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Tese

Atividade antibacteriana e antioxidante de extrato de araçá (*Psidium cattleianum* Sabine): influência da temperatura, do genótipo e estudo do mecanismo antiestafilocócico

Andréia Saldanha de Lima

Pelotas, 2021

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Resumo

Lima, Andréia Saldanha. **Atividade antibacteriana e antioxidante de extrato de araçá (*Psidium cattleianum* Sabine): influência da temperatura, do genótipo e estudo do mecanismo antiestafilocócico**. 2021. 85f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, Pelotas, 2021.

O araçá é uma fruta rica em compostos bioativos que tem seu potencial antimicrobiano ainda pouco explorado. O objetivo deste estudo foi avaliar o potencial antibacteriano e antioxidante de extratos de araçá (EA), verificar a influência do genótipo e da temperatura e elucidar o mecanismo de ação dos EA contra *Staphylococcus aureus*. Os EA foram preparados com genótipos de araçá amarelos e vermelhos. A atividade antibacteriana dos EA foi avaliada por testes de disco difusão em ágar, concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM), utilizando cepas ATCC de *S. aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Typhimurium e *Campylobacter jejuni.* A atividade antioxidante foi determinada pelo sequestro do radical DPPH. O teor de fenóis totais foi determinado por espectrofotometria e os compostos fenólicos individuais foram quantificados por HPLC-MS, com todos os ensaios realizados antes e após os tratamentos térmicos. A termoestabilidade dos EA foi avaliada pela exposição dos extratos a temperaturas de até 100 ºC (40, 60, 80 e 100), por 1 h em cada temperatura. O mecanismo de ação antiestafilocócio foi determinado pela cinética de ação antibacteriana, integridade da membrana celular bacteriana (extravasamento de macromoléculas e ácidos nucleicos, microscopia eletrônica de varredura e microscopia confocal a laser), bem como por testes de interação dos EA com o DNA bacteriano (espectrofotometria de fluorescência). Os EA apresentaram atividade contra todos os micro-organismos avaliados, com halos de inibição variando entre 11 e 17 mm de diâmetro e valores de CIM de 9 a 147 $mg.mL^{-1}$ e de CBM de 18 a 294 $mg.mL^{-1}$. Os menores valores de CIM e CBM foram observados para *S. aureus*. Não houve diferença na atividade antibacteriana entre os genótipos estudados e, em todos os EA, os valores de atividade antioxidante foram superiores a 90%, antes e após o tratamento térmico. O teor de fenóis totais oscilou entre 414 e 465 $mg.100\ g^{-1}$ e os principais compostos fenólicos identificados foram os ácidos gálico, ferúlico e cumárico. O tratamento térmico dos EA aumentou de 2 a 4 vezes os valores de CIM e CBM e ocorreu redução na concentração dos compostos fenólicos, com exceção dos ácidos ferúlico e cumárico nos EA vermelhos, onde não houve perdas significativas. Quanto ao mecanismo de ação antiestafilocócico, verificou-se que o micro-organismo foi eliminado em 24 h de contato quando se utilizou a CBM, havendo perda da integridade

da membrana celular, provocando a liberação de importantes constituintes celulares. Os EA também apresentaram capacidade de se intercalar ao DNA bacteriano, demostrando que a atividade antiestafilocócica ocorre por alteração na integridade da membrana celular e danos ao DNA de *S. aureus*. Os resultados obtidos neste estudo são inéditos e demonstram que os EA possuem potencial antibacteriano e antioxidante, independente do genótipo, podendo ser uma alternativa para auxiliar na conservação de alimentos, incluindo alimentos processados termicamente.

Palavras-chave: aditivos naturais; atividade antiestafilocócica; bactérias patogênicas em alimentos; compostos fenólicos; frutas nativas.

Abstract

Lima, Andréia Saldanha**. Antibacterial and antioxidant activity of araçá extract (*Psidium cattleianum* Sabine): influence of temperature, genotype and study of the antistaphylococcal mechanism**. 2021. 85f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, Pelotas, 2021.

Araçá is a fruit with high levels of bioactive compounds whose antimicrobial potential is still little explored. The aim of this study was to evaluate the antibacterial and antioxidant activities of araçá fruit extracts (AEs), verify the influence of genotype and temperature on their bioactivity and elucidate the mechanism of action of AEs against *Staphylococcus aureus*. The AEs were prepared with yellow and red araçá genotypes. The antibacterial activity of the AEs was evaluated by disk diffusion, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) tests using ATCC strains of *S. aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Typhimurium and *Campylobacter jejuni*. Antioxidant activity was determined using the DPPH radical scavenging method. Total phenolic content was determined by spectrophotometry and the quantification of individual phenolic compounds was performed by HPLC-MS, with all determinations carried out before and after heat treatments. The thermostability was evaluated by exposing the AEs to temperatures between and 100 ºC, for 1 h at each temperature. The antistaphylococcal mechanism of action was determined by kinetics of antibacterial action, cell membrane integrity bacterial (releasing of macromolecules and nucleic acids, scanning electron microscopy and confocal laser scanning microscopy), as well as by testing the interaction of AEs with bacterial DNA (fluorescence spectrophotometry). The AEs showed antibacterial activity against all microorganisms, with inhibition halos diameter ranging from 11 to 17 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranged between 9 and 147 mg mL^{-1} and 18 and 294 mg mL^{-1}, respectively. The lowest MIC and MBC values were found against *S. aureus*. There was no difference in antibacterial activity between yellow and red genotypes and the antioxidant activity was higher than 90%, before and after the heat treatment in all AEs evaluated. The total phenol content ranged from 414 to 465 mg 100 g^{-1}, with higher values in red AEs than in yellow AEs. Gallic, ferulic, and cumharic acids were idenfied in all AEs. The heat treatment of the AEs increased 2 to 4 times the MIC and MBC values and there was a reduction in the concentration of phenolic compounds. In addition, the heat treatment caused partial losses in phenolic compounds, with exception of ferulic and cumharic acids in red AEs, in which did not have significant losses. In the study of the mechanism of antistaphylococcal action of AEs the bacterial multiplication was inhibited by AEs within 24 h of contact when the

MBC was used. The bacterial cell membrane was compromised, losing its integrity, and releasing important cellular constituents. AEs also showed the ability to intercalate with bacterial DNA, indicating that its antistaphylococcal activity of AEs occurs by modification in cell membrane integrity and by causing damage to the DNA of *S. aureus*. This study shows promising and unpublished results and demonstrates that AEs have antibacterial and antioxidant potential, regardless of genotype to be used as a natural adjuvant in food preservation, including thermally processed foods.

Key-words: antistaphylococcal activity; native fruits; natural aditives; pathogenic bacteria in foods; phenolic compounds.

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Sumário

1 Introdução

Todos os anos, milhares de pessoas adoecem pelo consumo de alimentos contaminados. A Organização Mundial da Saúde (OMS) reportou uma estimativa de que 31 agentes, entre bactérias, vírus, parasitas, toxinas e produtos químicos, são responsáveis pelas doenças transmitidas por alimentos (DTA), em nível global e regional, com 600 milhões – quase uma em cada dez pessoas no mundo – de pessoas doentes pelo consumo de alimentos contaminados com esses agentes (WHO, 2017).

Nesse contexto, a industrialização se torna fundamental à conservação dos alimentos, evitando perdas decorrentes de um sistema de abastecimento deficiente e da sazonalidade. No entanto, o consumo em excesso de alimentos industrializados, quando adicionados de aditivos químicos sintéticos, como os conservantes, a longo prazo, parece ter um impacto negativo à saúde do consumidor (FLEMING-JONES, M. E.; SMITH, R. E., 2003; GARCIA-FUENTES et al., 2015; HRNCIROVA et al., 2019; YU CAO et al., 2020).

O uso de aditivos naturais na conservação de alimentos é uma forte tendência de mercado. Uma ampla revisão sistemática, realizada em diferentes países europeus, a qual envolveu mais de oitenta mil consumidores, relata que para a maioria dos consumidores de países desenvolvidos, a naturalidade em produtos alimentícios exerce grande importância na hora da escolha do alimento. Essa naturalidade a que a pesquisa se refere, é vista como o alimento ser o menos processado possível e livre de aditivos químicos sintéticos (ROMÁN; SANCHEZ-SILES; SIEGRIST, 2017).

As propriedades bioativas das plantas são conhecidas há muito tempo. Visando encontrar alternativas aos aditivos químicos sintéticos, estudos sobre o efeito antimicrobiano de óleos e extratos vegetais contra patógenos de origem alimentar têm sido realizados, com resultados promissores (DANNENBERG et al., 2016; GOKOGLU, 2018; HAUBERT et al., 2019; HUSSAIN et al., 2021; LIMA et al., 2020;

MAIA et al., 2017; MAIA et al., 2019; MENDONÇA, et al., 2018; ZANDONÁ et al., 2020). Nesse aspecto, o Brasil possui uma das maiores biodiversidades do planeta, com fontes inexploradas de frutíferas nativas, ricas em compostos bioativos. Dentre essas frutíferas, o araçá é uma fruta da família das Mirtáceas, encontrada entre os estados da Bahia e o Rio Grande do Sul, bem como em outros países da América do Sul, o qual é amplamente cultivado em pomares domésticos (BEZERRA et al., 2016).

A composição química e fitoquímica de aráças (*Psidium cattleianum* Sabine), já foi bem caracterizada e a bioatividade atribuída aos frutos deve-se, principalmente, ao alto conteúdo de compostos fenólicos, que são metabólitos secundários, com alta atividade antioxidante (BIEGELMEYER et al., 2011; CASTRO et al., 2015; DOS SANTOS PEREIRA et al., 2018; MEDINA et al., 2; 011; ROMBALDI et al., VINHOLES et al., 2017).

Com relação à atividade antimicrobiana, algumas pesquisas demonstraram que óleos e extratos de folhas de araçazeiro e de araçá apresentaram atividade contra algumas bactérias e fungos patogênicos (BOMBARDELLI et al., 2021; CASTRO et al., 2015; MEDINA et al., 2011; SCUR et al., 2016; ZANDONÁ et al., 2020). No entanto, até o momento, inexistem estudos sobre o mecanismo pelo qual ocorre a ação antibacteriana do araçá, e de como fatores externos, como o aumento da temperatura, interferem no potencial antibacteriano e antioxidante de extratos de araçá.

2 Objetivos

2.1 Objetivo Geral

- Estudar o potencial antibacteriano e antioxidante de extratos de araçá (EA), avaliar a influência do genótipo e da temperatura na sua bioatividade e elucidar o mecanismo de ação dos EA contra *Staphylococcus aureus*.

2.2 Objetivos específicos

- Determinar a atividade antibacteriana de EA contra *Escherichia coli*, *Salmonella* Typhimurium, *S. aureus, Listeria monocytogenes* e *Campylobacter jejuni*;
- Avaliar a atividade antioxidante *in vitro* e o teor de fenóis totais e individuais dos EA;
- Verificar a influência do genótipo no potencial antioxidante e antibacteriano dos EA;
- Estudar a influência da temperatura (40 a 100 ºC por 1 h) na composição fenólica e nas atividades antibacteriana e antioxidante dos EA;
- Estudar o mecanismo de ação antiestafilocócico dos EA.

3 Revisão bibliográfica

3.1 Araçá (*Psidium cattleianum* Sabine)

O gênero *Psidium* é originário da América tropical e subtropical e é constituído de cerca de 100 espécies de árvores e arbustos, das quais a mais importante é a goiabeira (*P*. *guajava* L.). O gênero engloba, também, inúmeras outras espécies, produtoras de frutos comestíveis e de madeira, sendo a espécie *P. cattleianum*, originária do sul do Brasil e distribuída geograficamente da Bahia até o Rio Grande do Sul, considerada a espécie que produz os melhores frutos entre as espécies de araçás (BEZERRA et al., 2016).

Em 1985, o Centro de Pesquisa Agropecuária de Clima Temperado da Empresa Brasileira de Pesquisa Agropecuária (CPACT/EMBRAPA) iniciou uma coleção de espécies frutíferas do RS, entre elas o araçá. O germoplasma foi coletado, principalmente, nos arredores de Pelotas e Rio Grande (zona litorânea e colonial), no Planalto Central do RS (Ijuí e Passo Fundo) e no sul do Paraná (RASEIRA, RASEIRA, 1996). Após a realização de um trabalho de melhoramento e seleção dos melhores araçazeiros (quanto a produção, tamanho e qualidade do fruto, flor e folha), o CPACT/EMBRAPA lançou as duas únicas cultivares de araçazerios nativos da espécie *P. cattleianum* conhecidas no Brasil: a ‘Ya-cy’ e a ‘Irapuã’ (BEZERRA et al., 2016, RASEIRA, RASEIRA, 1996).

A ‘Ya-cy’ produz frutos de película amarela, sabor doce, baixa acidez e a produção total é em torno de 4 kg de frutos por planta ao ano, ocorrendo em até três colheitas (dezembro até maio). Já a ‘Irapuã’, produz frutos de película avermelhada e sabor mais ácido, com leve adstringência, sendo mais adequados à confecção de doces em pasta do que ao consumo como fruta fresca. Apresenta produções crescentes que vão de 3,4 a 14 kg de frutos por planta ao ano, na idade adulta, com início da produção em fevereiro. Mais de 30 mil mudas dessa espécie já foram

distribuídos a produtores da região sul do Rio Grande do Sul (BEZERRA et al., 2016; RASEIRA et al., 2001; RASEIRA, RASEIRA, 2000a, b).

Os frutos do araçazeiro são popularmente conhecidos como araçá, araçá-amarelo, araçá-vermelho, araçá-rosa, araçá-de-comer, araçá-da-praia, araçá-de-coroa, araçá-do-campo. Apesar das diferenças de nome e cor entre as variedades de araçás, os frutos são caracterizados por um núcleo, com uma polpa translúcida cheia de sementes (Figura 1) (BIEGELMEYER et al., 2011; CASTRO et al., 2015; DOS SANTOS PEREIRA et al., 2018; FRANZON, 2009).

Figura 1: Araçás amarelos e araçás vermelhos
Fonte: Paulo Lanzetta, Portal EMBRAPA

Os araçás são bastante perecíveis, fato que dificulta a comercialização *in natura* dos frutos. A fabricação de doces e geleias, produzidos em pequenas unidades de base familiar, é a principal forma de aproveitamento dos araçazeiros nativos, sendo, ainda, utilizados no preparo de compotas, sucos, polpas congeladas, sorvetes e licores (BEZERRA et al., 2016).

A composição centesimal da fruta fresca é de 81,73 - 84,9 g de água, 0,75 - 1,03 g de proteínas, 0,63 - 1,50 g de minerais, 4,32 - 10,01 g de carboidratos, 0,42 -

0,55 g de lipídios, 3,87 - 6,14 g de fibras e 26,8 kcal de energia. Quando comparado com a maçã, a fruta mais consumida em todo o mundo, o araçá é menos calórico, com menor teor de carboidratos e maior teor de fibras (DOS SANTOS PEREIRA et al., 2018; MORTON, 1987).

O araçá é boa fonte de vitamina C, minerais, ácidos graxos, polissacarídeos, compostos voláteis, carotenoides e compostos fenólicos. A bioatividade atribuída ao araçá deve-se, principalmente, ao alto conteúdo de compostos fenólicos, que são metabólitos secundários, com alta capacidade antioxidante (BIEGELMEYER et al., 2011; CASTRO et al., 2015; DOS SANTOS PEREIRA et al., 2018; MEDINA et al., 2011; VINHOLES et al., 2017).

Os principais compostos fenólicos encontrados em araçás, provenientes da mesma localização geográfica dos avaliados neste estudo, são a epicatequina e o ácido gálico (MEDINA et al., 2011; ROMBALDI et al., 2016). A esses compostos, têm sido atribuídas propriedades quelantes, inibição da peroxidação lipídica, manutenção do sistema de defesa antioxidante endógeno, atividade anti-inflamatória, antiproliferativa e antimicrobiana (GUTIÉRREZ-GRIJALVAA et al., 2016; JAGAN et al., 2008; KAUR et al., 2009; MEDINA et al., 2011; ROMBALDI et al., 2016; ZANDONÁ et al., 2020).

3.2 Compostos fenólicos

Os processos oxidativos nos alimentos podem ser evitados através da modificação das condições ambientais ou pela utilização de substâncias antioxidantes com propriedade de impedir ou diminuir o desencadeamento das reações oxidativas. Os antioxidantes podem ser divididos em duas classes: com atividade enzimática e sem atividade enzimática. Na primeira classe, estão os compostos capazes de bloquear a iniciação da oxidação, ou seja, as enzimas que removem as espécies reativas ao oxigênio. Na segunda classe, estão moléculas que interagem com as espécies radicalares e são consumidas durante a reação. Nesta classificação, incluem-se os antioxidantes sintéticos e os naturais, como os compostos fenólicos (ÂNGELO & JORGE, 2007).

Os compostos fenólicos são os antioxidantes mais abundantes na dieta humana. Possuem uma diversidade estrutural considerável, caracterizada pelos grupos hidroxila nos anéis aromáticos (Figura 2). Quando presentes em vegetais podem estar na forma livre ou complexados a açúcares e proteínas (REMPE et al., 2017). Estes compostos são capazes de proteger os sistemas biológicos contra o excesso de radicais livres e espécies reativas de oxigênio (ÂNGELO & JORGE, 2007; MIKOŁAJCZAK et al., 2021; VUOLO et al., 2019).

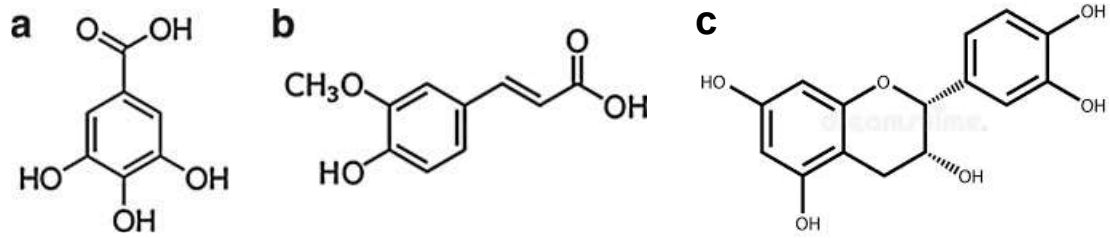

Figura 2: Exemplos de compostos fenólicos: (a) ácido gálico (b) ácido ferúlico (c) epicatequina

O metabolismo das plantas é classificado, principalmente, como primário e secundário. Compostos produzidos através do metabolismo primário, geralmente referidos como metabólitos primários, incluem açúcares, ácidos graxos, aminoácidos e ácidos nucleicos. Os metabólitos primários são necessários para a manutenção das células vegetais, enquanto metabólitos secundários são essenciais para o crescimento normal, desenvolvimento e defesa das plantas. Entre todos os metabólitos secundários, os antioxidantes fenólicos parecem ser os mais importantes, tendo mostrado atividade antioxidante promissora em investigações *in vivo* e *in vitro* (KASOTE et al., 2015). Os compostos fenólicos são essenciais no crescimento e reprodução dos vegetais e, normalmente, se formam em condições de estresse, como infecções, ferimentos, radiações UV, entre outros. Em alimentos, são responsáveis pela cor, adstringência, aroma e estabilidade oxidativa (KIM et al., 2016;

MIKOŁAJCZAK et al., 2021; NACZK & SHAHIDI, 2004; SHAHIDI et al.,1992; VUOLO et al., 2019).

A diversidade estrutural dos compostos fenólicos se deve a grande variedade de combinações que acontece na natureza e os compostos resultantes são chamados de polifenóis (ÂNGELO & JORGE, 2007). De acordo com o número de anéis fenólicos e os elementos estruturais que ligam os anéis uns aos outros, tais compostos são agrupados e classificados em várias classes (Tabela 1). Cada grupo possui diferentes mecanismos de ação correlacionados a uma especificidade estrutural, que confere propriedades antioxidantes aos compostos (VUOLO et al., 2019).

A estrutura e composição química dos compostos fenólicos afeta seu modo de ação e atividade antibacteriana, principalmente, no que se refere à posição e ao número de grupamentos hidroxila no anel fenólico (KIM et al., 2016; MIKOŁAJCZAK et al., 2021; VUOLO et al., 2019).

Tabela 1: Classes de compostos fenólicos em plantas

Classe	Estrutura
Fenólicos simples, benzoquinonas	C6
Ácidos hidroxibenzóicos	C6–C1
Acetofenol, ácidos fenilacéticos	C6–C2
Ácidos hidroxicinâmicos, fenilpropanóides	C6–C3
Nafitoquinonas	C6–C4
Xantonas	C6–C1–C6
Estilbenos, antoquinonas	C6–C2–C6
Flavonóides, isoflavonóides	C6–C3–C6

Fonte: Ângelo & Jorge, 2007

O mecanismo de ação antibacteriana dos compostos fenólicos provenientes de fontes naturais não é totalmente esclarecido. A maioria dos estudos com esses compostos mostraram ação na membrana celular bacteriana, tanto em bactérias Gram-positivas quanto em Gram-negativas (LI et al., 2014; LOU et al., 2012; PLAPER et al., 2003; TSOU et al., 2016; ZHANG et al., 2008). Alguns estudos mostraram, ainda, ação na parede celular, com rompimento da membrana citoplasmática e danos nas proteínas de membrana, extravasamento de conteúdo intracelular, coagulação do citoplasma e/ou depleção de prótons (BORGES et al., 2015; KIM et al., 2016; MIKOŁAJCZAK et al., 2021). Outros estudos avaliando esses compostos também

demostraram que ocorre atividade inibitória da DNA girase, inibição da atividade da helicase e inibição de bombas de efluxo (REMPE et al., 2017).

3.3 Doenças transmitidas por alimentos

As doenças transmitidas por alimentos (DTA) ocorrem pela ingestão de alimentos e/ou água contaminados por micro-organismos, parasitas, toxinas ou produtos químicos. É considerado surto de DTA quando duas ou mais pessoas apresentam doença ou sintomas semelhantes após ingerirem alimentos e/ou água da mesma origem, normalmente em um mesmo local (BRASIL, 2020).

No Brasil, a vigilância epidemiológica monitora os surtos de DTA, sendo notificados, em média, 600 surtos de DTA por ano, com envolvimento de 9 mil doentes e 10 óbitos. Entre 2016 e 2019, as principais bactérias envolvidas em surtos foram *Escherichia coli*, *Salmonella* spp. e *Staphylococcus aureus*, sendo que, na grande maioria dos casos, foi ignorado ou inconclusivo o alimento incriminado no surto. As principais fontes de contaminação identificadas foram água (28,4%), alimentos mistos (19,4%), múltiplos alimentos (12,2%), leite e derivados (9%), frutas, produtos de frutas e similares (5%), carne bovina *in natura*, processados e miúdos (4,1%), ovos e produtos à base de ovos (3,7%) e pescados, frutos do mar e processados (2,5%) (BRASIL, 2020).

O relatório do Banco Mundial (2018) sobre a carga econômica das DTA indicou que a perda total de produtividade associada a essas enfermidades, em países de baixa e média renda, foi estimada em US$ 95 bilhões por ano, e o custo anual do tratamento de DTA é estimado em US$ 15 bilhões. As DTA dificultam o desenvolvimento socioeconômico do país, sobrecarregam o sistema de saúde e prejudicam a economia nacional, o turismo e o comércio (WORLD BANK, 2018).

Na União Europeia (UE), *Campylobacter* spp., *Salmonella* spp. e *E. coli* foram os principais responsáveis pelas zoonoses em humanos reportadas no ano de 2019 (EFSA, 2021). Nos Estados Unidos da América (EUA), estima-se que, a cada ano, 48 milhões de pessoas (1 em cada 6) adoeçam pelo consumo de alimentos

contaminados. Semelhante ao que ocorre no Brasil, a ligação de doenças individuais a um determinado alimento raramente é possível, exceto durante um surto. De 2017 a 2019, *Campylobacter* spp., *Salmonella* spp. e *E. coli* foram os principais patógenos envolvidos em surtos naquele país. Com um número inferior de casos, porém, com maior necessidade de hospitalizações, a severidade de doenças causadas por *Clostridium botulinum, Listeria monocytogenes* e *Vibrio* spp., também coloca esses patógenos na lista de importantes causadores de DTA nos EUA (CDC, 2020).

3.4 Principais bactérias envolvidas em DTA

Campylobacter spp.

De acordo com a OMS, a campilobacteriose é a principal causa de gastroenterite no mundo, sendo *C. jejuni*, seguido por *C. coli*, as espécies mais frequentemente isoladas, responsáveis pela maior parte dos casos de campilobacteriose em humanos (WHO, 2020).

Campylobacter spp. está amplamente distribuído no ambiente, colonizando o trato gastrointestinal de animais silvestres e domésticos. As aves são consideradas reservatórios naturais dessas bactérias, possivelmente, pela temperatura corporal desses animais, que é similar à temperatura ótima para *Campylobacter* spp., que são micro-organismos termofílicos. Em humanos, a campilobacteriose geralmente está associada à ingestão de alimentos crus, carne de frango mal cozida e contaminação cruzada de alimentos consumidos *in natura* (KLEINUBING et al., 2021; WHO, 2020).

Os sintomas clínicos mais comuns da campilobacteriose incluem diarreia, dor abdominal, febre, dor de cabeça e vômitos, no entanto, complicações mais graves podem ocorrer, como hepatite, pancreatite, bacteremia e aborto espontâneo. As complicações pós-infecção podem incluir, ainda, distúrbios neurológicos como a síndrome de Guillain-Barré (WHO, 2020).

No sul do Brasil, estudos têm mostrado não somente a ocorrência e a diversidade genética entre os isolados de *Campylobacter* spp. na cadeia de frangos

de corte da região, como também a habilidade desses isolados em formar biofilmes, a resistência a antimicrobianos e a presença de genes associados à virulência (KLEINUBING et al., 2021; RAMIRES et al, 2020; SCHEIK et al., 2021; WÜRFEL et al., 2019), o que é motivo de grande preocupação e um risco à saúde pública.

Escherichia coli

A presença de *E. coli* nos alimentos é um indicador de manuseio higiênico-sanitário deficiente durante o processo de produção, condições de armazenamento inadequadas e contaminação pós-processamento (WHO, 2018). Existem várias categorias diferentes de *E*. *coli* diarreiogênica, e as mais importantes ou mais estudadas são *E. coli* enterohemorrágica (EHEC), enterotoxigênica (ETEC) e enteropatogênica (EPEC). Existem três outras categorias, incluindo *E. coli* enteroinvasiva (EIEC), enteroagregativa (EAEC) e difusamente aderente (DAEC) (MAIA et al., 2017).

Escherichia coli produtora de toxina shiga (STEC) tem sido bastante relacionada com surtos de DTA, principalmente o subgrupo *E. coli* enterohemorrágica (EHEC), tendo o sorotipo O157:H7 como um dos principais envolvidos (RAMIRES et al., 2019; WHO, 2018). Na maioria dos casos, os sintomas incluem cólicas abdominais e diarreia, com período de incubação de 3 a 8 dias, e a doença é autolimitante, porém, em algumas pessoas (principalmente crianças e idosos), o quadro pode se agravar, com ocorrência de síndrome hemolítico-urêmica (SHU). A SHU é caracterizada por insuficiência renal aguda, anemia hemolítica e trombocitopenia. Estima-se que, em torno de 10% dos pacientes contaminados por STEC podem desenvolver SHU, com taxa de letalidade variando de 3 a 5%, sendo a causa mais comum de insuficiência renal aguda em crianças (WHO, 2018).

As fontes primárias de surtos por STEC são produtos de carne crus ou mal cozidos, como hambúrgueres, leite não pasteurizado e vegetais *in natura*. No entanto, outros alimentos podem, também, veicular o patógeno. Em 2019, ocorreu o primeiro relato da presença de *E. coli* O157:H7 em sushi, um produto pronto para o consumo, demonstrando não apenas a necessidade de melhores práticas de higiene nos

estabelecimentos, quanto o risco à saúde dos consumidores, tendo em vista que esse é um alimento pronto para o consumo (RAMIRES et al., 2019).

Listeria monocytogenes

A listeriose é uma das doenças de origem alimentar mais severas. Afeta principalmente mulheres grávidas, recém-nascidos, idosos e pessoas imunodeprimidas, podendo ocasionar complicações como aborto, septicemia, meningite e até mesmo óbito, nos casos mais graves. Embora o número de casos de listeriose seja pequeno, quando comparado a outras DTA, a alta taxa de letalidade (20 a 30%) associada a essa infecção em determinados grupos de risco a torna particularmente preocupante em saúde pública (CDC, 2020). É causada pela bactéria *L. monocytogenes*, um micro-organismo ubíquo e psicrotrófico que, por sobreviver e se multiplicar em temperaturas de refrigeração, tem como principais alimentos envolvidos em surtos, aqueles com vida útil longa sob refrigeração, como salsichas, linguiças, patês, salmão defumado, laticínios (queijos de massa mole, leite não pasteurizado e sorvete), saladas preparadas (salada de repolho e broto de feijão), além de vegetais e frutas frescas (WHO, 2018).

No sul do Brasil, *L. monocytogenes* já foi isolada em alimentos produzidos e comercializados na região (carcaça ovina, linguiças frescais, queijos artesanais, carcaças de frango, carcaças bovinas, queijo e presunto fatiados), assim como no ambiente de processamento desses alimentos, tendo sido avaliada a diversidade genética desses isolados, os mecanismos de virulência, os mecanismos envolvidos na formação de biofilmes, a expressão de genes de virulência e a presença de genes de resistência a antimicrobianos e sanitizantes. Foram identificados os principais sorotipos associados à listeriose humana, além dos genes da internalina (indicando potencial de virulência), com alguns isolados multirresistentes a antimicrobianos e formadores de biofilme, possivelmente, já endêmicos no ambiente de processamento desses alimentos, representando um risco à saúde dos consumidores (ANTONIOLLO et al., 2003; HAUBERT et al., 2016; IGLESIAS et al., 2017; MAIA et al., 2019; PRATES et al., 2019; VON LAER et al., 2009).

Salmonella spp.

A salmonelose é a infecção alimentar causada por *Salmonella* spp., a qual é um problema de saúde pública mundial, sendo responsável por quase 94 milhões de DTA e 155.000 mortes por ano (SHU-KEE ENG et al., 2015). O quadro geralmente é caracterizado por dor abdominal, febre, diarreia, náuseas, com início dos sintomas entre 6 a 72 horas (geralmente de 12 a 36 horas) após a ingestão do alimento contaminado, e duração da doença de 2 a 7 dias. Os sintomas costumam desaparecer sem tratamento específico, na maioria dos casos. No entanto, em agumas pessoas (principalmente crianças e idosos), a desidratação associada ao quadro pode se tornar grave e até fatal (WHO, 2018). As infecções por *Salmonella* spp. que envolvem sorovares invasivos são frequentemente muito graves, necessitando de terapia antimicrobiana apropriada e eficaz (SHU-KEE ENG et al., 2015). Até o momento, mais de 2.600 sorovares de *Salmonella* spp. já foram identificados e mais da metade deles pertencem a espécie *Salmonella enterica* subsp. *enterica*, que causa a maioria das infecções por *Salmonella* spp. em humanos. O sorovar Enteritidis, seguido pelo sorovar Typhimurium, têm sido os mais frequentemente transmitidos de animais para humanos (WHO, 2018).

Em estudo realizado em dois frigoríficos no sul do Brasil, foi verificada a ocorrência de *Salmonella* spp. em carcaças bovinas, sendo o couro do animal um importante veículo de introdução desse patógeno na linha de abate. O estudo também mostrou a presença de sorovares frequentemente relacionados a surtos de DTA, com alguns isolados resistentes a mais de um agente antimicrobiano. Os dados evidenciaram a importância de um controle higiênico-sanitário rígido e sistemático durante todo o processo de abate (IGLESIAS et al., 2017).

Staphylococcus aureus

Staphylococcus aureus é um patógeno alimentar importante, devido a uma combinação de fatores, como virulência mediada por toxinas e resistência a diversos antimicrobianos e sanitizantes (KADARIYA; SMITH; THAPALIYA, 2014; KRONING et al., 2020). Além disso, é uma bactéria formadora de biofilme, podendo

se tornar endêmica em plantas de processamento de alimentos (BRIDIER et al., 2015; MAIA et al., 2020). Esse micro-organismo já foi identificado como agente responsável por inúmeros surtos de DTA, embora se saiba que casos e surtos de intoxicação alimentar estafilocócica (IAE) são bastante subestimados, especialmente pelo caráter autolimitante da doença, que raramente leva as pessoas envolvidas a procurar auxílio médico (CDC, 2016). No Brasil, *S. aureus* é um dos três principais patógenos envolvidos em surtos de DTA (BRASIL, 2018). Dados produzidos por 78 trabalhos científicos realizados em 21 países europeus, sobre a incidência de bactérias patogênicas em carne de frango, apontam *S. aureus* como o principal patógeno encontrado em carne de aves comercializadas na Europa (GONÇALVES et al., 2018).

Kroning et al. (2018), avaliaram a presença de genes de enterotoxinas estafilocócicas, resistência antimicrobiana e diversidade genética de *S. aureus* isolados de leite de vacas com mastite, em fazendas leiteiras do sul do Brasil. O estudo revelou a presença de genes de enterotoxinas clássicas e resistência a múltiplos agentes antimicrobianos. Foi observada diversidade genética entre os isolados de *S. aureus*, no entanto, foram identificados alguns clones em áreas geográficas distintas, revelando a disseminação de alguns grupos clonais entre rebanhos leiteiros, o que representa um risco à saúde animal e humana, principalmente, através de contaminação cruzada.

Diante do exposto, produzir, armazenar e distribuir alimentos de forma segura, além de atender as expectativas de um mercado consumidor exigente, minimizando os aditivos químicos sintéticos nos alimentos industrializados, tem sido um grande desafio, não apenas para a indústria de alimentos, mas também para a ciência de alimentos que tem buscado novas alternativas que sejam viáveis e possam contribuir para a conservação de alimentos seguros ao consumidor.

4 Manuscrito

Influence of heat treatment on antioxidant and antibacterial activities of araçá (*Psidium cattleianum* Sabine) fruit extracts

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Abstract

Araçá is a fruit with high levels of bioactive compounds. The aim of this study was to evaluate the antibacterial and antioxidant activities, as well as the thermostability of araçá fruit extracts (AEs). The AEs were prepared with yellow and red araçá genotypes, separately. The AEs showed antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Typhimurium, and *Campylobacter jejuni*, with inhibition halos diameter ranging from 11 to 17 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranged between 9 and 147 mg mL^{-1} and 18 and 294 mg mL^{-1}, respectively. The lowest MIC and MBC values were found against *S. aureus*. There was no difference in antibacterial activity between the yellow and red genotypes. The antioxidant activity was higher than 90% in all AEs evaluated, and the total phenol content ranged from 414 to 465 mg 100 g^{-1}, with higher values in red AEs than in yellow AEs. Gallic, ferulic, and cumharic acids were idenfied in all AEs. The AEs showed

thermostability after exposure to temperatures of up to 100 ºC for 1 h. The heat treatment of the AEs increased 2 to 4 times the MIC and MBC values. In addition, the heat treatment caused reduction in the concentration of the gallic, ferulic, and cumharic acids, with exception of ferulic and cumharic acids in red AEs, in which did not have significant losses. AEs showed bioactive properties, with high antioxidant, bactericidal and bacteriostatic activities, even when subjected to high temperatures, which makes them very promising as candidates for new natural additives in food preservation, including thermally processed foods.

Keywords: foodborne diseases; native fruits; natural aditives; phenolic compounds.

1 Introduction

Outbreaks and sporadic cases of foodborne diseases (FBDs) are common worldwide. It is estimated that around 10% of the world population gets ill annually from the consumption of contaminated food (WHO, 2017). In Brazil, between 2016 and 2019, the major pathogens involved in FBDs outbreaks were *Escherichia coli, Salmonella* spp., and *Staphylococcus aureus* (Brazil, 2020).

In the United States of America (USA), the major pathogens involved in FBDs outbreaks between 2017 and 2019 were *Campylobacter* spp., *Salmonella* spp. and *E. coli* (CDC, 2020). In European Union (EU), *Campylobacter* spp., *Salmonella* spp. and *E. coli* also were the most reported zoonoses in humans in 2019 (EFSA, 2021).

Plants synthesize several secondary metabolites (phytochemicals) as defense mechanisms and many of these molecules have been studied, with recognized beneficial health effects (Borges et al., 2013; Burt, 2004; Castro et al., 2015; Kaur et al., 2009; Moon et al., 2013; Rempe et al., 2017). In this context, many studies about essential oils and leaf extracts (basil, oregano, thyme, marjoram, rosemary and sage), flowers or buds (cloves), seeds (fennel, parsley and nutmeg), bulbs (garlic and onion), rhizomes (ginger) spices and fruits (cardamom, pepper, *butia odorata*), have been carried out, aiming the development of new natural antimicrobials, which can be used by the industry as food preserver (Dannenberg et al., 2018; Maia, Lopes & Silva, 2017; Maia et al., 2019; Mendonça et al., 2018).

The araçá (*Psidium cattleianum* Sabine) is a fruit of the Myrtaceae family, cultivated from the south to the northeast of Brazil, whose bioactivity potential is still little explored, being used mainly in the form of sweets and jellies, produced in small family-based units (Bezerra et al., 2016). Araçá fruits are sources of minerals, fatty acids, polysaccharides, volatile compounds, carotenoids and phenolic compounds (Biegelmeyer et al., 2011; Castro et al., 2015; dos Santos Pereira et al., 2018; Medina et al., 2011; Rombaldi et al., 2016; Vinholes et al., 2017). The reported bioactivity of araçá is mainly attributed to the high content of phenolic compounds, which are well known as secondary metabolites with high antioxidant capacity (dos Santos Pereira et al., 2018). However, few studies have evaluated the antibacterial activity and there are no studies evaluating their bioactivity after heat treatment. In this context, this study aimed to evaluate the antibacterial and antioxidant activity, as well as the thermostability of araçá extracts, in the search of new insights in this promising area of research.

2 Material and Methods

2.1 Samples

Red and yellow accessions araçá (*Psidium cattleianum* Sabine) were collected from a research orchard (germplasm collection of Embrapa Clima Temperado, Pelotas, RS, Brazil - geographical coordinates: 31º 40’ 47” S and 52º 26’ 24” W: 60 m of altitude) when fruit was ripe. The fruits were washed and sanitized, their seeds were removed, and fruits flesh with peel were frozen in liquid nitrogen and stored at −80 ºC until further analyses. All analyses described below were performed in triplicate.

2.2 Extracts

For the preparation of the araçá extracts (AEs), 100 g of yellow araçá and red araçá samples and 400 mL of 75% (v/v) ethyl alcohol (Synth, Brazil) were used,

producing two extracts: yellow araçá extract (YAE) and red araçá extract (RAE). The samples were macerated in a pistil grade, keptunder shaking for 2 h (200 rpm), at room temperature (23 ± 2 ºC) and protected from the light. After this, the mixture remained 15 min in an ultrasonic bath (Quimis, Brazil) (48 A) and was filtered in qualitative paper (Whatman, UK). The filtrate was centrifuged at 6000 x *g* for 20 min, and the supernatant was rotary-evaporated (Heidolph, Germany) at 40 °C to constant weight.

2.3 Thermostability

The evaluation of AEs thermostability was carried out exposing the AEs to temperatures up to 100 ºC (40, 60, 80 and 100 ºC), in a water bath, for 1 h at each temperature. AEs samples, without heat treatment, were used as controls. The heat-treated araçá extracts (AETs) were identified as YAET (heat-treated yellow araçá extract) and RAET (heat-treated red araçá extract).

2.4 Antimicrobial activity

2.4.1 Agar disk diffusion testing, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

In the agar disk diffusion testing, disks impregnated with 10 µL of AEs were placed on Mueller-Hinton agar (Oxoid, UK) Petri dishes, previously inoculated with the strains *S. aureus* ATCC 25923, *L*. *monocytogenes* ATCC 19114, *E*. *coli* ATCC 8739, *S.* Typhimurium ATCC 14020 or *C. jejuni* ATCC 33291. The inoculum was standardized at a concentration of 10^8 CFU mL^{-1} and the Petri dishes were incubated at 35 ºC for 20 h, with the exception of *C. jejuni*, where Mueller Hinton agar was used with 5% defibrinated horse blood, and incubated in a Permution® jar at 42 ºC, for 48 h, in microaerophilia (5% O_2, 10% CO_2, 85% N_2) (CLSI, 2018). Disks of ampicillin (Laborclin, Brazil) were used as positive control and disks with the same solution used in the preparation of AEs were taken as negative control. The inhibition zones were expressed in mm (CLSI, 2018).

For determination of MIC and MBC, the broth macrodilution technique was used. Firstly, an overnight culture was standardized to 10^5 CFU mL^{-1}. Dilutions of AEs were added in tubes containing the same volume of the inoculum, which were incubated at 35 ºC for 20 h, with the exception of *C. jejuni* which was incubated in a Permution® jar at 42 ºC, for 48 h, in microaerophilia (5% O_2, 10% CO_2, 85% N_2). As negative control, ultrapure water was used. The MIC was determined as the lowest concentration without visible bacterial multiplication. From the tubes without bacterial multiplication, 100 µL were removed and added to Brain Heart Infusion agar (BHIA) (Oxoid, UK) Petri dishes and Columbia agar (Oxoid, UK) Petri dishes for *C. jejuni*, to determine the MBC (CLSI, 2018).

2.5 Antioxidant activity

Antioxidant activity was determined using the DPPH radical scavenging method described by Brand-Williams et al. (1995), with some modifications. For this,10 µL of each extract was added to 3.990 µL of DPPH solution in methanol (60 µM) (Sigma–Aldrich, USA). The solution was then stirred and kept in a closed flask in the dark for 1 h. Control treatment was composed of methanol (Synth, Brazil) and ultrapure water. The absorbance readings were performed in UV/VIS IL-592 spectrophotometer (Kazuaki, Japan) at 515 nm. The radical scavenging capacity was expressed as % DPPH radical remaining according to the equation:

% Inhibition = (Control Absorbance - Sample Absorbance) x 100 / Control Absorbance

2.6 Total phenolic compounds

Phenolic compounds were extracted following protocols of Dewanto et al. (2002). For this, 125 µL of each AE were transferred to 0.5 mL of ultrapure water and 125 µL of Folin Ciocalteau 2 N reagent (Sigma-Aldrich, USA). After rest for 6 min, 1.25 mL of sodium carbonate 7% (Synth, Brazil) (m/v) was added, adjusting the final volume to 3 mL with ultrapure water. As control treatment was used ultrapure water replacing AEs. The mixture was kept at room temperature (20 ± 3 ºC) for 90 min in the dark. The

absorbance measurements were performed in a UV/VIS spectrophotometer IL-592 Kazuaki (Japan) at 760 nm. As standard, the gallic acid (relevant in araçá) was used to construct the calibration curve. The total phenolic content of AEs was expressed in mg of gallic acid equivalents per 100 g of fresh fruit pulp (mg 100 g^{-1} GA).

2.7 Individual phenolic compounds

To identify the individual phenolic compounds, the AEs were lyophilized and resuspended in 200 µL of ultrapure water acidified with acetic acid (98:2, v:v). Aliquots of 10 µL were injected in a high-performance liquid chromatograph (HPLC) (Shimadzu, Tokyo, Japan, CTO-20A) at a flow rate of 0.7 mL min^{-1} at 40 ºC. The separation of the phenolic compounds was performed using an NST C18 Column (250 mm x 4.6 mm, 5 µm) with mobile phase composed of ultrapure water acidified with acetic acid (98:2, v:v) and methanol at a ratio of 80:20 (v:v) in isocratic mode, with detection by diode array (PDA) (Shimadzu, Tokyo, Japan, SPD-M20A) and mass spectrometer (MS) (Shimadzu, Tokyo, Japan, LCMS - 2020). Individual phenolic compounds were identified by comparison to phenolic standards gallic acid, cumharic acid and ferulic acid (Sigma-Aldrich, USA). The linearity of the analytical curve was evaluated with 6 different levels of concentration injected in triplicate, and the determination coefficient (r^2) and the limit of detection (LOD) and quantification (LOQ) were calculated according to the parameters of the analytical curve. The areas mean values were used to calculate the coefficient of variation for the precision analysis.

2.8 Statistical analysis

All experiments were performed in triplicate, and the data were expressed as mean (± standard deviation). The results were submitted to statistical analysis of variance (ANOVA), comparison of means by Tukey's test ($p < 0.05$) and the correlation coefficient determined using the STATISTIX 8.0 program.

3 Results and discussion

3.1 Thermostability of araçá extracts

There was no significant difference in the size of the inhibition halos formed among the heat-treated extracts, at the different temperatures evaluated (40, 60, 80 and 100 ºC), therefore, just the results of the heat treatment at 100 ºC are showed in Table 1. In addition, just this treatment, for being the most severe, was used in subsequent tests, always performed in parallel with the AEs without heat treatment.

3.2 Antibacterial activity

All AEs showed antibacterial action, presenting inhibition zones diameter ranging from 11 to 17 mm in agar disk diffusion testing (Table 1).

Table 1: Antibacterial activity of araçá extracts and heat-treated araçá extracts (100 ºC/1 h) evaluated by the agar disk diffusion testing

Bacteria	YAE	YAET	RAE	RAET
	inhibition zone (mm)			
E. coli	11^{bA}	11^{aA}	14^{aB}	13^{bA}
L. monocytogenes	12^{bA}	12^{aA}	13^{bA}	13^{bA}
S. Typhimurium	12^{bA}	12^{aA}	14^{aA}	13^{bA}
S. aureus	14^{aA}	14^{aA}	17^{aA}	16^{aA}
C. jejuni	12^{bA}	12^{aA}	12^{bA}	13^{bA}

YAE: yellow araçá extract; YAET: yellow araçá extract heat treated; RAE: red araçá extract; RAET: red extract of heat treated araçá. Results expressed as averages. Different lowercase letters in the column indicate a significant difference ($p < 0.05$) between the bacteria for the same treatment. Different capital letters on the line indicate a significant difference ($p < 0.05$) between treatments for the same bacteria.

There was no significant difference related to the genotype (yellow or red), with the exception for *E. coli* strain, whose size of the halos was significantly higher with red AE than with yellow AE. Among the bacteria studied, *S*. *aureus* was the most susceptible to the AEs evaluated, showing significantly higher halos with YAE and RAET, and higher than *C. jejuni* and *L. monocytogenes* with RAE. The values of antimicrobial activity found in different studies with extracts vegetables are highly variable and mostly depend on the content of bioactive compounds, the method of extraction used, and the choice of solvente (Azmir et al., 2013). The agar disk diffusion testing allowed us to make a qualitative assessment of bacterial activity, however, it was only with the MIC and MBC evaluations this activity could be quantified.

Table 2 shows the values of MIC and MBC. There was a positive relationship between the agar disk diffusion test and MIC and MBC results for *S. aureus*, since this microorganism had the highest inhibition halos and the lowest MIC and MBC values.

Regarding the studied genotypes, there was no difference in MIC and MBC values between YAE and RAE. *Staphylococcus aureus*, followed by *E. coli*, were the most susceptible to AEs, with MIC and MBC significantly lower than the other bacteria. *Listeria monocytogenes* and *C. jejuni* showed no difference between them, while *S*. Typhimurium was the less susceptible bacteria to AEs. It is interesting to note that there was no linearity in the response, in relation to the action of AEs on Gram-positive and Gram-negative bacteria. It was observed that the best results were found to *S. aureus,* a Gram-positive bacterium, followed by *E. coli*, a Gram-negative microorganism.

Regarding the treated and not heat treated araçá extracts, there was difference between the MIC and MBC values obtained. The AETs showed MIC and MBC approximately twice as high for *E. coli*, *L. monocytogenes*, *S*. Typhimurium and *C. jejuni*, and four times higher for *S. aureus*, compared to extracts non-thermically treated, meaning that, when subjected to intense heat treatment, the AEs did not lose their bacterial action; however, a 2- to 4-fold higher concentration was required, compared to AEs without heat treatment, to obtain bacteriostatic/bactericidal activity.

The antimicrobial activity of phenolic acids depends on the concentration of non-dissociated acids, which cross the cell membrane by passive diffusion, damaging the cell membrane structure and possibly acidifying the bacterial cytoplasm, causing protein denaturation (Borges et al., 2013). The higher MIC and MBC obtained with the

thermically treated extracts can be justified by the partial loss of these acids, which dissociate with the increase of temperature.

Table 2: MIC and MBC values of araçá extracts and heat-treated araçá extracts (100 ºC/1 h)

Bacteria	MIC (mg mL^{-1})				MBC (mg mL^{-1})			
	YAE	YAET	RAE	RAET	YAE	YAET	RAE	RAET
E. coli	18.38^{a}	36.75^{b}	18.38^{a}	36.75^{b}	36.75^{b}	73.5^{c}	36.75^{b}	73.5^{c}
L. monocytogenes	36.75^{b}	73.5^{c}	36.75^{b}	73.5^{c}	73.5^{c}	147^{d}	73.5^{c}	147^{d}
S. Typhimurium	73.5^{c}	147^{d}	73.5^{c}	147^{d}	147^{d}	294^{e}	147^{d}	294^{e}
S. aureus	8.78^{d}	36.75^{b}	8.78^{d}	36.75^{b}	17.94^{a}	73.5^{c}	17.56^{a}	73.5^{c}
C. jejuni	36.75^{b}	73.5^{c}	36.75^{b}	73.5^{c}	73.5^{c}	147^{d}	73.5^{c}	147^{d}

YAE: yellow araçá extract; YAET: extract of yellow araçá heat treated; RAE: red araçá extract; RAET: heat treated red araçá extract; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; Values are presented as means (n = 3) ± standard deviation; Different letters indicate a significant difference ($p < 0.05$) by the Tukey test.

3.3 Antioxidant activity (*in vitro*)

All extracts showed high antioxidant activity, even after heat treatment (higher than 90%), which was measured by its ability to scavenge the DPPH radical, expressed as percentage of inhibition (Table 3). Medina et al. (2011) studied aqueous and acetonic extracts of araçá, from the same geographical area of this study, and found antioxidant activity values between 19.7 and 45.3% with the DPPH radical method and higher than 80% with the yeast method (Medina et al., 2011). In addition to the differences between genotypes and environmental conditions, the solvents were different from those used in this study (75% ethanol), which possibly influenced the phenolic composition and, consequently, the antioxidant activity of the extracts. Zhao and Hall (2008), compared extractions with different solvents in raisins and melons, and found that the best extraction values of the phenolic compounds were obtained using ethanol as solvent.

Table 3: Total phenolics (expressed as mg of gallic acid equivalent per 100 g of fresh fruit pulp) and antioxidant activity (expressed as % inhibition of DPPH radical) of araçá extracts and heat-treated araçá extracts (100 ºC/1 h)

Treatments	Phenolics	% inibition (DPPH)
YAE	439,64[a]	93,54[b]
YAET	413,76[b]	91,87[a]
RAE	464,80[c]	93,74[b]
RAET	456,50[d]	93,09[b]

YAE: yellow araçá extract; YAET: extract of yellow araçá heat treated; RAE: red araçá extract; RAET: heat treated red araçá extract. Values are presented as averages. Different letters in each column indicate a significant difference ($p < 0.05$) by the Tukey test.

3.4 Total phenolic compounds

The highest content of total phenols was found in RAE (464.80), followed by RAET (456.50). The YAE (439.64) and YAET (413.76) presented lower values. Biegelmeyer et al. (2011), Medina et al. (2011) and Rombaldi et al. (2016) also found higher levels of phenols in red araçá genotypes compared to yellow genotypes, with values ranging from 290 to 700 mg 100 g^{-1} GA. Rombaldi et al. (2016), studied the phytochemical profile of araçá genotypes from six consecutive harvest seasons (2008 – 2013), and attributed the great variation in the values of phytochemical compounds, mainly, to climatic variations.

Through regression analysis, it was observed a linear behavior between the content of total phenols and the ability of AEs to scavenge the free radical DPPH, with a value of r = 0.85, which is, mathematically, a good correlation (Milone, 2004). Several authors have demonstrated a positive relationship between the total phenol content and the antioxidant activity of vegetables (Azmir et al., 2013; Balasundram et al., 2006; Lou et al., 2015; Medina, et al 2011; Rempe et al., 2017; Vats, 2015). This relationship suggests that the contribution of phenolic compounds was relevant to antioxidant activity.

3.5 Identification of phenolic compounds

The retention times, in the best operating conditions, were 4.62, 27.39 and 35.38 min for gallic, cumharic and ferulic acids, respectively, and the total running time was 45 min. The detection and quantification limit values were 0.1 and 1.5 μg mL^{-1} for gallic acid and 10 and 60 μg mL^{-1} for cumharic and ferulic acids. The analytical curves were linear in the range of 1.5 to 45 μg mL^{-1} for gallic acid and 60 to 600 μg mL^{-1} for cumharic and ferulic acids, in a total of six points for each compound. The determination coefficients were higher than 0.97.

Table 4: Individual phenolic composition of araçá extracts and heat-treated araçá extracts (μg g^{-1})

Compound	YAE	YAET	RAE	RAET
Gallic acid	$2.987,26^{aA}$	$578,05^{bA}$	$2.695,07^{cA}$	$499,32^{dA}$
Ferulic acid	$1.247,05^{aB}$	$927,73^{bB}$	$184,23^{cB}$	$179,02^{cB}$
Cumharic acid	$101,7^{aC}$	$43,86^{bC}$	$83,91^{cC}$	$70,12^{cC}$

YAE: yellow araçá extract; YAET: extract of yellow araçá heat treated; RAE: red araçá extract; RAET: heat treated red araçá extract. Results expressed as averages. Different lowercase letters on the line indicate a significant difference ($p < 0.05$) of the compound in the different AEs. Different capital letters in the column indicate a significant difference ($p < 0.05$) between the compounds in the same AE.

The main phenolic compounds identified in the AEs evaluated in this study (gallic, ferulic and cumharic acids), have been extensively studied for their activities as anti-inflammatory, antioxidant, antidiabetic, neuroprotective, anticancer and antimicrobial, all of great interest in the areas of food and health (Borges et al., 2013; Fernandes & Salgado, 2015; Ibitoye & Ajiboye, 2019; Maurya & Devasagayam, 2010; Rakesh, Prabhakar & Mukesh, 2017; Wang et al., 2017). It interesting to note that gallic acid, the major compound in the AEs evaluated (Table 4), was the one that showed the highest losses after heat treatment, both in YAE and RAE. Ferulic and cumharic acids did not show significant losses in the thermicaly treated red araçá extracts.

Borges et al. (2013) found antibacterial activity of gallic and ferulic acids against *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *L. monocytogenes,* being ferulic acid the most effective compared to gallic acid, showing lower MIC and MBC values for all bacteria. The hydroxyl of ferulic acid in the ortho position with the electron donor methoxy group, is a factor that increases the stability of the phenoxyl radical and the antioxidant effectiveness of the compound

(Degáspari & Waszcznskyj, 2004), also contributing to lower losses, as was verified in this study.

The high temperatures (100 ºC for 1 h) to which the thermically treated extracs were subjected, may have formed new compounds with antioxidant and antimicrobial activities. Some studies have shown that darkening reaction products, especially intermediates, have antioxidant activity (Atrooz, 2008; Gu et al., 2009; Tonon et al., 2010) and these products may have compensated the degradation of some bioactive compounds in the heat treated extracts, maintaining its antioxidant and antibacterial activities. In a study with aqueous extract of immature kunquats (small citrus fruits, similar to orange), Lou et al. (2015) found an increase in antioxidant activity and total phenols using high temperatures (130 ºC for 1.5 h). The authors suggest that products resulting from the darkening reaction increased the antioxidant activity, and significant changes in the color of the residual water from dry extracts submitted to high temperatures have also been observed in relation to the water used in fresh extracts, reinforcing this hypothesis.

These results are very interesting, since the temperature and time used in this experiment were extremely high (100 ºC for 1 h), which is much higher than those used in most thermally processed foods.

4 Conclusion

Araçá fruit extracts showed bioactive properties, regardless of the genotype studied (red or yellow). Even when subjected to intense heat treatment (100 ºC for 1 h), the bioactive compounds were partially preserved, maintaining the antioxidant, bactericidal and bacteriostatic activities of the araçá extracts, making them promising candidates to new natural additives for foods, including thermally processed foods.

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Action mechanism of araçá (*Psidium cattleianum* Sabine) hydroalcoholic extract against *Staphylococcus aureus*

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Abstract

Araçá (*Psidium cattleianum* Sabine) is a native fruit of South America, with high levels of bioactive compounds as well as antimicrobial potential. The aim of this study was to evaluate the antibacterial activity of the hydroalcoholic extract of araçá (HEA) against *Staphylococcus aureus* and to evaluate its mechanism of action. HEA showed activity against *S. aureus*, with inhibition halos of 17 mm in diameter. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were 9 mg mL^{-1} and 18 mg mL^{-1}, respectively. The bacterial multiplication was inhibited by HEA exposure at the two concentrations evaluated (MIC and MBC), given that the microorganism was eliminated within 24 h of contact when the MBC was used. The bacterial cell membrane was

compromised, losing its integrity, and releasing important cellular constituents. HEA also showed the ability to intercalate with bacterial DNA, indicating that its antistaphylococcal activity can occur in more than one target in the bacterium. The antistaphylococcal activity of HEA occurs by modification in cell membrane integrity and by causing damage to the DNA of *S. aureus*. This study shows promising and unpublished results and demonstrates that HEA has the potential to be used as a natural adjuvant in food preservation.

Keywords: Antistaphylococcal activity; natural additives; scanning electron microscopy; confocal laser scanning microscopy; fluorescence spectrophotometry.

1 Introduction

Foodborne diseases (FBD) are a major cause of morbidity and mortality worldwide and, in recent decades, they have emerged as an increasing public health and economic concern in many countries (WHO, 2017). Among the major bacterial causes of FBD, *Staphylococcus aureus* stands out. It is an important foodborne pathogen whose pathogenicity is due to a complex set of virulence factors, including the production of toxins, as well as the presence of a resistance profile to several antimicrobials (Kadariya, Smith & Thapaliya, 2014).

In Brazil, between the years 2010 and 2017, *S. aureus* appeared as the third major pathogen involved in FBD outbreaks (Brazil, 2018). In the United States of America, more than 240,000 cases of SFP were reported in 2016, with 1067 hospitalizations and 6 deaths (CDC, 2016). The results of 78 scientific

studies performed in 21 European countries regarding the incidence of pathogenic bacteria in chicken meat indicated that *S. aureus* is a major pathogen found in poultry marketed in Europe (Gonçalves-Tenório, Silva, Rodrigues, Cadavez & Gonzales-Barron, 2018). In the food chain of products of plant origin, *S. aureus* is one of the three major pathogens involved in outbreaks related to the consumption of vegetables in several countries (New Zealand, Australia, USA, Canada, Japan, European Union) between the years 2010 and 2015 (Li et al., 2018).

The use of natural additives in food preservation is a significant market trend. According to a systematic review conducted in different European countries, involving more than 80,000 consumers, reveal preference for *in natura* food and, in the case of processed food, consumers are willing to accept food additives of natural origin more often than synthetic products (Román et al., 2017). In this context, many studies about essential oils and extracts from different plant organs have been performed to investigate bioactive compounds with antimicrobial activity for the utilization in food preservation (Azmir et al., 2013; Tiwari et al., 2011).

Brazil has one of the largest biodiversities in the world, with unexplored sources of native fruit with high levels of bioactive compounds. Among these fruits, the araçá (*Psidium cattleianum* Sabine) is notable. It is a fruit widely grown in domestic orchards belonging to the Myrtaceae family, found from the northeast to the south of Brazil as well as in other South American countries (Franzon, 2009). The araçá fruit is a source of vitamin C, minerals, fatty acids, polysaccharides, volatile compounds, carotenoids and phenolic compounds, and it is rich in nutrients and in phytochemicals with different biological functions. It is

noteworthy that the bioactivity of the araçá is mainly attributed to the high content of phenolic compounds, which are well-known secondary metabolites with high antioxidant capacity (dos Santos Pereira et al., 2018). Rempe, Burris, Lenaghan & Stewart (2017), summarized the current knowledge about mechanisms of antibacterial action of phenolic compounds of plants, including cell membrane disruption methods, enzyme inhibition assays and, more recently, DNA intercalation assays.

Regarding the antimicrobial activity of araçá, some studies have showed that oils and extracts of leaves and fruit demonstrated activity against some pathogenic bacterial and fungi (Castro et al., 2015; Medina et al., 2011; Scur et al., 2016). On the other hand, there are no studies about the araçá's potential to be used as new adjuvant in food preservation. Moreover, its mechanism of antimicrobial action remains largely unknown.

In this context, this study aimed to verify the antistaphylococcal activity of the hydroalcoholic extract of araçá (HEA), as well as to elucidate its mechanism of action against *S. aureus*.

2 Material and methods

2.1 Samples

Ripe fruits of araçá were collected from the bank of accessions at Embrapa Clima Temperado, Pelotas, RS, Brazil (geographical coordinates: 31 º 40' 47" S and 52 º 26' 24" W: 60 m of altitude). Fruits from two genotypes were used, which had been individually evaluated in a previous study and no difference in their antibacterial activity was detected (data not shown). These fruits were mixed, generating a single sample. Then, the fruits were washed and sanitized, their

seeds were removed, and fruits flesh with peel were frozen in liquid nitrogen and stored at −80 ºC until extract preparation.

2.2 Extract

For the preparation of the hydroalcoholic extract of araçá (HEA), the samples were macerated with a pistil grade. A quantity of 100 g of sample and 400 mL of 75% ethyl alcohol (v/v) (Synth, Brazil) was used, left under shaking for 2 h (200 rpm), at room temperature and protected from the light. After this, the mixture remained 15 min in an ultrasonic bath (Quimis, Brazil) (48 A) and was filtered in qualitative paper (Whatman, UK). The filtrate was centrifuged at 6000 x *g* for 20 min, and the supernatant was rotary-evaporated (Heidolph, Germany) at 40 °C to constant weight.

2.3 Antistaphylococcal activity

The antistaphylococcal activity was evaluated by agar disk diffusion testing, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In agar disk diffusion testing, disks impregnated with 10 µL of HEA were placed on Mueller-Hinton agar (Oxoid, UK) Petri dishes, previously inoculated with the strain *S. aureus* ATCC 25923, with incubation at 35 ºC for 20 h. The inoculum was standardized at a concentration of 10^8 CFU.mL^{-1}. Disks of ampicillin (Laborclin, Brazil) were used as a positive control and disks with the same solution used in the preparation of HEA were taken as a negative control. The inhibition zones were expressed in mm (CLSI, 2018).

For determination of MIC and MBC, the broth macrodilution technique was used. Firstly, an overnight culture was standardized to 10^5 CFU.mL^{-1}. Dilutions of

HEA were added in tubes containing the same volume of the inoculum, and these were incubated at 35 ºC for 20 h. As a negative control, ultrapure water was used. The MIC was determined as the lowest concentration without visible bacterial multiplication. From the tubes without bacterial multiplication, 100 µL were removed and added to Brain Heart Infusion agar (BHI) (Oxoid, UK) Petri dishes, to determine the MBC (CLSI, 2018).

2.4 Mechanism of action of HEA against *S. aureus*

2.4.1 Kinetics of antibacterial action

The kinetics of antibacterial action were performed according to Diao, Hu, Zhang & Xu (2014) with minor modifications. The MIC and MBC of HEA were added to tubes containing BHI broth and *S. aureus* inoculum (10^5 CFU.mL^{-1}), with incubation at 37 ºC under agitation (120 rpm) for 24 h. At time zero and after 4, 8, 12 and 24 h serial dilutions of the samples were performed in 0.1% (w/v) Peptone water (Oxoid, UK), followed by inoculation in BHI agar, with incubation at 37 ºC for 24 h. Tubes with inoculum without HEA were used as control.

2.4.2 Cell membrane integrity

Cell membrane integrity was evaluated by quantification of cell constituents that had released in the supernatant, by scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM).

2.4.2.1 Releasing of cell constituents

The releasing of macromolecules and nucleic acids was verified according to a protocol described by Diao, Hu, Zhang & Xu (2014), with minor modifications.

Staphylococcus aureus cells from an overnight culture were centrifuged for 10 min at 5000 x *g*, and washed three times with phosphate solution buffer (PBS) (Laborclin, Brazil). After that, the cells were resuspended in 20 mL of PBS, treated with HEA (MIC and MBC) with incubation at 37 ºC, under shaking (120 rpm), for 8 h. A new centrifugation was performed at 11000 x *g* for 5 min, and the supernatant was used for the analysis of proteins, reducing sugars and nucleic acids. Bacterial cells without HEA were prepared the same way, and used as control.

The concentration of proteins in the supernatant was determined according to Bradford (1976), with utilization of Coomassie G-250 Bright Blue Dye (Sigma-Aldrich, USA), and the proteins were quantified with a calibration curve of bovine serum albumin (Inlab, Brazil) with the absorption readings at 595 nm. Reducing sugars were evaluated by 3,5-dinitrosalicylic acid test according to Miller (1959). A glucose calibration curve was used to quantify the concentration of reducing sugars, with readings at 550 nm. The determination of nucleic acids was performed with a reading at 260 nm of supernatant (diluted 10x) in UV/VIS IL-592 spectrophotometer (Kazuaki, Japan).

2.4.2.2 Scanning electron microscopy (SEM)

To verify possible morphological alterations in *S. aureus* cells caused by HEA exposure, SEM was used. The microorganism was incubated until logarithmic phase (10^8 CFU.mL^{-1}), and HEA was added in two concentrations (MIC and MBC) with incubation at 37 ºC, 120 rpm, for 8 h. After that, cells with the extract in two concentrations (MIC and MBC) were centrifuged at 6000 x *g* for 10 min, washed three times with PBS and incubated with glutaraldehyde solution

2.5% (v/v) (Dinâmica, Brazil), overnight at 4 ºC. The control without HEA was used in the same way. Following this, cells were washed three times with PBS and dehydrated with increasing concentrations of ethyl alcohol (30, 50, 70, 80, 90, and 100%). They were then lyophilized, fixed in SEM support and sprayed with gold for visualization by S-4800 SEM (Hitachi High-Technologies, Japan).

2.4.2.3 Confocal laser scanning microscopy (CLSM)

For discrimination between viable and dead cells, CLSM was used with a LIVE/DEAD® BacLight 7012 bacterial viability kit (Thermo Fisher Scientific, USA). The microorganism was incubated until logarithmic phase (10^8 CFU.mL^{-1}), and HEA was added in two concentrations (MIC and MBC) with incubation at 37 ºC, 120 rpm, for 8 h. Next, cells were prepared according to manufacturer's instructions from the LIVE/DEAD® kit, stained with SYTO 9 and propidium iodide (1:1) for 15 min and observed in Leica TSC SP8 Confocal Microscope, at a magnification of 400x (Leica Microsystems, Germany).

2.4.3 HEA effect on genomic DNA

Genomic DNA of *S. aureus* was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The concentration was determined by reading the absorbance at 260 nm, using UV-VIS NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, USA).

HEA's effect on bacterial DNA was determined in the fluorescence spectrophotometer F-7000 (Hitachi High-Technologies, Japan), using the protocol described by Ning et al. (2017), with modifications. The extracted DNA was diluted in Tris-HCl 10 mM (Sigma-Aldrich, USA) until the concentration of 30

µg mL^{-1} and the same volume of HEA (MIC or MBC) or Tris-HCl (control) was added to DNA. The microplates were incubated at 37 ºC, in the dark, for 10 min. The fluorescence was measured using an excitation of 280 nm and scanning emission of 300-445 nm.

2.4.4 HEA interaction with genomic DNA

HEA interaction with genomic DNA was evaluated by competitive binding assays with ethidium bromide (EtBr) (Sigma-Aldrich, USA), according to Tang et al. (2009), with minor modifications. In microplates, the same volume of DNA was added at a concentration of 30 µg.mL^{-1} and HEA at concentrations of MIC and MBC. As control, Tris-HCl was used. The microplates were incubated at 37 ºC, in the dark, for 10 min. After that, EtBr solution was added (2 µg.mL^{-1}) with incubation at 37 ºC, in the dark, for 10 min. The fluorescence was measured using an excitation of 535 nm and scanning emission of 560-650 nm.

2.5 Statistical analysis

All experiments were performed in triplicate, and data were expressed as mean (± standard deviation). The results were submitted to statistical analysis of variance (ANOVA), comparison of means by Tukey's test ($p < 0.05$) determined with the program STATISTIX 8.0.

3 Results and discussion

3.1 Antistaphylococcal activity

HEA showed antistaphylococcal activity, observed by formation of an inhibition zone of 17 mm diameter in agar disk diffusion testing. The MIC value was 9 mg mL^{-1}, and MBC value was 18 mg mL^{-1}. The values of antimicrobial activity found in different studies with extracts and essential oils of vegetables are highly variable and mostly depend on the content of bioactive compounds, the method of extraction used, and the choice of solvent (polarity); among these, methanol and ethanol are the most used solvents, pure or in hydroalcoholic solutions (Azmir et al., 2013). In this study, the use of a 75% ethanoic solution was chosen as the solvent for future application of HEA in foods.

The major phenolic compounds identified in this extract, quantified by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS), were gallic (2841 µg g^{-1}) and ferulic acids (716 µg g^{-1}), a hydroxybenzoic acid and a hydroxycinnamic acid, respectively. Borges, Ferreira, Saavedra & Simões (2013) investigated the action of these two phenolic acids against *Escherichia coli*, *Pseudomonas aeruginosa*, *S. aureus,* and *Listeria monocytogenes* and found antibacterial activity against all the evaluated pathogens, with MIC values varying from 100 to 2000 mg mL^{-1} and MBC ranging from 500 to 5500 mg mL^{-1}. The MIC and MBC values found for *S. aureus* were 1750 mg mL^{-1} and 5250 mg mL^{-1} using gallic acid, and 1250 mg mL^{-1} and 5000 mg mL^{-1} using ferulic acid. These MIC and MBC values are higher than those found in the present study; however, Borges et al. (2013) evaluated the individual and pure molecules. Although these two phenolic acids were the main bioactive substances identified in the HEA, the presence of other phytochemicals in the extract (even in a smaller

proportion), plus a possible synergism between the extract contents, may have potentiated the antistaphylococcal activity of HEA.

3.2 Mechanism of action of HEA against *S. aureus*

3.2.1 Kinetics of antibacterial action

It was verified that in the first 4 h of contact with HEA, there was a difference of at least 4 logarithmic cycles in *S. aureus* growth in comparison to the control (Fig. 1). However, since HEA was added, inhibition already occurred in the microorganism growth, and there was no difference ($p > 0.05$) between the times 0, 4 and 8 h of incubation with HEA, while a significant reduction in the microorganism occurred after 8 h. The major concentration of HEA (MBC) was effective in eliminating the microorganism, while the lowest concentration (MIC) was able to prevent *S. aureus* growth above 10^5 CFU per g or mL (concentration in which it produces staphylococcal enterotoxins) (Schelin et al., 2017). This result is important, since even if HEA at the MIC values does not eliminate *S. aureus* from a contaminated food, it would avoid the synthesis of enterotoxins, being able to keep this food safe for a longer time.

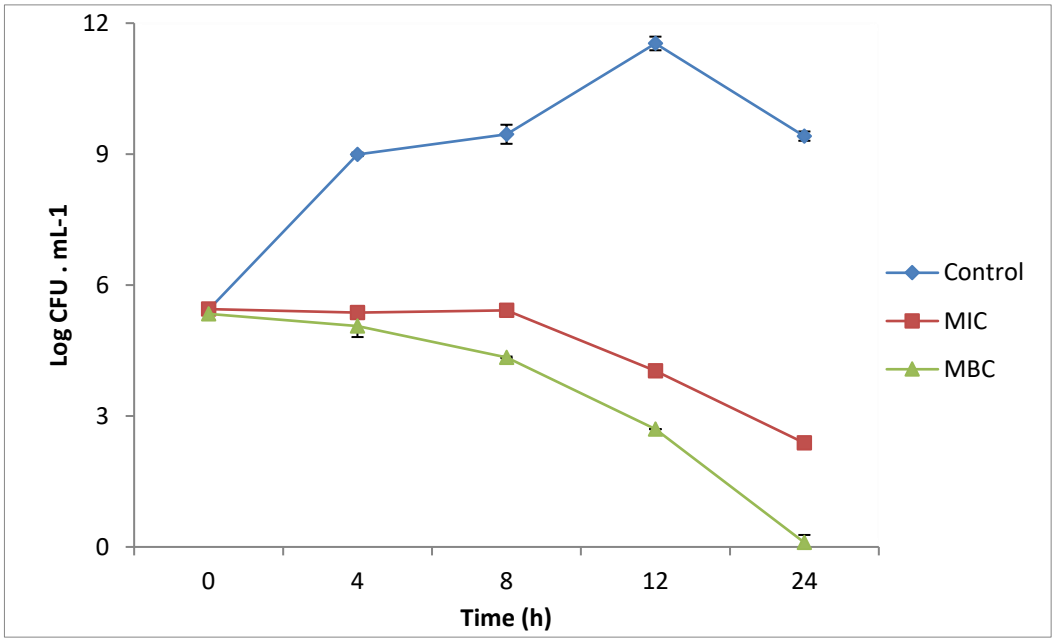

Fig. 1. Kinetics of antibacterial action of hydroalcoholic extract of araçá (HEA) against *Staphylococcus aureus* ATCC 2593. Values represent the averages of three reproducible experiments. Bars represent the standard deviation ($p < 0.05$).

3.2.2 Cell membrane integrity

3.2.2.1 Releasing of cell constituents

The damage caused by HEA to *S. aureus* cell membrane treated for 8 h, with two extract concentrations (MIC and MBC), was evaluated by quantification of released cell constituents through cell membrane (proteins, reducing sugars and nucleic acids).

Table 1 shows that the releasing of cell constituents significantly increased in relation to the control, and proportionally the concentration of HEA.

The release of a small quantity of intracellular components can be tolerable and does not affect the viability of the bacteria; however, an increase in the releasing becomes crucial, causing the death of the microorganism (Burt, 2004). The results found in this study demonstrate that HEA promotes *S. aureus* cell

membrane damage, with liberation of important cell constituents, where this loss is dependent on the HEA concentration used.

Table 1. Effect of hydroalcoholic extract of araçá (HEA) on extravasation of cell components through *S. aureus* cell membrane.

Concentrations	Cell constituents' release		
	Protein (µg/mL)	Reducing sugar (µg/mL)	Cell constituents (OD260nm)
Control	2.1 ± 1.6c	2.2 ± 0.2c	0.188 ± 0.008c
MIC	38.06 ± 0.4b	25.4 ± 5.6b	0.454 ± 0.001b
MBC	62.99 ± 2.1a	47.5 ± 2.2a	0.498 ± 0.005a

Values represent means of three independent replicates ± SD. Different letters within a column indicate statistically significant differences between the means ($p < 0.05$).

3.2.2.2 Cell morphology

The modifications in *S. aureus* morphology caused by HEA exposure were evaluated by SEM. Regular morphology was seen in *S. aureus* not treated with HEA, with uniform cells in size and distribution, as well as a flat surface. On the other hand, cell membrane rupture was observed, with releasing of intracellular components, in cells with contact with HEA (Fig. 2). With the increase in HEA concentration (MBC), the cell membrane collapsed or even ruptured, with accumulation of cellular debris, as indicated by the arrows in Fig. 2 (E and F).

These alterations in cell morphology corroborate previous results, in which it was verified that releasing of macromolecules and nucleic acids took place by loss of integrity of the *S. aureus* cell membrane after exposure to HEA.

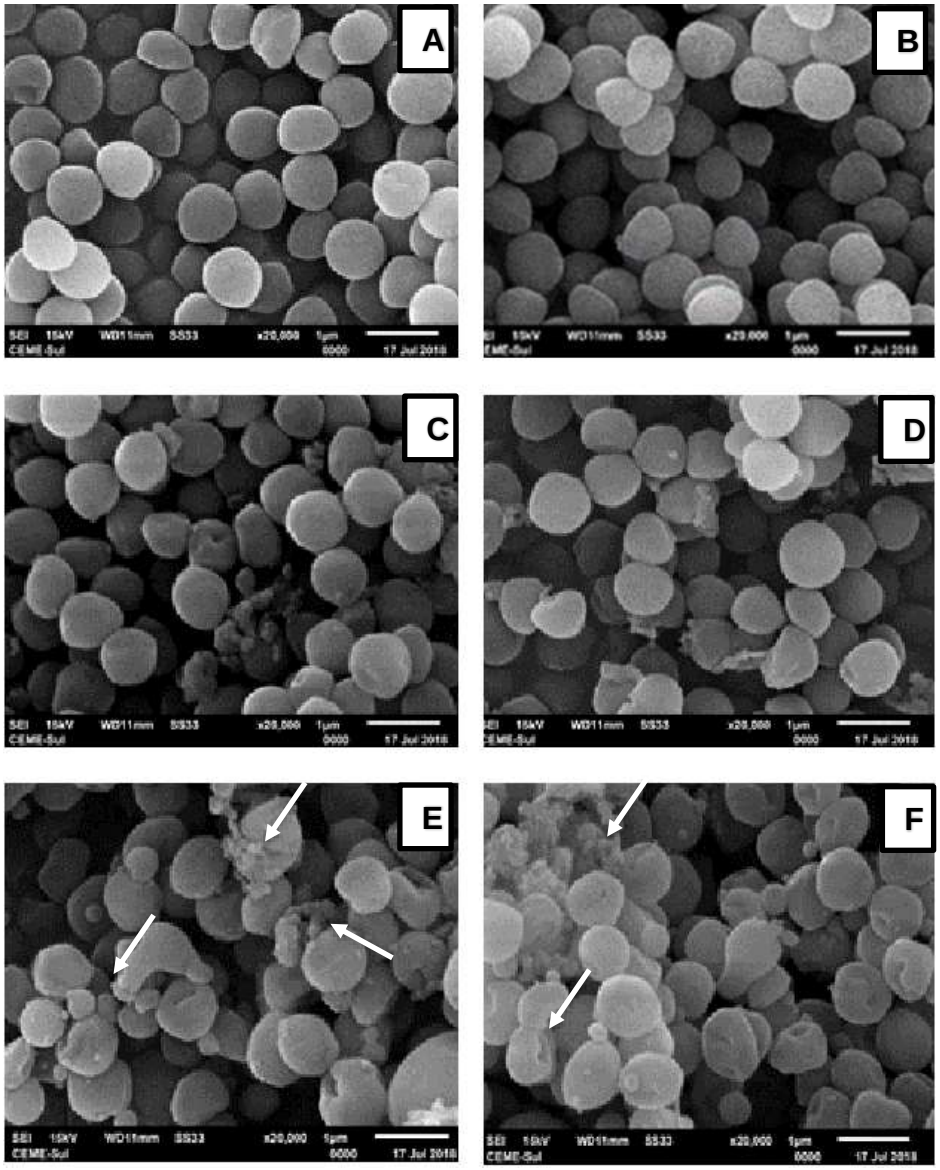

Fig. 2. Scanning electron microscopy of *Staphylococcus aureus*, treated for 8 h with hydroalcoholic extract of araçá (HEA). A and B: *S. aureus* without treatment; C and D: using MIC concentration; E and F: using MBC concentration.

3.2.2.3 Bacterial cell viability

The bacterial cell viability was evaluated by CLSM, which is a technique that allows the differentiation of cells with an intact cytoplasmic membrane (viable cells show green fluorescence) and cells with compromised cytoplasmic membrane (dead cells show red fluorescence) (Haugland, 2005).

As demonstrated in Fig. 3, in control treatment (A, without HEA contact), almost all *S. aureus* cells showed green fluorescence (viable cells). In the treatment with HEA using MIC (B), after 8 h of contact with bacteria plus HEA, an equilibrium was observed between cells with green and red fluorescence. However, in the treatment using MBC of HEA (C), almost all *S. aureus* cells showed red fluorescence, in view of the damage to the cytoplasmic membrane, causing cell death.

The simultaneous utilization of SEM and CLSM techniques allowed morphological features of viable, compromised and dead cells by HEA exposure to be evaluated, as well as estimating the proportion in which the action of the extract occurred. The results obtained in the cell membrane integrity assays of *S. aureus* demonstrate that the cell membrane was one of the targets of action of HEA in this microorganism.

Borges et al. (2013) studied the action mechanism of gallic and ferulic acids (major phenolic compounds in the HEA used in this study) against pathogenic bacteria. They found irreversible alterations in cell membrane properties, such as alterations in hydrophobicity, a decrease in the negative surface charge and local rupture or formation of pores in the cellular membranes, with consequent releasing of intracellular constituents, as occurred in the present study. The mechanism of antibacterial action of phenolic compounds present in

plants is not fully understood; however, bacterial cell membrane rupture is one of the main mechanisms reported in both Gram-positive and Gram-negative bacteria (Rempe, Burris, Lenaghan, & Stewart, 2017), which reinforces the hypothesis that the antistaphylococcal action found in HEA is largely due to the presence of these compounds.

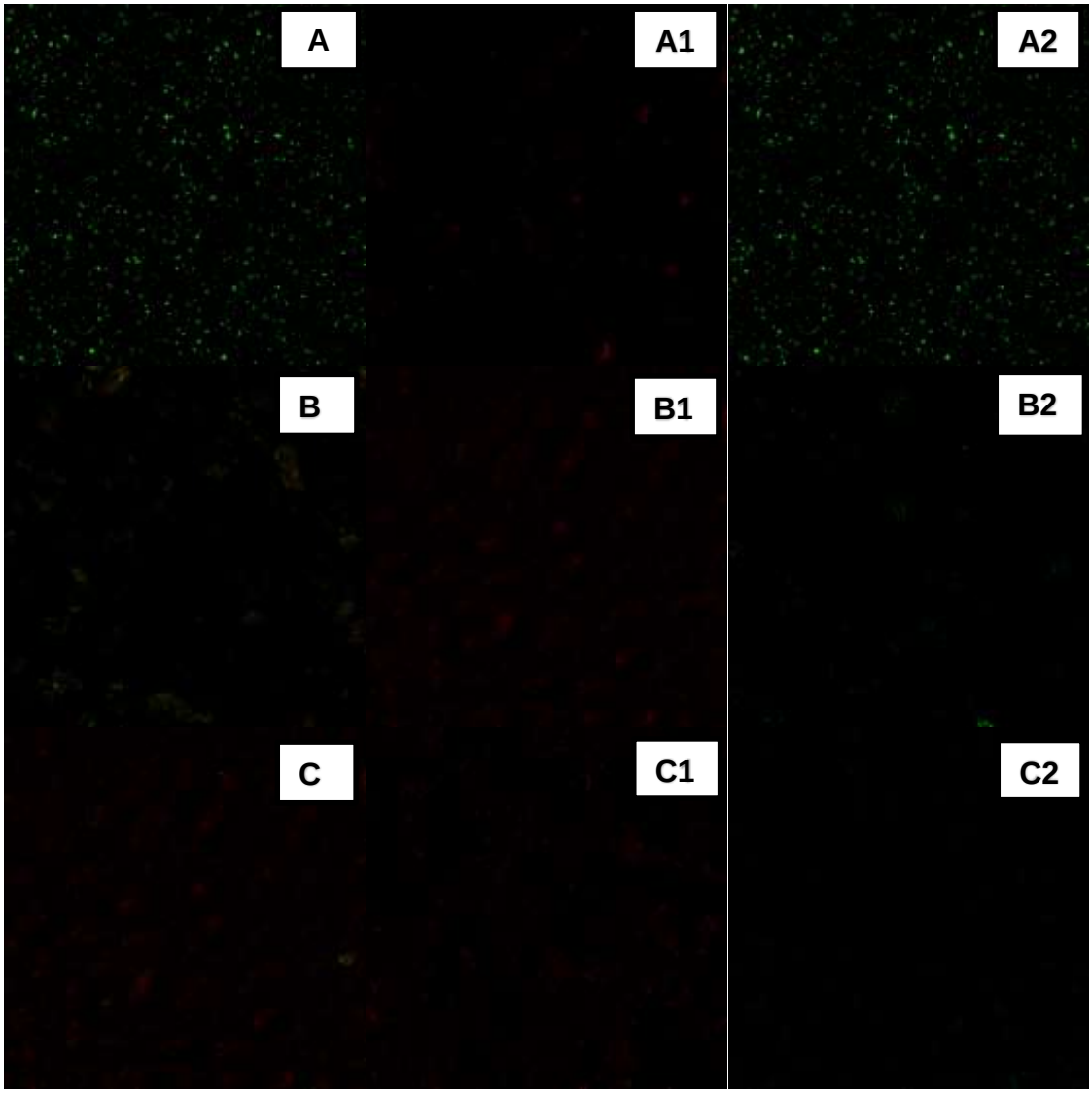

Fig. 3. Confocal laser scanning microscopy of *S. aureus* treated for 8 h with hydroalcoholic extract of araçá (HEA). A: *S. aureus* without treatment; B: using MIC concentration; C: using MBC concentration; A1, B1, and C1: dead cells in each treatment; A2, B2, and C2: viable cells in each treatment.

3.2.3 HEA effect on genomic DNA

Regarding studies about the mechanism of action of an antimicrobial, the evaluation of bacterial DNA damage is of great importance, since it can impair gene expression, leading to the blockage of the normal synthesis of enzymes and receptors, causing the death of the bacterium (Ning et al., 2017).

The loss of cell membrane integrity and rupture of *S. aureus* cells caused by HEA may facilitate the extract to reach the internal structure of bacterial cells. In this way, the HEA effect on bacterial DNA was investigated using fluorescence spectrophotometry, one of the most sensitive techniques for studies with this nucleic acid (Li et al., 2017; Tang et al., 2009). Fig. 4 shows that the addition of HEA caused a significant reduction in DNA fluorescence, in the range of 300 to 360 nm. The HEA concentration had an influence only in the range of 305 to 315 nm, where a greater reduction was observed in the emission of fluorescence when the highest extract concentration was used (MBC). These results suggest that there was some binding of HEA to bacterial DNA, leading to changes in its conformation and structure and consequent decrease in emitted fluorescence.

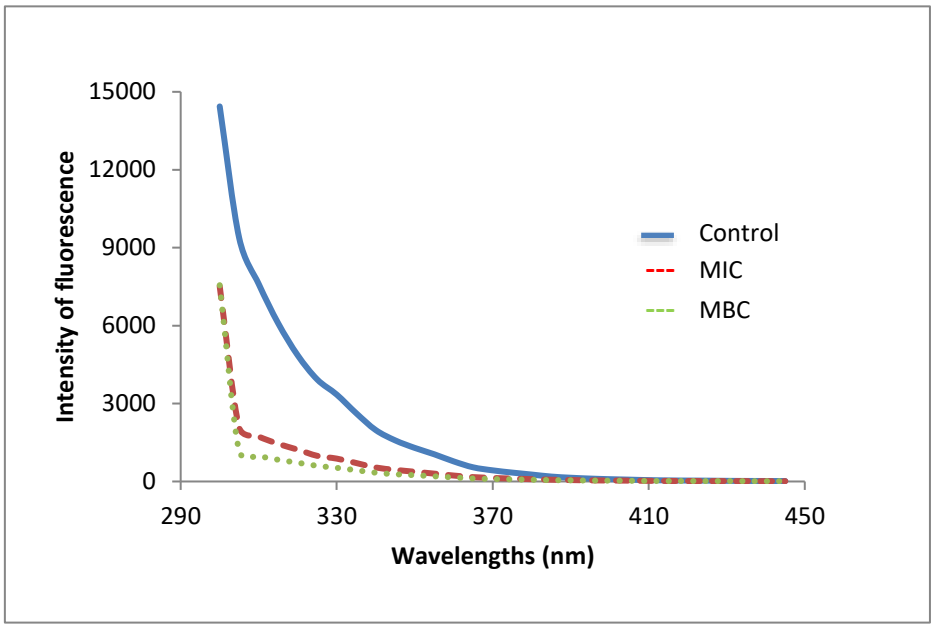

Fig. 4. Fluorescence spectrum of hydroalcoholic extract of araçá (HEA) in *S. aureus* DNA. Values represent the averages of three reproducible experiments ($p < 0.05$).

3.2.4 HEA interaction with genomic DNA

The competitive assay between HEA and EtBr was evaluated by fluorescence spectrophotometry in order to examine whether HEA can displace EtBr from the DNA helix and interact with this nucleic acid. EtBr is a cationic conjugated planar molecule that is well known as a DNA intercalator. The fluorescence intensity of EtBr is generally weak; however, it increases with the addition of DNA. Thus, EtBr has been used as a probe for the spectroscopic study of the interaction between DNA and potent intercalating species. When another compound exhibits similar binding mode to that of EtBr with the DNA, the fluorescence is decreased or even extinguished (Ebrahimipour et al., 2015).

Fig. 5 shows that the addition of HEA caused a decrease in fluorescence emission, which was significant ($p < 0.05$) between the wavelengths of 635 and 645 nm, when the MBC of HEA was used. This result suggests that EtBr molecules, combined in the DNA base pairs, were replaced by HEA molecules

when a higher concentration of the extract was used, indicating that intercalation was one of the forms of HEA binding to bacterial DNA. Similar results were found by Lou et al. (2012), researching the mechanism of action of p-coumaric acid, a phenolic acid found in a wide variety of vegetables, against *Shigella dysenteriae* 51302. The authors verified a decrease in fluorescence when increasing concentrations of p-coumaric acid were added to the DNA-EtBr complex, suggesting that this acid replaced some EtBr molecules, to intercalate with the DNA base pairs (hydrophobic medium), releasing the EtBr in aqueous solution (hydrophilic medium), with a consequent decrease in excitation.

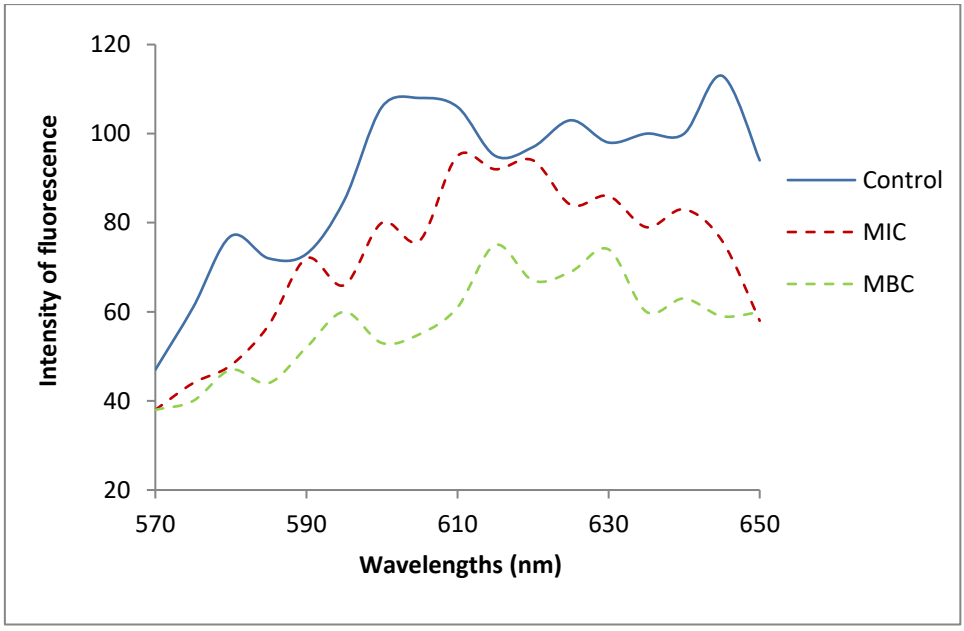

Fig. 5. Fluorescence spectrum of HEA-DNA complex titered with EtBr. Values represent the averages of three reproducible experiments ($p < 0.05$).

In the DNA intercalation complex, an aromatic system is inserted between the base pairs, while a cationic substituent binds in the major groove and the other substituent interacts with the minor DNA groove (Strekowski & Wilson, 2007). The major phenolic compounds present in the HEA (gallic and ferulic

acids), have the same aromatic group as all the phenolic compounds and, alone or together with other phytochemical constituents present in lower concentrations, may have been responsible for this intercalation with bacterial DNA and the consequent death of *S. aureus* cells.

4 Conclusion

HEA shows antistaphylococcal activity presenting more than one action site in the *S. aureus* cell. The extract causes cell membrane damage, with loss of integrity and release of nucleotides and macromolecules. Moreover, after overcoming the barrier formed by the cell membrane, when used at the MBC, the extract is capable of intercalating with bacterial DNA, inhibiting basic cell functions, with consequent death of the microorganism. This study shows promising and unpublished results and demonstrates that HEA has potential to be used as a natural adjuvant in food preservation.

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6 Considerações finais

O estudo realizado possibilitou verificar que:

- Frutas nativas, como o araçá, são ainda, pouco aproveitadas comercialmente, sendo até mesmo desconhecidas por parte da população urbana, no entanto, possuem importantes constituintes fitoquímicos, com potencial benéfico tanto para o consumo *in natura* quanto para o preparo de extratos naturais;
- Os EA possuem atividade antibacteriana e antioxidante, mesmo após submetidos à intenso tratamento térmico, com preservação de parte dos compostos fenólicos e, possível formação de novos compostos com atividade antioxidante;
- A atividade antibacteriana e antioxidante dos EA não foi dependente do genótipo estudado, podendo-se otimizar a produção dos EA (com o uso de um mix de araçás amarelos e vermelhos no preparo dos EA, resultando em maior quantidade de EA);
- A atividade antiestafilocócica dos EA ocorre por alteração na integridade da membrana celular e dano ao DNA do micro-organismo;
- Este estudo apresenta dados inéditos que podem servir de embasamento para futuros experimentos científicos, que possibilitem avaliar a aplicação dos EA em alimentos, incluindo alimentos tratados termicamente.

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