and $OD_{600}$ was adjusted to 0.35. Next, another 1:9 (v/v) dilution was performed and 800 μL of the final cell suspension was transferred to 9-cm Petri dishes and mixed with the blood agar. Then, holes were made in the plates and 50 μL of distilled water (control) or PgTeL sub-inhibitory concentrations (1/2 × $MIC_{50}$, 1/4 × $MIC_{50}$, 1/8 × $MIC_{50}$, and 1/16 × $MIC_{50}$) were added in the holes and incubated for 24 h at 37 °C. The appearance of a hemolysis halo around the hole was observed. Three experiments were performed in triplicate.

### 2.10. Static biofilm assay

The assay was performed as described by Nielsen et al. [36] with minor modifications. Starter cultures of 8325–4 and LAC USA300 in TSB with $OD_{600} = 0.5$ were used. From this culture, 50 μL was withdrawn and 10-fold diluted in 0.9% NaCl. Next, 5 μL was inoculated into wells of a 96-well microtiter plate containing 200 μL of TSB. PgTeL was added to attain final concentrations ranging from 0.78 to 200 μg/mL. Distilled water was added in the control. The plates were incubated for 20 h at 37 °C without shaking. The biofilm was washed twice with 0.9% NaCl (200 μL), air-dried in a LAF-bench, and stained with 0.1% (w/v) crystal violet (125 μL) for 30 min followed by three washing steps with 0.9% NaCl (200 μL). For quantification purposes, the stain bound to the biofilm was solubilized in 96% ethanol and the absorbance measured at 590 nm using a microplate reader. Three experiments were performed in triplicate.

### 2.11. Qualitative β-galactosidase reporter plate assay

The possible influence of PgTeL on the expression of virulence factors controlled by the *agr* system was evaluated by the β-galactosidase reporter plate assay according to Bojer et al. [35]. Firstly, the 8325–4 strain was used to obtain supernatants containing the autoinducer peptide (AIP). The bacteria were inoculated into 5 mL TSB in 15-mL tubes and incubated overnight (16 h) at 37 °C under shaking at 225 rpm. After this period, the cultures were spun down (10,000 ×g, 5 min) and the supernatants were passed through 0.22 μm sterile filters.

In the β-galactosidase plate assays, the strains PC203 (*spa*::*lacZ*), PC322 (*hla*::*lacZ*), and SH101F7 (*rnaIII*::*lacZ*) were used. The bacteria were separately cultivated in TSB overnight and dilutions in 0.9% NaCl were prepared to reach $OD_{600} = 0.0035$. The diluted culture was inoculated (800 μL) in Petri dishes containing melted TSA and cooled down (45 min) to 45 °C and supplemented with 5 μg/mL erythromycin and 150 μg/mL of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), the β-galactosidase substrate. Holes in each plate were made with a sterile cork borer and the agar plugs were removed. PgTeL at different concentrations (1/2 × $MIC_{50}$, 1/4 × $MIC_{50}$, 1/8 × $MIC_{50}$, 1/16 × $MIC_{50}$) and the AIP-containing supernatant were added to each well. Distilled water was used in the negative control. In the positive control, the supernatant of a *Staphylococcus schleiferi* 30743 culture, a strain with inhibitory effect on *agr* system, was added [15]. The samples were allowed to diffuse into the agar and the plates were incubated at 37 °C until blue color development, which is derived from X-gal hydrolysis. The plates were inspected visually for any up- or down-regulation of color. Three experiments were performed in triplicate.

### 2.12. Statistical analyses

Data are expressed as the mean or the percent mean ± standard deviation (SD) and statistical differences were determined using Tukey's range test; a p value < 0.05 was considered statistically significant.

## 3. Results and discussion

In recent years, there has been a growing interest in the search for new drugs active against microorganisms that are resistant to conventional antibiotics. In addition, the modulation of virulence by antimicrobial agents has been the subject of study, since severe damage to host body can be induced by virulence factors released by bacteria [37].

The interactions between lectins and glycoconjugates on cell surfaces can induce different cellular responses; in addition, these proteins can be internalized by endocytosis [24,38,39]. It has been reported that these proteins can affect cellular pathways linked to bacterial growth and survival, as well as can disturb the QS circuits involved in the formation of biofilms [19,40]. Therefore, we evaluated whether PgTeL would be able to interfere with the growth, survival, aggregation, hemolytic, and biofilm-forming abilities of *S. aureus* clinical isolates, as well as influence the expression of virulence-linked genes.

PgTeL was isolated following the protocol previously established by Silva et al. [25] and showed specific hemagglutinating activity of 13,205, confirming the functionality of the carbohydrate-binding sites. The lectin showed antibacterial activity against 8325–4 and LAC USA300 strains by interfering with both growth ($MIC_{50}$ of 6.25 and 12.5 μg/mL, respectively) and survival (MBC values of 25.0 and 50.0 μg/mL, respectively). PgTeL was previously reported to be bacteriostatic against a standard strain of *S. aureus* (ATCC-6538) with an $MIC_{50}$ of 50 μg/mL [25]. Interestingly, it was more active against the clinical isolates evaluated here. PgTeL was more efficient in killing *S. aureus* isolates than the lectins isolated from *Cladonia verticillaris* lichen (ClaveLL) and *Moringa oleifera* seeds (WSMoL), which showed MBCs of 459.9 and 300 μg/mL, respectively [41,42]; but it was less active than lectin from *Schinus terebinthifolius* leaves (SteLL) that showed an MIC of 7.18 μg/mL [43].

The growth curves of 8325–4 (Fig. 1A) and LAC USA300 (Fig. 1B) incubated with PgTeL at different concentrations were established in order to monitor the kinetics of the inhibitory effect. Growth of both strains was inhibited from the third hour of incubation in all the treatments with PgTeL, denoting a strong bacteriostatic effect. For the 8325–4 strain, growth in the presence of PgTeL started only at the sixth hour with 1/4 × $MIC_{50}$, at the ninth hour with 1/2 × $MIC_{50}$ and $MIC_{50}$, and at the eleventh hour with 2 × $MIC_{50}$. For LAC USA300, there was no significant growth with treatments at 1/2 × $MIC_{50}$ and 1/4 × $MIC_{50}$ until the tenth hour and with the $MIC_{50}$ and 2 × $MIC_{50}$ concentration until the fourteenth and nineteenth hours, respectively. At 4 × $MIC_{50}$, no growth was detected for both strains throughout the assay, which corroborates the fact that this corresponds to the MBC.

The effects of PgTeL on bacterial structure were evaluated through three-dimensional images of cells treated with the $MIC_{50}$ and 2 × $MIC_{50}$ obtained by SEM. The images obtained for 8325–4 and LAC USA300 cells from the control treatments (Fig. 2A and B, respectively) showed them to have a preserved shape, regular size, and smooth surface, organized in grape-like clusters characteristic of the species. In the treatments at the $MIC_{50}$, a reduction in the number of cells can be observed, which presented deformations in the surface, as well as small groups of 8325–4 cells (Fig. 2C) and no clustering of LAC USA300 (Fig. 2D). With the 2 × $MIC_{50}$ treatments, only the isolated 8325–4 cells can be observed, with severe shrinkage degree (Fig. 2E). For LAC USA300, it can be mainly observed as a large amount of ruptured cell debris (Fig. 2F). These data reveal that PgTeL causes strong damage to the structure of both *S. aureus* strains.

The structural alterations observed in the cells treated with PgTeL at the $MIC_{50}$ prompted the evaluation of their viability after treatment. As expected, the amount of cells present was very low, as corroborated by the highest volume necessary to reach the established number of events in flow cytometry analysis, in comparison with the control (data not shown). However, most of the remaining cells of 8325–4 (Fig. 3A) and LAC USA300 (Fig. 3B) were still viable after PgTeL treatment at the $MIC_{50}$, with only some injured cells (2.4–3.3%) being detected. This result indicates the predominance of a bacteriostatic effect at this concentration and suggests that the surface alterations observed were not enough to impair the permeability of most cells. Conversely, the treatment at the MBC resulted in a predominance of dead cells for 8325–4 (53.6%, Fig. 3A) and injured cells for LAC USA300 (65.3%, Fig. 3B). In both the cases, the remaining intact live cells (12.4–13.7%) were not able to maintain colony viability. Thus, it is possible to clearly discern two types of PgTeL effect, depending on the concentration: it can be a predominantly bacteriostatic agent, without promoting a high level of cell death, or it can act as a bactericidal drug.

Multicellular organization is involved in many aspects of *S. aureus* infection pathogenesis, including nonsurface-attached behaviors like aggregation [44]. The anti-aggregation effect of PgTeL was evaluated and the results can be seen in Fig. 4. In comparison with control, there was a gradual increase in $OD_{600}$ in the supernatants with an increase in lectin concentration, with the treatments from 1/4 × $MIC_{50}$ to $MIC_{50}$, evidencing a greater presence of single cells in the suspension. This result is in accordance with the microscopy analysis. In the treatments at 2 × $MIC_{50}$ and 4 × $MIC_{50}$ there was a drastic decrease in $OD_{600}$ in the supernatant, probably due to the strong action of the lectin as previously demonstrated. It has been shown that, in comparison with planktonic cells, *S. aureus* aggregated cells are more metabolically active, have increased mutation frequency, and are less sensitive to antibiotics [45]. Thus, the blocking of aggregation by an antibacterial agent is an additional characteristic that can have important impact on the virulence and development of resistance. Baldry et al. [28] showed that the compound norlichexanthone, which is produced by fungi and lichens, has a dose-dependent anti-aggregating action, similar to PgTeL.

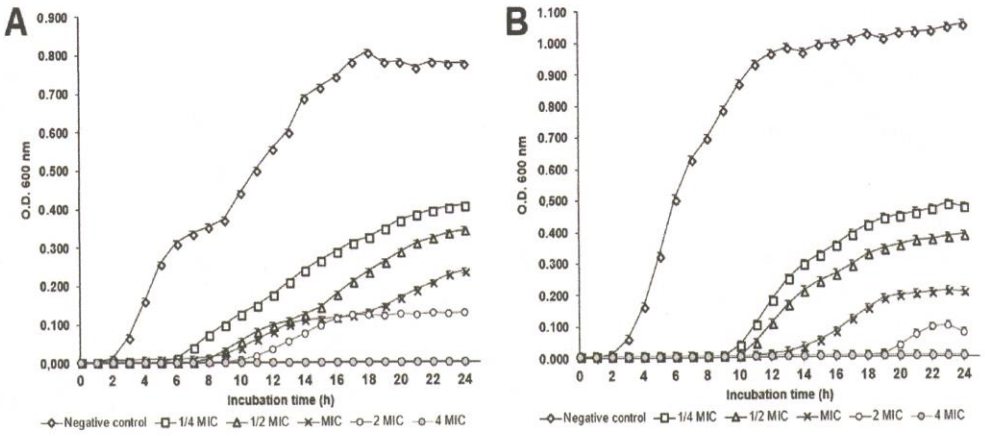


**Fig. 1.** Growth curves (24-h incubation) of *Staphylococcus aureus* 8325–4 (A) and LAC USA300 (B) in the absence (negative control) and presence of PgTeL at different concentrations. Data are expressed as the mean ± standard deviation (SD) of three experiments.

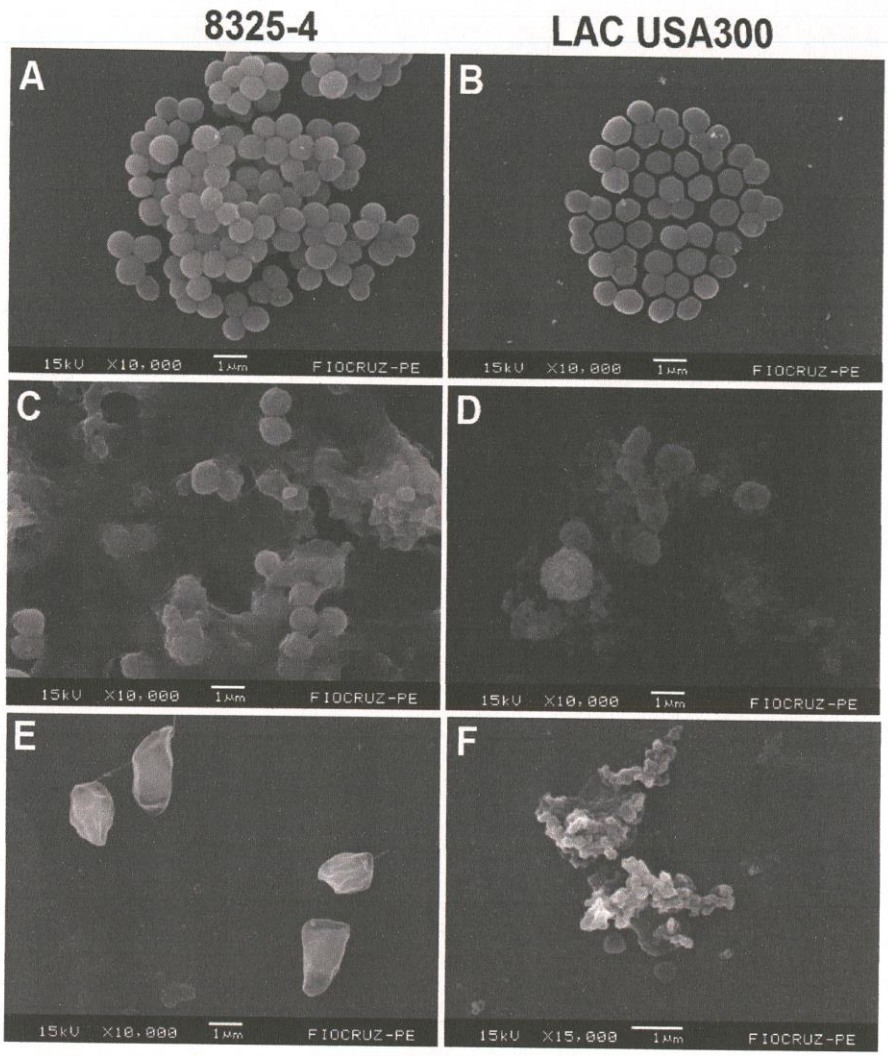


**Fig. 2.** Scanning electron microscopy (SEM) of *S. aureus* 8325–4 and LAC USA300 cells from the control (A and B) or after exposure to PgTeL at the $MIC_{50}$ (C and D) or 2 × $MIC_{50}$ (E and F). Control cells showed preserved morphology and smooth surface and were organized in grape-like clusters (A and B). For the treatments at $MIC_{50}$, a reduction in the number and clustering of cells can be observed, which presented deformations in the surface (C and D). For the 2 × $MIC_{50}$ treatment, 8325–4 cells showed severe shrinkage degree (E) and ruptured cell debris can be seen for LAC USA300 (F).

Another virulence factor of *S. aureus* is the hemolytic property, conferred by toxins like alpha-hemolysin encoded by the *hla* gene controlled by the *agr* system. The bacterial cells promote the lysis of erythrocytes to obtain the heme iron [46]. To investigate if PgTeL would be capable of inhibiting the secretion of hemolytic toxins, LAC USA300 cells incubated with or without the lectin were evaluated for the ability of lysis in blood agar. However, the halos produced by the cells exposed to PgTeL were not significantly different ($p > 0.05$) from those from the control cells, indicating no interference with the hemolytic activity (Fig. 5). However, indole [3,2-*b*]quinoline derivatives, norlichexanthone, and *Vetiveria zizanioides* root extract were able to inhibit the hemolysis by *S. aureus* [28,47,48].

Bacterial biofilms are the underlying cause of many persistent and recurrent infections. Similar to the aggregation ability, the formation of biofilms is also associated with increased mutation frequency and lower susceptibility to antibiotics; however, biofilms usually have low metabolic activity. In addition, biofilm formation is associated with fibronectin-binding proteins, while aggregation is mediated by adhesins. Apart from this, protein A encoded by *spa* is associated with both biofilm formation and aggregation [19,45]. PgTeL demonstrated antibiofilm activity against both the isolates. For the 8325–4 strain, biofilm inhibition by >50% was detected only at the concentration of 200 μg/mL and there was a stimulating effect at the lowest concentrations (Fig. 6A). This stimulus for the transition of planktonic to biofilm form can be a defensive response of the bacterial cells after detecting the presence of the lectin at concentrations still unable to cause significant effects on growth [26]. In the LAC USA300 strain, there was inhibition of biofilm formation at all concentrations and the values were higher than 50% at concentrations from 25 to 200 μg/mL (Fig. 6B). Thus, the lectin was more effective in inhibiting the biofilm formation of the MRSA isolate.

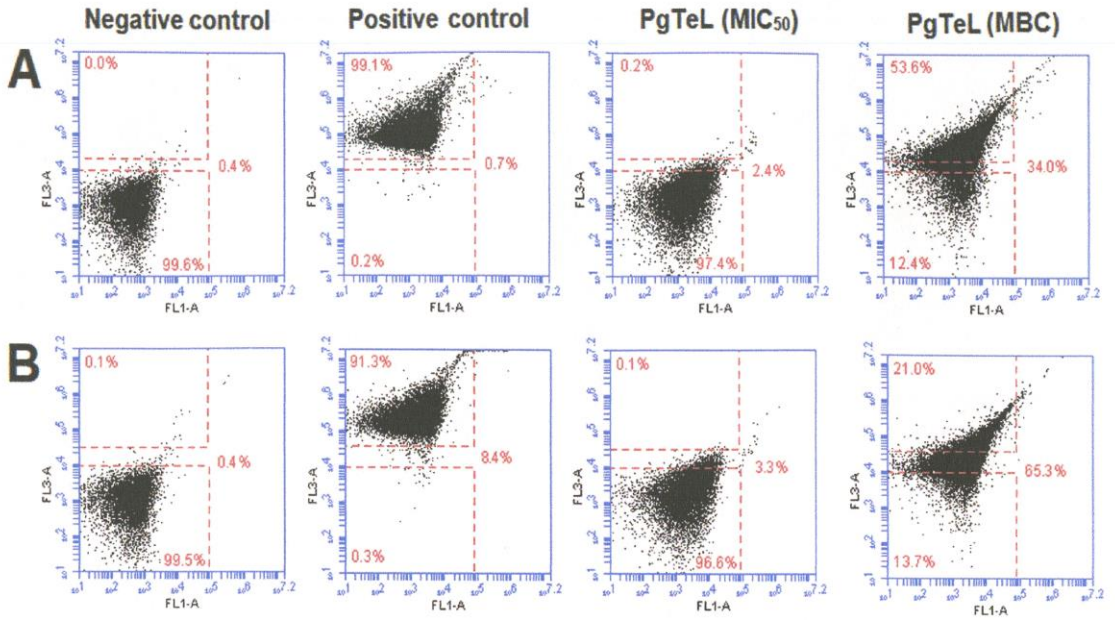


**Fig. 3.** Flow cytometry analysis of viability of *Staphylococcus aureus* 8325–4 (A) and LAC USA300 (B) isolates treated with or without PgTeL at the $MIC_{50}$ or MBC. In the negative control (NC), distilled water was used instead of lectin. Isopropyl alcohol (70%, v/v) was used as positive control (PC). The cells were stained with thiazol orange (FL1 channel) and propidium iodide (FL3 channel). The FL1 vs. FL3 dot plots were gated by scatter using NC and PC treatments as references for live cells (lower gate) and dead cells (upper gate), respectively. Between these gates, a population of injured cells can be observed.

The lectin from *Calliandra surinamensis* leaf pinnulae showed antibiofilm activity against the oxacillin-resistant *S. aureus* and the compound alpha-mangostin from the peels of *Garcinia mangostana*, as well as the *Vetiveria zizanioides* root extract were effective against biofilm formation by MRSA [28,40,48,49]. Different mechanisms are involved in the antibiofilm effect of lectins, such as the binding of these proteins to polysaccharides on the bacteria cell wall, preventing cell attachment and/or interrupting EPS polymerization, impairment of the viability of biofilm cells, and alteration in the expression of some genes related to biofilm formation and virulence [19].

The Agr system of *S. aureus* is considered a model of QS regulator in gram-positive bacteria. It consists of two units, RNAII and RNAIII, whose transcription is driven by the P2 and P3 promoters, respectively. The RNAII contains four genes: *agrB*, *agrD*, *agrC*, and *agrA*; the proteins AgrB and AgrD constitute an autoinducer peptide (AIP) and the AgrC corresponds to a transmembrane receptor for AIP [50]. The AIP can bind to AgrC present in the cell that produced it or in other cells, activating the DNA-binding protein AgrA, which can exert a positive feedback effect resulting in P2 activation. AIP can activate P3 as well, which regulates the transcription of alpha-hemolysin encoded by *hla* and of RNA

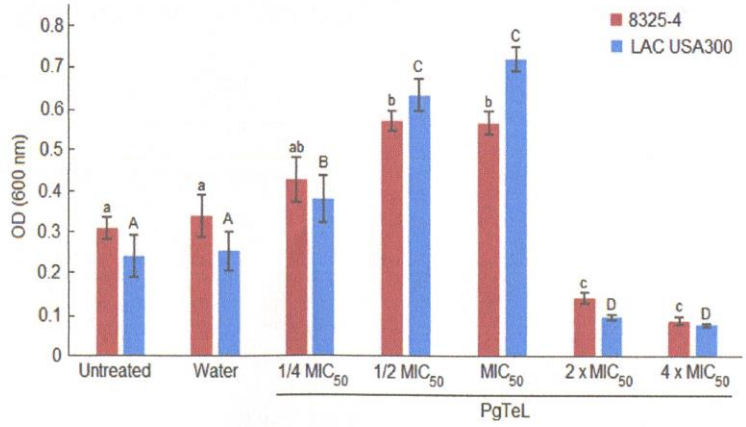


**Fig. 4.** Evaluation of PgTeL effect on the aggregation ability of *Staphylococcus aureus* 8325–4 and LAC USA300 strains. Quantification of aggregation was performed by measuring optical density (OD) at 600 nm of supernatants from the untreated and treated cultures after sedimentation. Distilled water was used in the control. Each bar represents the mean ± standard deviation of three replicates. Different letters indicate significant difference ($p < 0.05$) in comparison with the untreated sample. Lowercase letters were used for comparisons involving 8325–4 and uppercase letters for assays with LAC USA300.

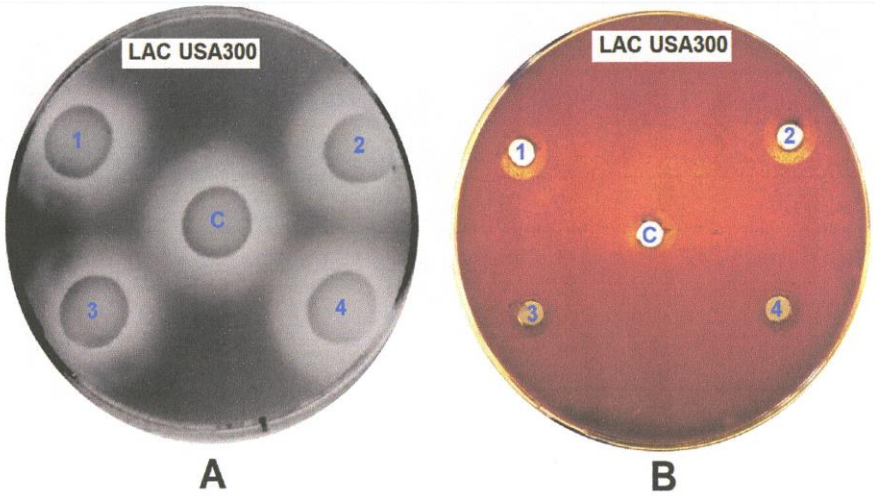


**Fig. 5.** Evaluation of PgTeL effect on the hemolytic ability of *Staphylococcus aureus* LAC USA300 (MRSA strain). (A) Suspensions of LAC USA300 together with PgTeL (1: 1/2 × $MIC_{50}$; 2: 1/4 × $MIC_{50}$; 3: 1/8 × $MIC_{50}$; 4: 1/16 × $MIC_{50}$) or distilled water (control, C) were dropped at different points on a blood agar plate. The hemolysis halo around the drops can be observed. (B) LAC USA300 culture was mixed with the blood agar and then holes were made in the plates. Distilled water (control, C) or PgTeL (1: 1/2 × $MIC_{50}$; 2: 1/4 × $MIC_{50}$; 3: 1/8 × $MIC_{50}$; 4: 1/16 × $MIC_{50}$) was added in the holes and incubated for 24 h at 37 °C. The appearance of a halo around the holes indicate hemolysis. This figure is representative of one set of screening plates.

III; in turn, RNA III activates the expression of virulence proteins (e.g. delta-hemolysin, as well as other extracellular toxins and enzymes) and suppresses the expression of surface-associated proteins such as the protein A encoded by *spa*, which is able to bind to immunoglobulins and impair host defense [51–53].

PgTeL did not interfere with the expression of the virulence genes *hla* (Fig. 7A) and *rnaIII* (Fig. 7B). Similar to these results, the expression of *spa* (which is down-regulated by RNAIII) was not activated in the treatments with the lectin (Fig. 7C). As expected, the addition of *S. schleiferi* 30743 supernatant in the positive control inhibited the expression of *hla* and *rnaIII* and activated the expression of *spa*. Despite the fact that PgTeL did not affect hemolysin and RNAIII generation, it can be noticed that this lectin did not activate the expression of *spa*, which enables *S. aureus* to evade host immune responses through its IgG-binding ability. The absence of alteration in *hla* expression agrees with the inability of PgTeL to modulate the hemolytic action of *S. aureus*. In contrast to PgTeL, solonamide B (a cyclodepsipeptide isolated from marine bacterium *Photobacterium halotolerans*) was able to reduce the expression of RNAIII [54] and lactam hybrid analogs of solonamide B are AgrC inhibitors [18]. Our results suggest that PgTeL action mechanisms against aggregation and biofilm formation by *S. aureus* are different from that involving interference with the expression of virulence genes evaluated and involve the binding to cell wall and membrane glycoconjugates.

## 4. Conclusion

PgTeL showed antibacterial activity against the clinical isolates of *S. aureus*, including a MRSA strain, exerting multiple damaging effects, affecting growth, structure, viability, aggregation, and biofilm formation.

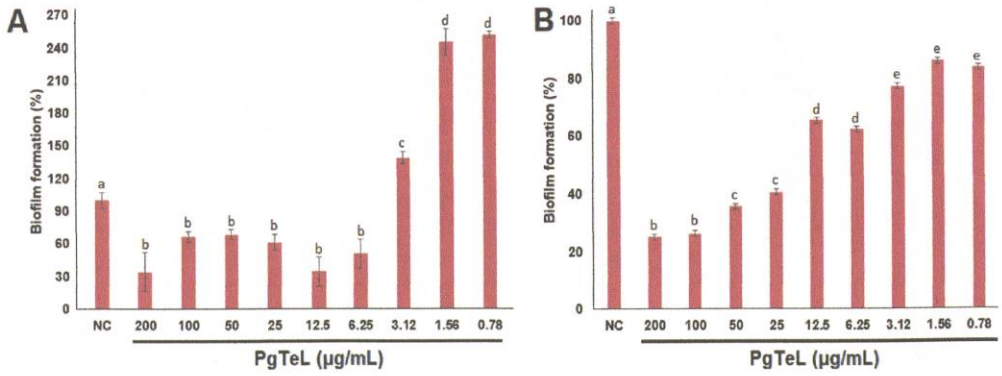


**Fig. 6.** Antibiofilm activity of PgTeL against *Staphylococcus aureus* 8325–4 (A) and LAC USA300 (B) strains. The cells were treated for 24 h with the lectin and the biofilm formation was evaluated by the crystal violet method and measurement of the optical density at 540 nm. Cells treated with distilled water corresponded to the negative control (NC). Different letters indicate statistical difference ($p < 0.05$) between the treatments and NC.

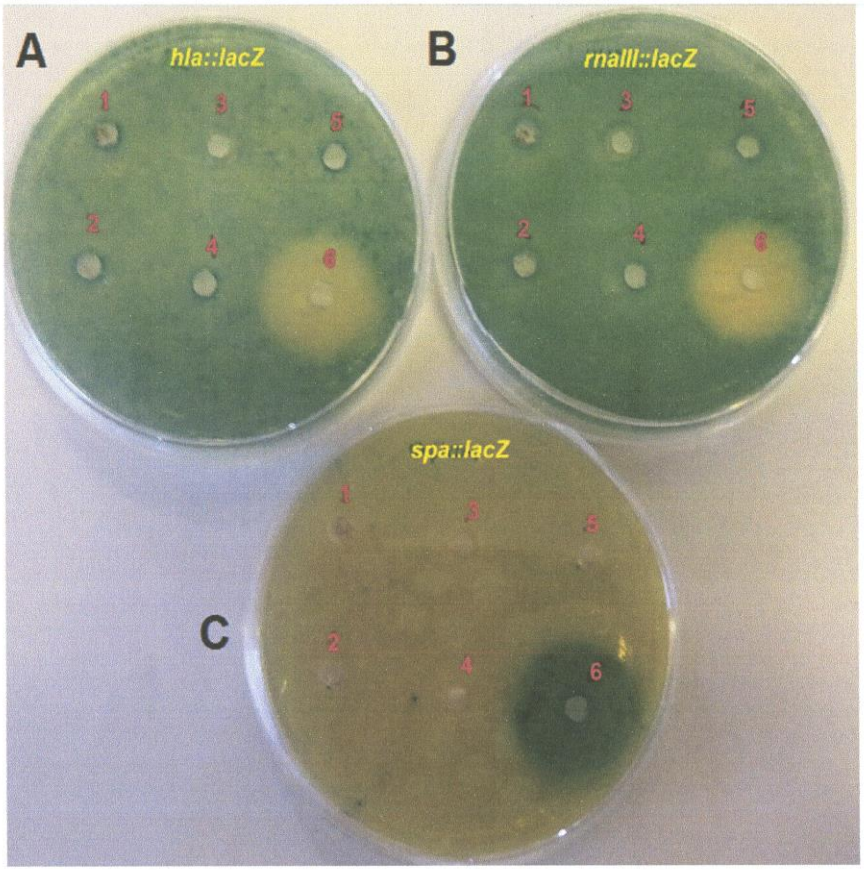


**Fig. 7.** Evaluation of virulence gene expression by *Staphylococcus aureus* in the absence or presence of PgTeL, by qualitative β-galactosidase reporter plate assay. Shown are TSA plates containing erythromycin and X-gal (β-galactosidase substrate), containing the strains PC322 *hla::lacZ*, SH101F7 *rnalII::lacZ*, and PC203 *spa::lacZ*. PgTeL was applied in the plate holes (1: 1/2 × $MIC_{50}$; 2: 1/4 × $MIC_{50}$; 3: 1/8 × $MIC_{50}$; 4: 1/16 × $MIC_{50}$) together with a staphylococcal culture supernatant containing auto-inducer peptide (AIP). The AIP presence activates the expression of *hla* and *rnalII* genes of the *agr* system; whereas, the RNAIII inhibits the expression of *spa* gene. Distilled water was used in negative control (hole 5). In the positive control (hole 6), the supernatant of a *Staphylococcus schleiferi* 30743 culture, a strain with inhibitory effect on *agr* system, was added. Zones appearing around the holes corresponded to β-galactosidase activity and indicates expression of the fusion gene. This figure is representative of one set of screening plates.

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## 3.3 ARTIGO 3 - *Punica granatum* SARCOTESTA LECTIN (PgTeL) HAS ANTIBACTERIAL ACTIVITY AND SYNERGISTIC EFFECTS WITH ANTIBIOTICS AGAINST β-LACTAMASE-PRODUCING *Escherichia coli*

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### Abstract

The sarcotesta of *Punica granatum* fruit contains an antimicrobial lectin called PgTeL. In this work, we evaluated the antibacterial activity of PgTeL against five drug-resistant *Escherichia coli* isolates able to produce β-lactamases. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations were determined by broth dilution. Morphometric and viability analyses were performed by flow cytometry, and ultrastructural changes were evaluated by scanning electron microscopy. Potential synergistic effects of PgTeL with antibiotics and anti-biofilm effect were also evaluated. PgTeL showed antibacterial activity against all isolates with MIC and MBC values ranging from 12.5 to 50.0 μg/mL and from 25.0 to 100.0 μg/mL, respectively. For most isolates, PgTeL postponed the growth start by at least ten hours. At the MIC, the lectin caused alterations in size, shape and structure of bacterial cells. The

combination PgTeL-ceftazidime showed a synergistic effect for all isolates. Synergy was also detected with ampicillin (one isolate), carbenicillin (one isolate), cefotaxime (one isolate), cephalexin (four isolates) and cefuroxime (three isolates). PgTeL exhibited anti-biofilm activity against all isolates, causing ≥50% inhibition of biofilms at or above 6.25 μg/mL. The antibacterial effect of PgTeL and its synergy with antibiotics indicate that this fruit-derived molecule may have potential for future treatment of multidrug-resistant infections.

**Keywords:** microbial resistance*;* pomegranate; fruit lectin; antibacterial activity; synergy.

## 1. Introduction

Microbial resistance has become one of the biggest health threats worldwide, being a leading cause of morbidity and mortality. One of the most widespread resistance mechanisms in bacteria is the production of β-lactamases, which are enzymes that hydrolyze β-lactam antibiotics. The β-lactamases are classified according to their molecular properties and/or amino acid sequences into distinct groups: the enzymes belonging to the A, C, and D classes use a serine residue in the active site for β-lactam hydrolysis and some examples are the extended-spectrum β-lactamases (ESBL), AmpC β-lactamases and oxacillinases, respectively. Class B includes the metallo-β-lactamases, whose catalysis mechanism involves zinc ions present at the active site [1–3].

The spread of Enterobacteriaceae strains producing β-lactamases is of great concern because they can hydrolyze the majority of β-lactams. In this scenario, there is a concentration of efforts on the discovery of new antibiotics and β-lactamase inhibitors, for being used alone or in combination with standard drugs, in order to minimize the impact of resistance [4–7]. *Escherichia coli* is a commensal bacterium of the gut in humans and animals. However, some strains have acquired virulence factors that make them capable of causing diseases like urinary tract infections, enteritis and meningitis [8].

One virulence factor that increases the resistance of bacteria to antibiotics is the formation of biofilms. Biofilm is a microbial life style characterized by a high density of cells synthesizing an exopolysaccharide matrix that maintains the cohesion between cells and allows them to adhere to biotic or abiotic surfaces. The physical protection conferred by this matrix contributes to higher resistance of the bacteria residing in biofilms in comparison with

planktonic forms. Furthermore, bacteria in biofilms have an increased exchange of genetic DNA facilitating the spreading of antibiotic resistance genes [9, 10].

Plants are broadly used in folk medicine to treat diseases, including infections [11]. A lot of plant compounds have antimicrobial properties such as the lectins isolated from leaves of *Schinus terebinthifolius*, *Moringa oleifera* seeds, *Alpinia purpurata* inflorescence, and *Portulaca elatior* root [12–15]. The lectins are bioactive proteins that possess a carbohydrate-binding domain that is capable of interacting in a reversible and non-covalent way with free sugars as well as with those attached to proteins and lipids, even found in cell surfaces [16–17].

Earlier studies reported that the sarcotesta of *Punica granatum* contains a chitin-binding lectin called PgTeL, which is a 26-kDa protein with sequence similarities (37%) with a class III chitinase from *P. granatum* seeds; this lectin stands out for its thermostability as it does not undergo unfolding even when heated at 100 ºC [18, 19]. Furthermore, it has antibacterial activity against *Aeromonas* sp., *Enterococcus faecalis*, *E. coli*, *Klebsiella* sp., *Micrococcus luteus*, *Salmonella enterica* serovar. Enteritidis, *Serratia marcescens*, *Streptococcus mutans*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and can also reduce adhesion and invasion of human cells by *Aeromonas* sp., *S. aureus*, *S. marcescens* and *S. enterica* [18]. This lectin showed bacteriostatic and bactericidal properties against non-resistant *S. aureus* and methicillin-resistant strains (MRSA), causing remarkable alterations in cell morphology, and impairing the aggregating and biofilm-forming abilities [20]. In another study, it was demonstrated that PgTeL has antifungal activity against *Candida albicans* and *Candida krusei* by promoting oxidative stress, energetic collapse, and damage to the cell wall [19].

In the present work, the effects of PgTeL on growth, viability, morphology and biofilm-forming ability of drug-resistant *E. coli* isolates producing different types of β-lactamase (CTX-M, CMY, and metallo-β-lactamases) were determined. In addition, a potential synergistic effect between PgTeL and antibiotics, to which the isolates are resistant, was evaluated.

## 2. Materials and methods

### *2.1. Microorganisms and growth conditions*

The *E. coli* isolates used in the present work were 2011-60-1493-3 (from caecum samples from pigs collected at slaughter in Denmark), 2011/25/62 (human urinary tract infection, collected in USA), C-24716 (dog wound, collected in Denmark), and C-26036 (dog ear infection, collected in Denmark), which produce the enzymes CTX-M-14, CMY-2, CTX-M-14/CMY-2, CTX-M-1, respectively. In addition, we included the isolate 1184b (healthy pig, collected in Denmark), which is a suspected metallo-betalactamase-producer based on its resistance profile. We also included the ATCC 25922 reference strain, which does not produce β-lactamase. The bacteria were provided by the Department of Veterinary and Animal Sciences at the University of Copenhagen, Denmark. The bacteria were grown overnight in Luria-Bertani (LB) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in a 1:10 volume/flask ratio, at 37 ˚C for 24 h, under shaking at 200 rpm. Next, bacteria were cultured on LB agar overnight at 37 °C for 24 h. For use in the experiments, the optical density of culture at 600 nm ($OD_{600}$) was adjusted with sterile saline solution (0.15 M NaCl) in order to reach the cell density required in each assay as described in the next sections.

*2.2. Lectin isolation*

Pomegranate fruits were collected at Glória do Goitá (Pernambuco, Brazil) under authorization of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of Environment (licence number 36301). The access was recorded (A9F23D0) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). PgTeL was isolated according to Silva et al. [18]: the sarcotesta was passed through a sieve (1-mm mesh), mixed with 0.15 M NaCl (in a proportion of 9:1, v/v), and homogenized during 6 h under magnetic stirring. After filtration and centrifugation (3000 *g*, 15 min, 4 ºC), the supernatant was treated with ammonium sulphate at 30% saturation [21]. After centrifugation, the supernatant was dialyzed against distilled water (4 h) followed by 0.15 M NaCl (2 h) and loaded (4.0 mg of protein) onto a chitin (Sigma-Aldrich, USA) column (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl. PgTeL was recovered by elution with 1.0 M acetic acid and dialyzed against distilled water (4 h) for eluent removal.

*2.3. Protein concentration and hemagglutinating activity (HA) of PgTeL*

Protein concentration was determined according to Lowry et al. [22] using a standard curve of bovine serum albumin (31.25–500 μg/mL). The HA was determined using a suspension of rabbit erythrocytes (2.5% v/v) previously treated with glutaraldehyde [23]. Collection of erythrocytes was approved by the Ethics Committee on Animal Experimentation of the *Universidade Federal de Pernambuco* (process 23076.033782/2015-70). In a 96-well V-bottomed microplate, PgTeL (50 μL) was serially two-fold diluted in 0.15 M NaCl followed by addition of 50 μL of the erythrocyte suspension to each well. For negative controls, erythrocytes were incubated only with 0.15 M NaCl. After incubation for 45 min at 28 ºC, the number of HA units (HAU) was determined as the reciprocal of the highest dilution of the sample that promoted full agglutination of rabbit erythrocytes. The specific HA was defined as the ratio between the HAU and the protein concentration (mg/mL).

*2.4. Determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations*

The antibacterial activity assay was performed according to guidelines from the Clinical and Laboratory Standards Institute [24]. In a row of a 96-well flat-bottomed microplate, 100 μL of Mueller Hinton broth (MHB) was added to all wells except the first (sterility control), which was filled with 200 μL of this culture medium. Next, 100 μL of PgTeL (0.5 mg/mL) was added to the third well and subjected to a two-fold serial dilution up to the twelfth well. The bacterial suspension (100 μL at 3 x $10^6$ colony-forming units, CFU, per mL) was added from the second until the twelfth well. The second well corresponded to the growth control. The microplates were incubated at 37 °C. The $OD_{600}$ was measured at time zero and after 24 h using a Epoch microplate reader (BioTek, Winooski, VT, USA). The MIC was defined as the lowest PgTeL concentration able to promote total inhibition, in comparison with the 100% growth control. To determine the minimal bactericidal concentration (MBC), inoculations (10 μL) from the wells containing the lectin at concentrations higher or equal to the MIC were transferred to Mueller Hinton Agar plates, which were incubated at 37 °C for 24 h. The MBC corresponded to the lowest PgTeL concentration able to reduce 99.9% of bacteria in the initial inoculum. Each assay was conducted in triplicate and three independent experiments were performed.

*2.5. Growth curves*

Twenty-four-hour growth curves in the absence or presence of PgTeL were established for all five *E. coli* isolates in assays performed as described in the previous section. In the microplate row, the first well corresponded to the sterility control; the second well corresponded to the 100% growth control; and the third to seventh wells corresponded to treatments at 4×MIC, 2×MIC, MIC, ½MIC, and ¼MIC. The microplate was incubated at 37 ºC and the $OD_{600}$ was determined every hour. Three independent experiments were performed in triplicate.

*2.6. Flow cytometry analysis*

The shape, size and viability of bacterial cells treated or not with PgTeL were evaluated by flow cytometry in a BD Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA). The strains (3 x $10^6$ CFU/mL) were incubated as described in section 2.4 with PgTeL at the MIC. The negative control was prepared by adding distilled water instead of PgTeL. The samples were centrifuged (10,000 × *g*, 10 min, 25 °C) and the cell pellets were washed three times with 0.1 M PBS pH 7.0. The Cell Viability Kit of BD Biosciences was used to analyze viability, starting by adding 42 µM thiazole orange (TO, 5 µL) and 4.3 mM propidium iodide (PI, 5 µL) to the samples, which were then vortexed and incubated for 5 min at 25 ºC. Data were acquired in the cytometer with an SSC threshold of 200 and stopped after getting 20,000 events for each sample. Analysis was performed in the BD Accuri C6 Software. The results were presented as FSC vs. SSC dot plots and mean FL3 fluorescence (PI staining).

*2.7. Microscopy analysis*

The structure of bacterial cells with or without PgTeL was evaluated by scanning electron microscopy (SEM). Bacterial cells (1.2 mL; $10^8$ CFU/mL) were incubated with MHB (0.6 mL) and PgTeL (0.4 mL) at the respective MIC in Milli-Q water. The negative control was established by adding Milli-Q water instead of lectin. After incubation (24 h at 37 °C),

the samples were centrifuged (300 × *g*; 10 min, 25 °C) and the precipitated cells were washed three times in 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 28 ºC. After washing with the same buffer, the cells were allowed to adhere to stubs (Ø 12.7 mm, 9 mm length; Ted Pella Inc., Redding, CA, USA) and post-fixed for 1 h with 1% osmium tetroxide. The cells were dehydrated in graded acetone, critical-point-dried with $CO_2$, coated with a 20 nm-thick gold layer, and observed with a JEOL JSM-5600 LV microscope (JEOL, Peabody, MA, USA). Cells without treatment were used as controls.

*2.8. Synergy assay*

Synergistic effects between PgTeL and antibiotics (amoxicillin, ampicillin, carbenicillin, cefotaxin, ceftazidime, cephalexin, cefuroxime, penicilin G) were evaluated using the method described by Pillai et al. [25]. Each experiment corresponded to two rows of a 96-well microplate. The antibiotic was added (80 µL, 200 µg/mL) to the fourth well of the first row and serial two-fold dilution in sterile Milli-Q water was performed until the penultimate well of the second row. Next, PgTeL was added (80 µL at 8×MIC) to the penultimate well of the second row, and two-fold serial dilution was carried out in the opposite direction until the fourth well of the first row. The third well of the first row contained only the drug (100 µg/mL), and the last well of the second row contained only PgTeL (4×MIC). MHB (40 µL) was added to all wells, except the first, which contained 200 µL of culture medium (sterility control). The second well corresponded to the 100% growth control. Each well, except the first, was inoculated with microbial suspension (80 µL at $3\times10^6$ CFU/mL) and incubated at 37 ºC. The experiment was followed by measuring the $OD_{600}$ at time zero and after 24 h. The evaluation of the interaction between the different treatments was performed by determining the fractional inhibitory concentration index (ΣFIC), as follows: ΣFIC = (MIC of PgTeL in combination/MIC of PgTeL alone) + (MIC of antibiotic in combination/MIC of antibiotic alone). The combinations were classified as synergistic (ΣFIC≤0.5), additive (ΣFIC > 0.5–1.0), indifferent (ΣFIC > 1.0–≤ 4.0) or antagonistic (ΣFIC > 4.0).

*2.9. Static biofilm assay*

The effects of PgTeL on biofilm formation were determined according to Nielsen et al. [26] with minor modifications. Fifty μL of a bacterial culture in MHB ($OD_{600}$=0.5) were 10-fold diluted in 0.15 M NaCl, and then 5 μL were inoculated into wells of a 96-well microtiter plate containing 200 μL of MHB. PgTeL was added to achieve final concentrationsfrom 1.56 to 200 μg/mL. Distilled water was added in the control. The plates were incubated for 24 h at 37 ˚C without shaking. After this period, the planktonic cells and culture medium were removed and the attached biofilm was washed twice with 0.9% NaCl (200 μL), dried in LAF-bench and stained with 0.1% crystal violet (125 μL) for 30 min followed by three washing steps with 0.9% NaCl (200 μL). For quantification purposes, the stain bound to the biofilm was solubilized in 96% ethanol and the absorbance measured at 590 nm using a microplate reader. Three independent experiments were performed in triplicate.

*2.10. Statistical analysis*

The data were expressed as the mean or the percent mean ± standard deviation (SD) and statistical differences were determined using Tukey's range test; a p-value < 0.05 was considered statistically significant.

## 3. Results and discussion

In recent decades, community- and hospital-acquired infections caused by β-lactamase-producing bacteria have increased worldwide with β-lactam hydrolysis being the most common mechanism of resistance of Gram-negative bacteria against third-generation cephalosporins. This scenario has prompted the use of last-resort antibiotics with the inherent risk of selecting for even more resistant bacteria such as carbapenemase-producing Gram-negatives [5, 27]. Thus, there is a growing need for new antimicrobial agents, and lectins isolated from plants have been reported as alternative antimicrobial agents [17].

In this work, the antibacterial action of PgTeL against drug-resistant *E. coli* isolates producing different types of β-lactamases was evaluated. PgTeL showed bacteriostatic and bactericidal effects against all tested isolates with MIC ranging from 12.5 to 50.0 and MBC from 25.0 and 100.0 μg/mL (Table 1). It was more active on the ATCC strain and less active

on the CTX-M-14-producing isolate. In previous work, it was reported that PgTeL at 100 µg/mL did not have cytotoxic effects on human peripheral blood mononuclear cells (PBMCs) [19].

The MIC and MBC values of PgTeL against ATCC strain of *E. coli* were lower than those determined for a chitin-binding *S. terebinthifolia* leaf lectin (28.75 and 115 µg/mL, respectively), which was also isolated from a plant used against infections in folk medicine [12]. Although the chitin-binding lectin from *Myracrodruon urundeuva* heartwood was a more effective bacteriostatic agent (MIC of 1.17 µg/mL) than PgTeL against standard *E. coli* strain, this lectin did not show bactericidal effect [28].

Our results indicate a new antimicrobial agent present in the pomegranate fruit that is able to inhibit the growth of β-lactamase-producing bacteria, in addition to some secondary metabolites previously reported. For example, methanol extract from *P. granatum* pericarp presented action against Gram-negative bacilli that produce ESBL and metallo β-lactamase [29]. Caffeic and ellagic acids, epigallocatechin-3-gallate and quercetin isolated from the pomegranate juice showed action against β-lactamase-producing *Klebsiella pneumoniae* [30]. In addition, ethanolic and methanolic extracts from *P. granatum* pericarp were also active against ESBL-producing *E. coli* [31].

Figure 1 shows the growth curves of *E. coli* isolates during 24-h incubation in absence or presence of PgTeL. Growth of all bacteria was inhibited from the first hour of incubation in the treatments with PgTeL at 2×MIC and 4×MIC corroborating with MBC values. In the treatments at MIC, the growth started only after 10–18h of incubation, except for the CTX-M-14/CMY-2-producing strain (Figure 1C), which started to grow after one hour, although slower than negative control. Interestingly, the growth of all isolates in the presence of PgTeL at the ½MIC showed to be delayed. The results reveal that PgTeL not only reduces the bacterial count but also postpones the start of cell replication.

Flow cytometry analysis was employed to evaluate alterations in the shape and viability of *E. coli* cells treated with PgTeL at MIC. The FSC vs. SSC dot plots (Figure 2) show that, in PgTeL treatments, there were displacements of the cells in direction of higher FSC and SSC values, indicating morphometric changes regarding shape. This result can also be indicative of cell aggregation. In spite of this, the cells were still viable after PgTeL treatment as indicated by the FL3 fluorescence (Figure 2). These results indicate the predominance of a bacteriostatic effect at this concentration.

The effects of PgTeL on bacterial structure were evaluated through three-dimensional images of cells treated with PgTeL at MIC (Figure 3). Control cells showed a preserved shape (bacilli with rounded ends), regular size, and smooth surface. In all the treatments with the lectin, a reduction in the number of cells was observed along with severe alterations in shape and size. These data reveal that PgTeL causes strong damage to the structure of all isolates, corroborating the evidence of bigger cells from flow cytometer analysis. The bacteria cell surface is rich in teichoic and teichuronic acids, lipopolysaccharides and peptidoglycan. Interactions between lectins and these components can lead to agglutination, growth inhibition, changes in cellular permeability and interactions with membrane receptors that trigger intracellular responses. The lectins can induce pore formation in the cell wall and cell membrane, thus leading to leakage of cytoplasmic content [17, 32].

New antimicrobial agents are being searched not only to be used alone but also in combination with standard drugs in order to overcome the phenomenon of antimicrobial resistance. Plant compounds have been considered as good alternatives because they can act by distinct action mechanisms and have less side effects. Thus, we evaluated the action of PgTeL in combination with conventional antibiotics. The MIC values for the antibiotics used in the synergy assay can be seen in Table 2. According to the Clinical and Laboratory Standards Institute [33], all isolates were considered resistant to all antibiotics tested, except those producing CTX-M-14 and CMY-2 enzymes, which were sensitive to ceftazidime. The standard strain (ATTC 25922) was sensitive to all the drugs.

Table 2 shows that PgTeL presented a synergistic effect together with ampicillin and carbenicillin (against CMY-2-producing isolate), cefotaxime (suspected metallo-β-lactamase-producing isolate), ceftazidime (all isolates), cephalexin (CTX-M-14-, CMY-2-, CTX-M-14/CMY-2 and suspected metallo-β-lactamase-producing isolates) and cefuroxime (CTX-M-14-, CTX-M-1, and suspected metallo-β-lactamase-producing isolates). No synergy was detected in combinations with amoxicillin and penicillin G. An additive effect was detected for PgTeL-ampicillin and PgTeL-cephalexin against the CTX-M-1-producing isolates and PgTeL-carbenicillin and PgTeL-cefuroxime against the CTX-M-1/CMY-2-producing isolate. Synergy of lectin-antibiotic combinations were also detected by Ferreira et al. [14], evaluating the effects of *A. purpurata* inflorescence lectin against oxacillin-resistant isolates of *S. aureus*. Alkaloids extracted from *Sophorea alopecuroides* showed synergistic effects with cefotaxime and ceftazidime against ESBL-producing *E. coli* isolates [34] and *Periploca laevigata*

essential oil also showed synergistic effect in combination with cefexime, ciprofloxacin and gentamicin against *E. coli* [35]. This is the first study reporting the synergistic action between a lectin and standard drugs against β- lactamase-producing *E. coli*.

Biofilms provide protection against antibiotic therapy and host immune responses, and facilitate the transfer of genes linked to resistance [36]. PgTeL inhibited biofilm formation by CTX-M-14-producing isolate at concentrations of 50 to 200 µg/mL (Figure 4A). For the CMY-2-producing strain, there was inhibition ≥ 50% at concentrations from 6.25 to 200 µg/mL (Figure 4B), while for the isolates producing CTX-M-14/CMY-2 (Figure 4C) and CTX-M-1 (Figure 4D), biofilm inhibition in more than 50% was detected at lectin concentration ranges of 25–200 µg/mL. Biofilm production by the strain producing suspected metallo-β-lactamase was reduced in more than 50% at treatments from 12.5 to 200 µg/mL (Figure 4E). The ATCC strain had biofilm formation impaired since the treatment with PgTeL at 3.12 µg/mL (Figure 4F). Antibiofilm activity of PgTeL was also reported against *S. aureus* and *Candida albicans* [19, 20].

Lectins have been reported as antibiofilm agents such as the *Bauhinia variegata* lectins (native and recombinant forms), which inhibited biofilm formation on a saliva-coated surface by *Streptococcus mutans* and *Streptococcus sanguis* [37]. The lectin from the marine sponge *Aplysina fulva* reduced the biofilm production by *S. aureus*, *S. epidermidis* and *E. coli* [38], and the water-soluble *Moringa oleifera* seed lectin impaired biofilm development by *S. marcescens* and *Bacillus* sp. when immobilized in a glass surface [39]. The *A. purpurata* lectin reduced growth and biofilm formation in *S. aureus* [14]. Lectins can interfere with biofilm development by affecting bacterial cell viability, reducing the expression of genes related with quorum sensing mechanisms and biofilm formation, and interacting with surfactants, enzymes and polysaccharides involved in biofilm production, for example [17, 32]. The antibiofilm agents have great biotechnological importance because biofilms are cause of high rates of chronic infections in humans, mainly those associated to medical devices, as well as large losses in many industries, such as those producing food and petroleum derivatives [9].

## 4. Conclusion

PgTeL showed antibacterial activity against clinical and drug-resistant isolates of *E. coli* producers of β-lactamases by exerting damaging effects on growth, cell structure and biofilm formation. In addition, the lectin showed synergistic effect with antibiotics to which the isolates displayed resistance. *In vivo* studies are required to determine if the promising antibacterial effect of PgTeL is reproducible in infection models, either alone or in combination with currently available antimicrobial drugs.

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**Figure captions**

**Figure 1.** Growth curves (24-h incubation) of β-lactamase-producing *Escherichia coli* isolates in the absence (negative control) and presence of PgTeL at different concentrations. It is presented the curves for the isolates producers of the enzymes CTX-M-14 (A), CMY-2 (B), CTX-M-14/CMY-2 (C), CTX-M-1(D) and a suspected metallo β-lactamase (E) as well as of the standard strain ATCC 25922 (F). Data are expressed as the mean ± standard deviation (SD) of three experiments. MIC values are presented in Table 1.

**Figure 2.** Flow cytometry analysis of morphology and viability of of β-lactamase-producing *Escherichia coli* cells incubated for 24-h in the absence (control) or presence of PgTeL at the MIC. In the negative control (NC), distilled water was used instead of lectin. The isolates analyzed are producers of the enzymes CTX-M-14 (A), CMY-2 (B), CTX-M-14/CMY-2 (C), CTX-M-1(D) and a suspected metallo β-lactamase (E). The left and central images shows the FSC vs. SSC dot plots. In the overlay histograms at right it can be seen the fluorescence in the FL3 channel (staining with prodidium iodide). The images are representative of three experiments.

**Figure 3**. Scanning electron microscopy (SEM) of β-lactamase-producing *Escherichia coli* cells incubated for 24-h in the absence (control) or presence of PgTeL at the MIC. It can be seen images of the isolates producers of the enzymes CTX-M-14 (A), CMY-2 (B), CTX-M-14/CMY-2 (C), CTX-M-1(D) and a suspected metallo β-lactamase (E). PgTeL reduced the number of cells, which presented strong alteration in the shape as well as cells surface damage.

**Figure 4.** Biofilm formation by β-lactamase-producing *Escherichia coli* isolates in the absence (negative control) and presence of PgTeL at different concentrations. The isolates evaluated ate producers of the enzymes CTX-M-14 (A), CMY-2 (B), CTX-M-14/CMY-2 (C), CTX-M-1(D) and a suspected metallo-β-lactamase (E). A standard strain ATCC 25922 (F) was also tested. The cells were treated for 24 h with the lectin, and the biofilm formation was evaluated by the crystal violet method and measurement of the optical density at 540 nm.

Cells treated with distilled water corresponded to the negative control (NC). Different letters indicate statistical difference ($p < 0.05$) between the treatments and NC.

**Table 1.** Antibacterial activity of PgTeL against β-lactamase-producing *Escherichia coli* isolates.

| Isolate | Enzymes produced | PgTeL (μg/mL) | |
|---|---|---|---|
| | | MIC | MBC |
| 2011-60-1493-3 | CTX-M-14 | 50 | 100 |
| 2011/25/62 | CMY-2 | 25 | 50 |
| C-24716 | CTX-M-14/CMY-2 | 25 | 50 |
| C-26036 | CTX-M-1 | 25 | 50 |
| 1184b | Metallo β-lactamase | 25 | 100 |
| ATCC 25922 | - | 12.5 | 25 |

MIC: minimal inhibitory concentration. MBC: minimal bactericidal concentration.

**Table 2.** Evaluation of antibacterial activity of PgTeL and antibiotics (separated or in combination) against β-lactamase-producing *Escherichia coli* isolates.

| Isolates | Enzymes produced | MIC (µg/mL) | | | | ΣFIC | Effect |
|---|---|---|---|---|---|---|---|
| | | Alone | | In combination | | | |
| | | PgTeL | Antibiotic (MIC) | PgTeL | Antibiotics | | |
| 2011-60-1493-3 | CTX-M-14 | 50 | Amoxicillin (>200) (R) | 50 | Amoxicilin (>200) | 2.0 | Indifferent |
| | | | Ampicilin (>200) (R) | 50 | Ampicilin (>200) | 2.0 | Indifferent |
| | | | Carbenicilin (>200) (R) | 50 | Carbenicilin (>200) | 2.0 | Indifferent |
| | | | Cefotaxin (50) (R) | 50 | Cefotaxin (50) | 2.0 | Indifferent |
| | | | Ceftazidime (6.25) (S) | 0.024 | Ceftazidime (1.56) | 0.25 | Synergism |
| | | | Cephalexin (100) (R) | 12.5 | Cephalexin (0.00305) | 0.25 | Synergism |
| | | | Cefuroxime (>200) (R) | 0.78 | Cefuroxime (0.048) | 0.015 | Synergsim |
| | | | Penicilin G (>200) (R) | 50 | Penicilin G (> 200) | 2.0 | Indifferent |
| 2011/25/62 | CMY-2 | 25 | Amoxicillin (>200) (R) | 25 | Amoxicilin (>200) | 2 | Indifferent |
| | | | Ampicilin (>200) (R) | 6.25 | Ampicilin (0.0061) | 0.25 | Synergism |
| | | | Carbenicilin (>200) (R) | 0.00305 | Carbenicilin (12.5) | 0.063 | Synergism |
| | | | Cefotaxin (25) (R) | 0,00038 | Cefotaxin (100) | 4.0 | Indifferent |
| | | | Ceftazidime (6.25) (S) | 0.048 | Ceftazidime (0.78) | 0.13 | Synergism |
| | | | Cephalexin (200) (R) | 0.024 | Cephalexin (1.56) | 0.0088 | Synergism |
| | | | Cefuroxime (100) (R) | 25 | Cefuroxime (100) | 2.0 | Indifferent |
| | | | Penicilin G (>200) (R) | 25 | Penicilin G (> 200) | 2.0 | Indifferent |
| C-24716 | CTX-M-14/CMY-2 | 25 | Amoxicillin (>200) (R) | 25 | Amoxicilin (>200) | 2.0 | Indifferent |
| | | | Ampicilin (>200) (R) | 25 | Ampicilin (>200) | 2.0 | Indifferent |
| | | | Carbenicilin (50) (R) | 0.00076 | Carbenicilin (50) | 1.0 | Additive |
| | | | Cefotaxin (200) (R) | 25 | Cefotaxin (200) | 2.0 | Indifferent |
| | | | Ceftazidime (50) (R) | 0.024 | Ceftazidime (1.56) | 0.032 | Synergism |
| | | | Cephalexin (200) (R) | 0.195 | Cephalexin (0.195) | 0.0088 | Synergism |
| | | | Cefuroxime (100) (R) | 12.5 | Cefuroxime (0.0305) | 0.5 | Additive |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Penicilin G (>200) (R) | 25 | Penicilin G (> 200) | 2.0 | Indifferent |
| C-26036 | CTX-M-1 | 25 | Amoxicillin (>200) (R) | 25 | Amoxicilin (>200) | 2.0 | Indifferent |
| | | | Ampicilin (200) (R) | 0.00038 | Ampicilin (100) | 0.5 | Additive |
| | | | Carbenicilin (50) (R) | 25 | Carbenicilin (50) | 2.0 | Indifferent |
| | | | Cefotaxin (200) (R) | 25 | Cefotaxin (200) | 2.0 | Indifferent |
| | | | Ceftazidime (100) (R) | 0.00305 | Ceftazidime (12.5) | 0.125 | Synergism |
| | | | Cephalexin (50) (R) | 0.00153 | Cephalexin (25) | 0.5 | Additive |
| | | | Cefuroxime (>200) (R) | 0.048 | Cefuroxime (0.78) | 0.0058 | Synergism |
| | | | Penicilin G (>200) (R) | 25 | Penicilin G (> 200) | 2.0 | Indifferent |
| 1184b | Suspected metallo β-lactamase | 25 | Amoxicillin (>200) (R) | 25 | Amoxicilin (> 200) | 2.0 | Indifferent |
| | | | Ampicilin (>200) (R) | 25 | Ampicilin (> 200) | 2.0 | Indifferent |
| | | | Carbenicilin (>200) (R) | 25 | Carbenicilin (> 200) | 2.0 | Indifferent |
| | | | Cefotaxin (100) (R) | 0.097 | Cefotaxin (0.39) | 0.0078 | Synergism |
| | | | Ceftazidime (50) (R) | 0.097 | Ceftazidime (0.39) | 0.0117 | Synergism |
| | | | Cephalexin (200) (R) | 0.097 | Cephalexin (0.39) | 0.0058 | Synergism |
| | | | Cefuroxime (100) (R) | 0.78 | Cefuroxime (0.048) | 0.0316 | Synergism |
| | | | Penicilin G (200) (R) | 25 | Penicilin G (200) | 2.0 | Indifferent |

MIC: minimal inhibitory concentration that promotes inhibition ≥50%. R: resistant. S: sensitive. ΣFIC: Sum of the fractional inhibitory concentration (FIC) indexes.

**Figure 1**

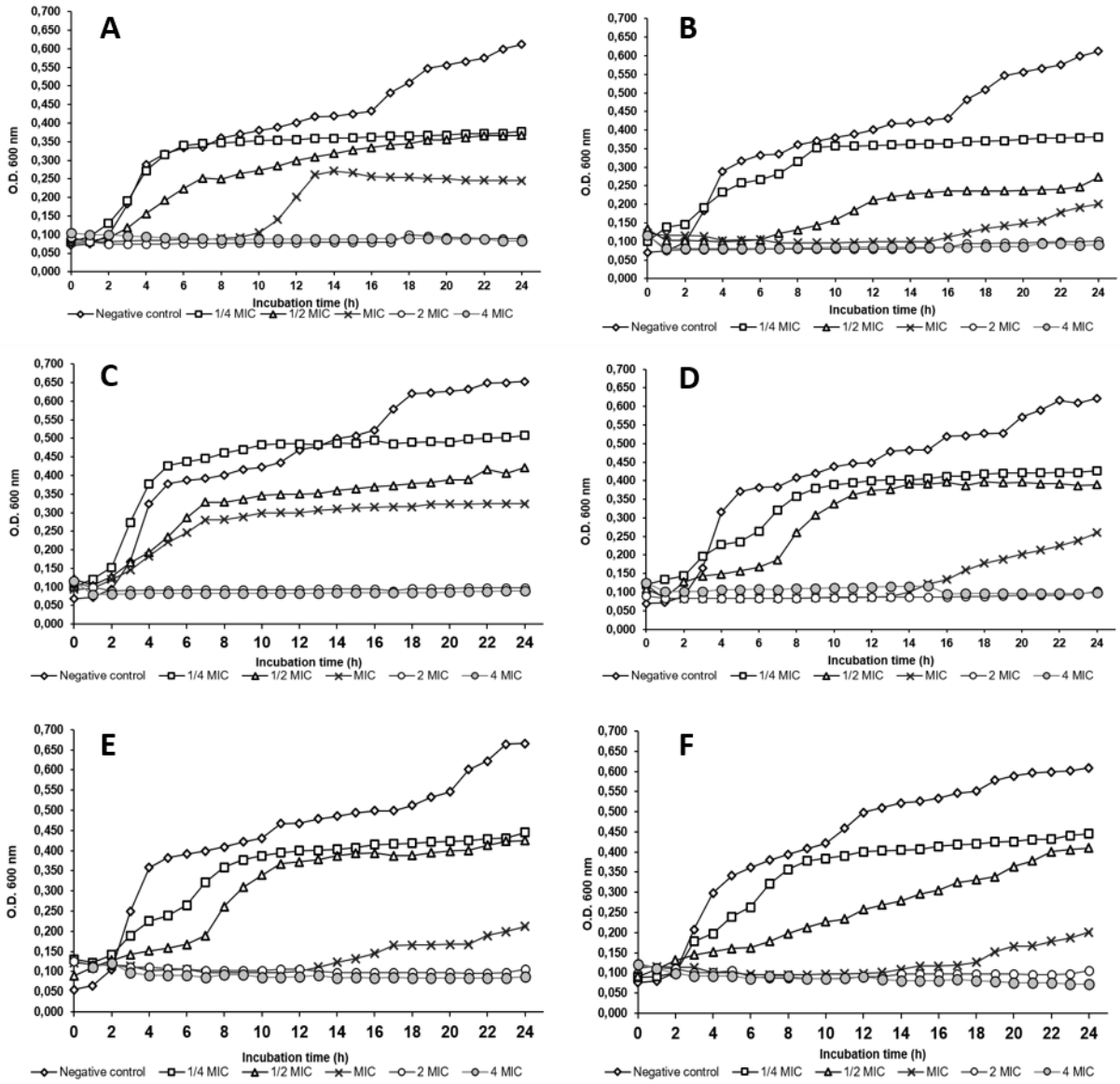

A
B
C
D
E
F
O.D. 600 nm
Incubation time (h)
Negative control
1/4 MIC
1/2 MIC
MIC
2 MIC
4 MIC

**Figure 2**

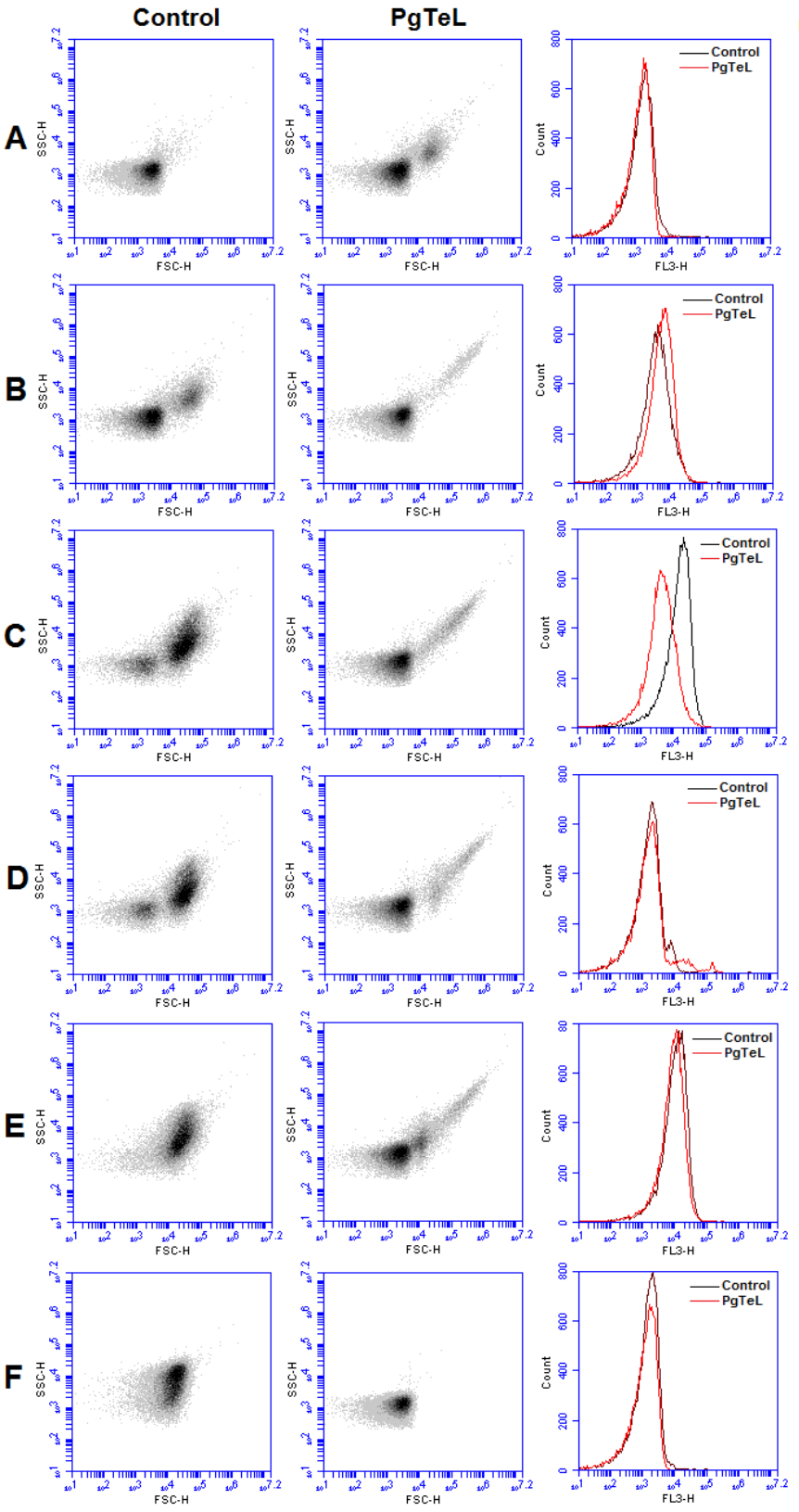


**Figure 3**

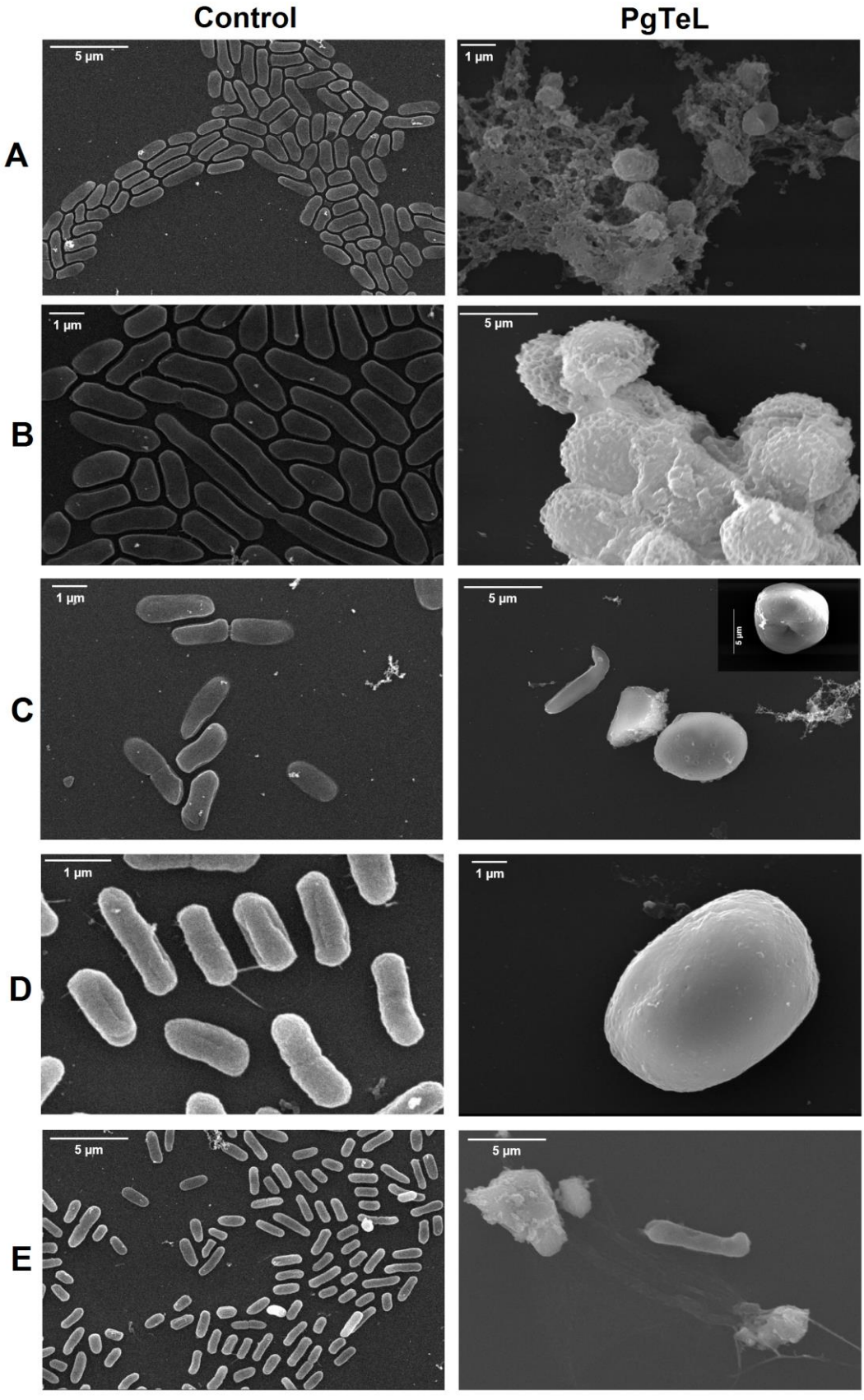
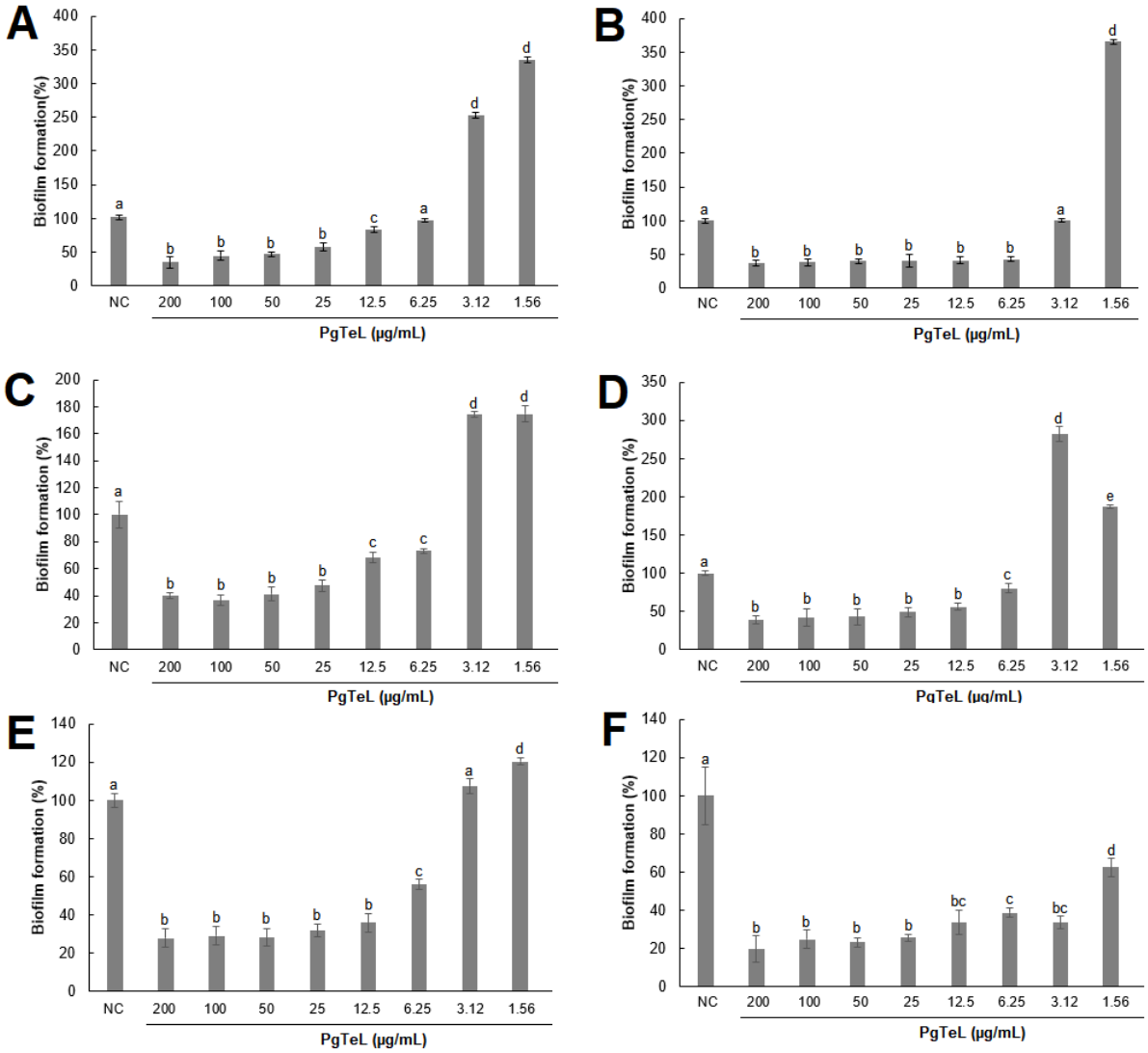

Control
PgTeL
A
B
C
D
E
5 µm
1 µm
1 µm
5 µm
1 µm
5 µm
5 µm
1 µm
1 µm
5 µm
5 µm

**Figure 4**


A
Biofilm formation(%)
PgTeL (μg/mL)
B
Biofilm formation(%)
PgTeL (μg/mL)
C
Biofilm formation (%)
PgTeL (μg/mL)
D
Biofilm formation (%)
PqTeL (μq/mL)
E
Biofilm formation (%)
PgTeL (μg/mL)
F
Biofilm formation (%)
PgTeL (μg/mL)
NC
200
100
50
25
12.5
6.25
3.12
1.56

# 4 CONCLUSÕES

- PgTeL, uma lectina termoestável e não-tóxica para células de sangue humano, apresentou atividade antifúngica contra espécies de *Candida* e atividade antibacteriana contra isolados clínicos de *S. aureus* (não-resistente e MRSA) e isolados de *E. coli* produtores de β-lactamases.
- A atividade antifúngica da lectina envolve indução de estresse oxidativo, colapso energético e rompimento da parede celular.
- Além de inibir o crescimento e causar a morte dos isolados de *S. aureus*, PgTeL também é capaz de interferir na agregação das células dessa espécie.
- A atividade antimicrobiana de PgTeL também se deve aos danos estruturais nas células de *Candida*, *S. aureus* e *E. coli*.
- A lectina também demonstrou potencial para inibir a formação de biofilme por *C. albicans* e pelos isolados bacterianos.
- Adicionalmente, a lectina apresentou potencial sinérgico junto a antibióticos para os quais os isolados de *E. coli* são resistentes.
- Os resultados estimulam a realização de estudos in vivo para determinar se os promissores efeitos antimicrobianos da PgTeL são reprodutíveis em modelos de infecção.

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