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CARACTERIZAÇÃO QUÍMICA E BIOLÓGICA DE EXTRATOS DE PRÓPOLIS VERMELHA DO NORDESTE DO BRASIL OBTIDOS POR DIFERENTES MÉTODOS DE EXTRAÇÃO

JOÃO HENRIQUE DE OLIVEIRA REIS

Salvador – BA

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Orientadora: Profª Dra. Janice Izabel Druzian

Co-Orientador: Prof. Dr. Alex Alisson Bandeira Santos

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TERMO DE APROVAÇÃO

A TESE:

"CARACTERIZAÇÃO QUÍMICA E BIOLÓGICA DE EXTRATOS DE PRÓPOLIS VERMELHA DO NORDESTE DO BRASIL OBTIDOS POR DIFERENTES MÉTODOS DE EXTRAÇÃO"

Elaborada por:

JOÃO HENRIQUE DE OLIVEIRA REIS

Foi aprovada por todos os membros da banca examinadora e aceita pelo Programa de Pós-Graduação em Biotecnologia da Renorbio como requisito parcial à obtenção do título de

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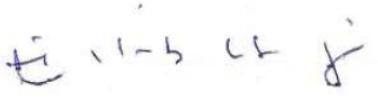

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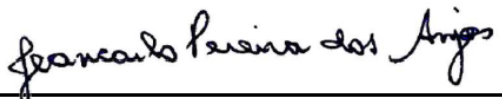

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DEDICATÓRIA

Aos meus queridos familiares, principalmente a minha mãe por todo amor e suporte dado, a minha esposa Bruna, pelo amor, paciência e por sempre estar comigo em todos os momentos. A minha irmã e amigos que torceram para que essa etapa fosse concluída. A minha orientadora Pró Janice, por toda a confiança depositada e permitir a conquista deste sonho. Tenho certeza que a senhora ficará muito feliz em ver mais um aluno seu Doutor! Obrigado por todo o cuidado que teve comigo desde quando entrei na Faculdade de Farmácia (UFBA). Obrigado pela confiança! Obrigado por ter se dedicado tanto a Ciência! A senhora Pró Janice, SEMPRE será um exemplo para mim e para todos os seus alunos. Não tenha dúvidas que somos as suas sementes! Aproveito para agradecer o meu co-orientador o Prof. Dr. Alex Alisson pela confiança e imensas contribuições. Não tenha dúvidas de que a sua participação foi essencial para o desenvolvimento e finalização desse trabalho. O meu mais sincero obrigado! As queridas colegas de laboratório, e em especial a Gabriele Barreto e Larissa Fonseca por toda ajuda e dedicação para realização desse trabalho.

"*Faça o teu melhor, na condição que você tem, enquanto não tem condições melhores, para fazer melhor ainda*".

(Mario Sergio Cortella)

Sumário

Resumo

A própolis é um composto natural de grande aplicação nas áreas alimentícia, farmacêutica e cosmética sendo uma resina complexa produzida pelas abelhas através da mistura de exsudatos de diferentes plantas, cera e secreções salivares. As variações na composição química, e consequentemente, na atividade biológica de extratos de própolis, estão associadas a muito fatores, como tipo, origem geográfica, sazonalidade, entre outros. Dentro desse contexto, o objetivo desse estudo foi avaliar a influência de diferentes métodos de extração na composição química e biológica de extratos de própolis vermelha de diferentes origens geográficas, bem como determinar as condições de processo para a obtenção de extratos de própolis vermelha utilizando a tecnologia de extração com fluído supercrítico (SFE). Inicialmente, os métodos de extração convencional (etanólica) e assistida por ultrassom foram empregados para extrair compostos ativos de própolis de diferentes regiões do Nordeste do Brasil (Sergipe, Alagoas, Bahia, e Rio Grande do Norte). Teores de compostos fenólicos, flavonoides, atividade antioxidante *in vitro*, concentração de marcadores (formononetin e kaempferol) e a citotoxidade para linhagens de células tumorais humanas (HCT116 – human colon, HL60 – leukemia, PC3 – carcinoma of prostate e SNB19 – glioblastoma) foram avaliados comparativamente para os doze extratos obtidos a partir de seis diferentes amostras. Adicionalmente, foram obtidos extratos de própolis vermelha por SFE de uma amostra de Alagoas empregando diferentes condições de processo. Para a determinação dos parâmetros de processo aplicando a SFE, foram estudados e obtidos a Curva Global de Extração (*OEC - Overall Extraction Curves*), o S/F (massa de CO_2/massa de própolis), o percentual de co-solvente (etanol – 0, 1, 2 e 4%) e as isotermas de rendimento global (*GYI - Global Yield Isotherms*) em função das diferentes pressões (250, 350 e 450 bar) e temperaturas (31,7, 40 e 50°C). Foram investigados os compostos fenólicos totais, atividade antioxidante e o teor de formononetina, naringenina e kaempferol nos extratos obtidos nas diferentes condições empregadas (SFE). Como resultados deste estudo, foram identificadas variações significativas ($p>0,05$) no teor dos compostos investigados nos extratos de própolis vermelha, confirmando que a composição química varia de acordo com a região de coleta das amostras. A maior concentração dos compostos de interesse e a maior atividade antioxidante *in vitro* foram exibidas pelos extratos obtidos em amostras do estado de Alagoas (A1, A2, B1 e B2), que atualmente é a única no Brasil que apresenta certificação de origem. Os biomarcadores formononetina e kaempferol foram identificados em todas as amostras, independente da origem ou do método de extração empregado. As maiores concentrações de formononetina foram identificadas nos extratos obtidos por ultrassom, indicando uma maior seletividade para a extração desse composto por este método. Em relação à atividade citotóxica, para a linhagem HCT-116, todos os extratos apresentaram inibição superior a 90%, enquanto para as linhagens HL-60 e PC3 a menor inibição identificada foi de 80%. Em geral, não houve diferença significativa ($p>0,05$) no potencial antiproliferativo quando comparados os métodos de extração. Os resultados mostraram que a composição da própolis vermelha brasileira varia significativamente dependendo da origem geográfica e que o método utilizado influencia os compostos resultantes que estão presentes na própolis. No que se refere a aplicação da SFE como método extrativo, dentro dos parâmetros investigados, as melhores condições encontradas foram um S/F de 131 e o uso de etanol na maior concentração (4%), o que resultou em maiores rendimentos de extrato e maior teor de compostos antioxidantes. A formononetina, principal biomarcador da própolis vermelha, foi o composto encontrado em maiores quantidades nos extratos obtidos por SFE em todas as condições empregadas. Como esperado, as condições de temperatura e pressão também influenciaram no rendimento do processo, sendo 350 bar e 40°C as melhores condições para a obtenção de compostos bioativos a partir de uma amostra de própolis vermelha. Os novos resultados para a própolis vermelha encontrados neste estudo mostram que é possível obter extratos com alto potencial antioxidante utilizando tecnologias alternativas, bem como que a origem geográfica, o tipo de método empregado e as condições de processo influenciam na qualidade do extrato obtido.

Palavra-chave: Própolis vermelha, extração com fluido supercrítico, ultrassom, origem geográfica, citotoxicidade, atividade antioxidante.

Abstract

Propolis is a natural compound of wide application in the food, pharmaceutical and cosmetic areas, being a complex resin produced by bees through the mixture of exudates from different plants, wax and salivary secretions. Variations in the chemical composition, and consequently, in the biological activity of propolis extracts, are associated with many factors, such as type, geographic origin, seasonality, among others. Within this context, the objective of this study was to evaluate the influence of different extraction methods on the chemical and biological composition of red propolis extracts from different geographic origins, as well as to determine the process conditions for obtaining red propolis extracts using the technology extraction with supercritical fluid (SFE). Initially, conventional (ethanolic) and ultrasound-assisted extraction methods were used to extract active compounds from propolis from different regions of Northeastern Brazil (Sergipe, Alagoas, Bahia, and Rio Grande do Norte). Contents of phenolic compounds, flavonoids, in vitro antioxidant activity, concentration of markers (formononetin and kaempferol) and cytotoxicity to human tumor cell lines (HCT116 - human colon, HL60 - leukemia, PC3 - carcinoma of prostate and SNB19 - glioblastoma) were evaluated comparatively for the twelve extracts obtained from six different samples. Additionally, red propolis extracts were obtained by SFE from a sample from Alagoas using different process conditions. To determine the process parameters applying SFE, the Overall Extraction Curves (OEC - Overall Extraction Curves), the S/F (CO_2 mass/propolis mass), the percentage of co-solvent (ethanol – 0, 1, 2 and 4%) and the global yield isotherms (GYI - Global Yield Isotherms) as a function of different pressures (250, 350 and 450 bar) and temperatures (31.7, 40 and 50°C). The total phenolic compounds, antioxidant activity and the content of formononetin, naringenin and kaempferol in the extracts obtained under different conditions (SFE) were investigated. As a result of this study, significant variations ($p>0.05$) were identified in the content of the compounds investigated in red propolis extracts, confirming that the chemical composition varies according to the region where the samples were collected. The highest concentration of the compounds of interest and the highest in vitro antioxidant activity were shown by extracts obtained from samples from the state of Alagoas (A1, A2, B1 and B2), which is currently the only one in Brazil that has certification of origin. The biomarkers formononetin and kaempferol were identified in all samples, regardless of origin or extraction method used. The highest concentrations of formononetin were identified in the extracts obtained by ultrasound, indicating a greater selectivity for the extraction of this compound by this method. Regarding cytotoxic activity, for the HCT-116 strain, all extracts showed inhibition greater than 90%, while for the HL-60 and PC3 strains, the lowest inhibition identified was 80%. In general, there was no significant difference ($p>0.05$) in the antiproliferative potential when comparing the extraction methods. The results showed that the composition of Brazilian red propolis varies significantly depending on the geographic origin and that the method used influences the resulting compounds that are present in the propolis. Regarding the application of SFE as an extractive method, within the parameters investigated, the best conditions found were a S/F of 131 and the use of ethanol in the highest concentration (4%), which resulted in higher yields of extract and higher content of antioxidant compounds. Formononetin, the main biomarker of red propolis, was the compound found in greater amounts in the extracts obtained by SFE under all conditions used. As expected, the temperature and pressure conditions also influenced the process yield, with 350 bar and 40°C being the best conditions for obtaining bioactive compounds from a red propolis sample. The new results for red propolis found in this study show that it is possible to obtain extracts with high antioxidant potential using alternative technologies, as well as that the geographic origin, the type of method used and the process conditions influence the quality of the extract obtained.

Keywords: Red propolis, supercritical fluid extraction, ultrasound, geographic origin, cytotoxicity, antioxidant activity.

1.0 Apresentação

O presente trabalho de tese de doutorado foi organizado no formato de capítulos para uma melhor apresentação e entendimento do mesmo, possuindo no total sete capítulos. Cada capítulo refere-se a um item específico do trabalho, conforme descrito a seguir:

O Capítulo 1 constitui a introdução geral da Tese. Neste capítulo são abordados, de forma resumida, vários aspectos relacionados a própolis, a aplicação científica e industrial de seus extratos, o potencial biológico da própolis, e mais especificamente da própolis vermelha, e por fim a justificativa para aplicação de diferentes técnicas de extração para obtenção de extratos de alto valor agregado dessa matriz natural.

O Capítulo 2 constitui os objetivos da Tese. Neste capítulo são abordados o objetivo geral do trabalho e detalhados os objetivos específicos.

O Capítulo 3 se refere ao tópico de Revisão de Literatura e constitui uma revisão científica sobre a própolis, tipos, origem geográfica, métodos de extração. Também é apresentado um levantamento e avaliação dos principais artigos científicos e documentos de patentes envolvendo a tecnologia de extração com fluído supercrítico (SFE) aplicada à própolis de diferentes tipos e origens geográficas. Este estudo intitulado de "*Extraction of propolis using supercritical carbon dioxide*" foi publicado como capítulo do livro "*Green Sustainable Processes for Chemical and Environmental Engineering and Science* – Supercritical Carbon Dioxide as Green Solvent, 1st Edition 2020.

O Capítulo 4 constitui o artigo publicado na Revista *Plos One* (2019) intitulado de "*Evaluation of the antioxidant profile and cytotoxic activity of red propolis extracts from different regions of northeastern Brazil obtained by conventional and ultrasound assisted extraction"*. Neste capítulo foram avaliados extratos de própolis vermelha obtidos por extração convencional (etanólica) e extração assistida por ultrassom de seis amostras de diferentes regiões do Nordeste do Brasil. Os compostos fenólicos totais e flavonoides, atividade antioxidante *in vitro*, concentração de formononetina e kaempferol e a

citotoxicidade contra quatro linhagens de células tumorais humanas foram determinados para todos os doze extratos obtidos.

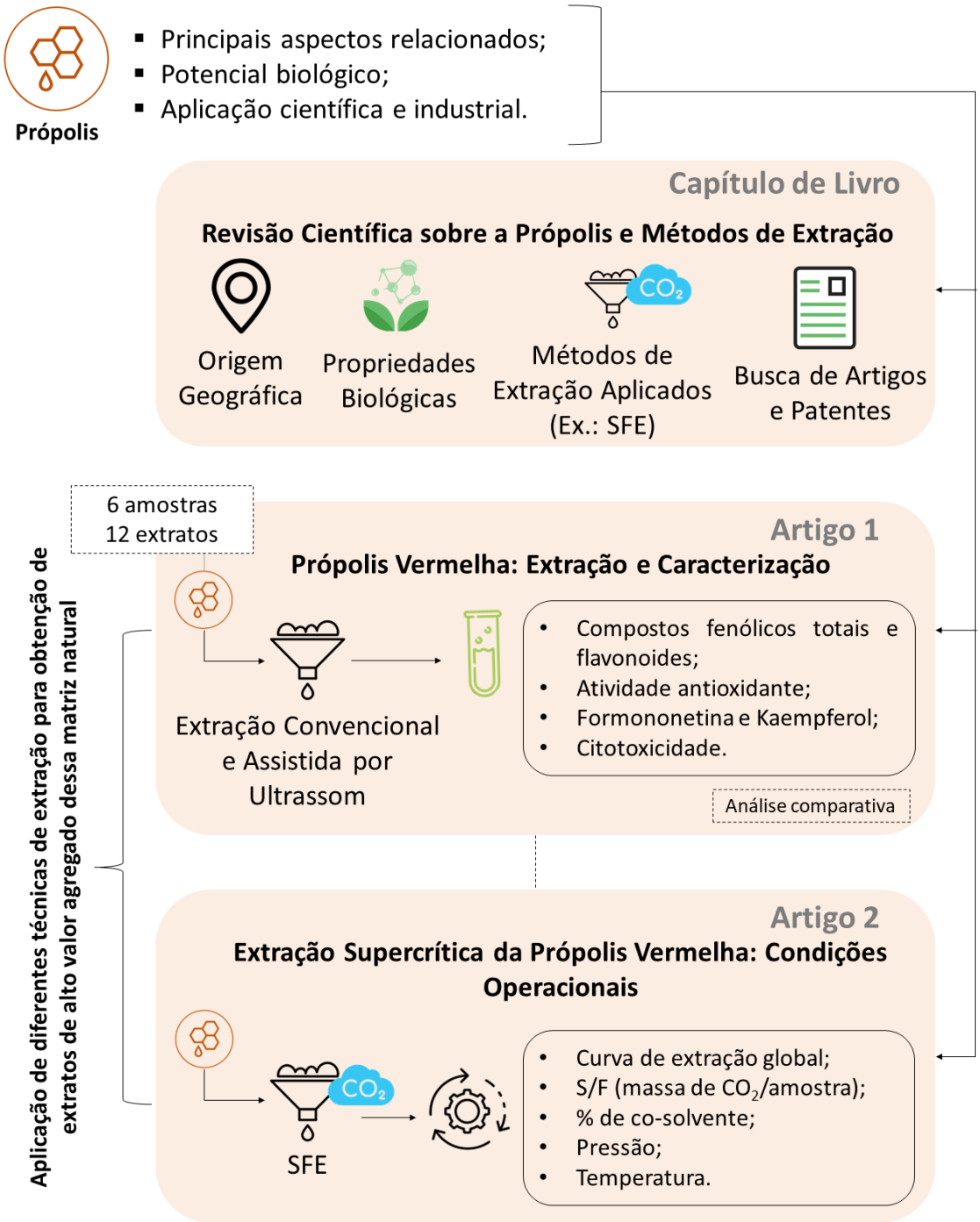
O Capítulo 5 constitui o artigo publicado na revista *Molecules* (2020) intitulado de "*Supercritical Extraction of Red Propolis: Operational Conditions and Chemical Characterization*". Neste capítulo foram determinadas as melhores condições operacionais para a obtenção do extrato de própolis vermelha com alto potencial antioxidante por meio da tecnologia de extração com fluido supercrítico (SFE), utilizando dióxido de carbono (CO_2) como fluido supercrítico e etanol como co-solvente. Os seguintes parâmetros foram estudados: curva de extração global, S/F (massa de CO_2/massa da amostra), porcentagem de co-solvente (0, 1, 2 e 4%) e isotermas de rendimento global em função de diferentes pressões (250, 350 e 450 bar) e temperaturas (31,7, 40 e 50 ° C).

No Capítulo 6 são apresentadas as Conclusões do presente trabalho, bem como, a perspectiva para desenvolvimento de trabalhos futuros.

Por fim, no Capítulo 7 está listada toda a produção científica desenvolvida durante todo o período do doutorado.

Na Figura 1 é apresentado uma representação esquemática da estratégia metodológica utilizada neste estudo inter-relacionando os trabalhos desenvolvidos com os objetivos e resultados alcançados.

Figura 1. Representação esquemática da estratégia metodológica utilizada neste estudo.



Capítulo 1

2.0 Introdução

A própolis é um composto natural de grande aplicação na área alimentícia, farmacêutica e cosmética (Irigoiti et al., 2021; Mendez-Pfeiffer et al., 2021; Picolotto et al., 2019) sendo definida como uma mistura resinosa complexa produzida pelas abelhas através da mistura de exsudatos de diferentes plantas, cera e secreções salivares (Marcucci et al., 1998; Park et al., 2002). Numerosos estudos têm comprovado as atividades antioxidantes (Alencar et al., 2007; Righi et al., 2011), antimicrobiana (Graikou et al., 2016; Regueira et al., 2017), anti-inflamatórias (Bueno-Silva et al., 2013; Lima Cavendish et al., 2015), anticâncer (Bueno-Silva et al., 2013; Lima Cavendish et al., 2015) e anti-nociceptiva (Lima Cavendish et al., 2015) de extratos de própolis obtidos de diferentes origens e métodos extrativos.

As atividades biológicas da própolis são atribuídas a compostos como ácidos fenólicos, flavonoides, terpenos e sesquiterpenos (Andrade et al., 2017; Awale et al., 2008; Sena-Lopes et al., 2018). Destaca-se que o processo de extração alcoólica (método convencional) é o mais empregado para a obtenção desses biocompostos , apesar de apresentar algumas desvantagens no processo extrativo quando comparada com métodos de extração alternativos (Šuran et al., 2021), como a extração assistida por ultrassom (Cavalaro et al., 2019; Oroian et al., 2020) e extração com fluidos supercríticos (*Supercritical Fluid Extraction* – SFE) (Machado et al., 2019; Reis et al., 2020).

Com base nas propriedades físico-químicas (cor, textura, composição química) e origem geográfica, as própolis brasileiras foram inicialmente classificadas em 12 tipos (Park et al., 2002). Um tipo relativamente novo de própolis, chamada de própolis vermelha devido a sua cor intensa (tipo 13), vem despertando a atenção da indústria, devido às propriedades farmacológicas bastante promissoras de alguns de seus compostos isolados, como por exemplo, vestitol, neovestitol, quercetina, medicarpina, formononetina, entre outros (Aldana-Mejía et al., 2021; Lima et al., 2022; Silva et al., 2020). Devido a sua composição química bastante distinta quando comparada aos outros tipos de própolis, alguns estudos já evidenciam que a própolis vermelha e seus

compostos isolados (principalmente isoflavonas) afetam uma ampla gama de alvos biológicos e podem ter um impacto contra numerosas doenças como um agente antimicrobiano, anti-inflamatório, imunomodulatório, antioxidante, antitripanossomal, antileishmania e anti-proliferativo (Freires et al., 2016; Regueira-Neto et al., 2018; Santos et al., 2021).

A composição química da própolis, e consequentemente sua atividade biológica, varia de acordo com a origem geográfica, fonte botânica, época de coleta e condições climáticas da região (de Oliveira et al., 2021; Hosoya et al., 2021; Ristivojević et al., 2018; Sampaio et al., 2016; Sawaya et al., 2010; Sforcin et al., 2000), devido a isso, diferentes trabalhos tem investigado a influência de diferentes fatores na constituição química da própolis (Anđelković et al., 2017; Falcão et al., 2013; Miguel et al., 2010; Pierini et al., 2016; Valencia et al., 2012). Entretanto, poucos são os trabalhos disponíveis que investigaram comparativamente amostras de própolis vermelha coletadas em diferentes regiões do Brasil em relação a sua composição antioxidante, bem como atividade citotóxica frente a diferentes linhagens de células tumorais.

Estudos, avaliando as atividades biológicas da própolis vermelha, realizados por Machado et al., (2016), Dantas Dantas Silva et al., (2017) e Teles et al., (2015) demonstraram diferenças na capacidade antimicrobiana e antitumoral; antioxidante e antiparasitária; e atenuantes de hipertensão e danos renais, respectivamente, para amostras obtidas de diferentes origens. Regueira et al., (2017) investigaram o efeito da sazonalidade sobre a atividade antibacteriana e composição química de uma amostra de própolis vermelha brasileira sendo determinado uma importante variação nas concentrações dos compostos investigados, e, consequentemente na atividade antibacteriana dos extratos, em relação ao período de coleta das amostras (estação seca e chuvosa).

É importante destacar que vários métodos são empregados em todo o mundo para extração dos componentes presentes em própolis, e conforme anteriormente mencionado, a extração por infusão utilizando etanol como solvente é usualmente o método mais empregado (Cao et al., 2017). Diferentes estudos descrevem composições químicas e atividades biológicas distintas para extratos de própolis a depender do

método de extração empregado, demonstrando que as condições de extração, bem como, o solvente extrator utilizado, influenciam diretamente no rendimento e na seletividade de alguns compostos (Biscaia & Ferreira, 2009; Chimie et al., 2016; Cottica et al., 2015; Jug et al., 2014; Machado et al., 2016; Song et al., 2021; Taddeo et al., 2016; Trusheva et al., 2007; Yen et al., 2017), e consequentemente, no potencial biotecnológico dos extratos obtidos. Dessa forma, apesar de a extração etanólica ser o método mais usualmente empregado pela indústria para obtenção de extratos de própolis de diferentes tipos, esse método apresenta como desvantagens: a baixa seletividade e baixo rendimento na extração de alguns compostos de interesse e longos períodos de extração (Cottica et al., 2015). Assim, outros métodos têm sido usados para aumentar a eficiência da extração de componentes bioativos de interesse de amostras de própolis, como a extração assistida por ultrassom e por micro-ondas e extração com fluidos supercríticos (Cao et al., 2017; Catchpole et al., 2004; Paviani et al., 2012; Pellati et al., 2013).

Neste âmbito, a extração assistida por ultrassom representa uma alternativa confiável às técnicas tradicionais de extração e tem sido amplamente aplicada na extração de compostos a partir de diferentes matrizes naturais (Briones-Labarca et al., 2015; Sallet et al., 2019; Stanisavljević et al., 2009; Zhong et al., 2018). No estudo realizado por Tan et al., (2018) foi demonstrado uma maior eficiência de extração do óleo de abacate utilizando a extração assistida por ultrassom e com fluido supercrítico quando comparado aos métodos convencionais empregados. De Figueiredo et al., (2018) demonstraram a maior eficiência da extração assistida por ultrassom para obtenção de fito esteróis em óleos vegetais.

É importante destacar que apesar das vantagens apresentadas pela tecnologia de ultrassom para obtenção de compostos de interesse em menor tempo, maior rendimento e menor consumo de solvente quando comparado aos métodos de extração convencionais, poucos são os estudos que investigaram a obtenção de extratos de própolis utilizando essa tecnologia (Andrade et al., 2017, 2018; Sadhana et al., 2017). Dessa forma, este estudo teve como um dos objetivos avaliar comparativamente o perfil antioxidante e atividade citotóxica *in vitro* de extratos obtidos por extração convencional e assistida por ultrassom de seis amostras de própolis vermelha coletadas em diferentes

regiões do nordeste do Brasil para obtenção de extratos com alto potencial biotecnológico.

Ainda dentro do contexto de métodos modernos utilizados em processos extrativos, a extração com fluidos supercríticos destaca-se como um dos mais promissores, especificamente com o uso de dióxido de carbono (CO_2) como o fluido extrator (Machado et al., 2013). Esta tecnologia tem se mostrado eficaz para aplicações em processos químicos, petroquímicos, farmacêuticos, ambientais e alimentícios devido principalmente por ser considerada uma tecnologia limpa (Catchpole et al., 2004; Devequi-Nunes et al., 2018; Paviani et al., 2012). A SFE é capaz de manter as propriedades antioxidantes dos extratos obtidos devido à utilização de baixas temperaturas, e esta característica é de extrema importância para as indústrias farmacêuticas e de alimentos (De Zordi et al., 2014; Machado et al., 2016).

Fluidos supercríticos apresentam viscosidade baixa como a de um gás, alta densidade como os líquidos e difusão intermediária entre gases e líquidos, variando com a sua densidade. Um solvente para a indústria de alimentos deve ter alto coeficiente de distribuição, alta seletividade para o soluto de interesse, ser atóxico, estável e inerte, não ser miscível com o alimento, não ser inflamável e ser ambientalmente seguro (GRAS = *Generally recognized as safe*) e barato. O dióxido de carbono é o solvente mais utilizado como fluido supercrítico devido as suas características de baixo custo, facilmente disponível em alta pureza, não ser tóxico, não inflamável e não explosivo, e ainda, considerado como ideal para a extração de materiais lipofílicos. Outra vantagem apresentada é que o dióxido de carbono é um gás à temperatura e pressão ambiente, sendo, portanto, fácil de ser removido após o processo de extração (Herrero et al., 2010). A solubilidade e a seletividade dos compostos de interesse podem ser aumentadas adicionando pequenas quantidades de outros solventes (co-solventes) como etanol, por exemplo (Devequi-Nunes et al., 2018; Di Capua et al., 2018).

Vários estudos têm demonstrado as diversas vantagens de usar fluidos supercríticos, particularmente CO_2, para extrair diferentes tipos de substâncias em uma larga variedade de matrizes (Herrero et al., 2010; Kakehashi et al., 2016; Tan et al., 2018). Adicionalmente, a escolha das condições operacionais no processo de extração para cada

matriz deve ser considerada tendo em vista que o uso dos valores otimizados para as diferentes condições (temperatura, pressão, tempo, tipo e percentual de modificadores ou co-solventes, tamanho da partícula amostra, entre outras) pode melhorar significativamente o rendimento e a recuperação de um composto alvo. Neste sentido, trabalhos têm sido publicados destacando os parâmetros experimentais, avaliação da viabilidade econômica e industrial de alguns processos desenvolvidos com a utilização da SFE (Alvarez-Henao et al., 2022; Dauber et al., 2021; Fachri et al., 2020; Jingfu et al., 2022; Monton et al., 2022).

A partir da pesquisa realizada, nenhum trabalho foi identificado na literatura técnica e/ou científica que relata o processo de definição de parâmetros para a obtenção de compostos de interesse da própolis vermelha utilizando a tecnologia de SFE. Os poucos trabalhos identificados relatam o estudo de diferentes condições de processo para a obtenção apenas de extratos de própolis verde (Biscaia & Ferreira, 2009; Fachri et al., 2020; Machado et al., 2015). É importante destacar que a própolis verde possui composição e características físico-químicas bem distintas da própolis vermelha (Hirata et al., 2021; Touzani et al., 2021), e por isso, um estudo que defina as condições ideais para a obtenção de extratos de própolis vermelha por SFE é de grande importância para a comunidade científica e industrial, tendo em vista principalmente as vantagens apresentadas por essa técnica, bem como, pelo diferencial químico e potencial biológico da própolis vermelha, considerada atualmente como o novo tipo de própolis para os mercados farmacêutico, cosmético e alimentício.

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Capítulo 2. Objetivos

3.0 Objetivos

3.1 Objetivo Geral

O objetivo geral desse estudo foi avaliar a influência de diferentes métodos de extração na composição química e biológica de extratos de própolis vermelha de diferentes origens geográficas do Nordeste do Brasil, bem como determinar as condições de processo para a obtenção de extratos de própolis vermelha utilizando a tecnologia de extração com fluído supercrítico (SFE).

3.2 Objetivos Específicos

a) Obter extratos a partir de seis amostras de própolis vermelha de diferentes origens geográficas do nordeste do Brasil (Bahia, Alagoas, Rio Grande do Norte e Sergipe) por extração convencional (extração etanólica) e extração assistida por ultrassom;
b) Caracterizar e avaliar comparativamente os 12 extratos pelos dois métodos de extração, considerando o total de compostos fenólicos e flavonoides, atividade antioxidante *in vitro*, concentração de dois marcadores (formononetin e kaempferol) e a citotoxidade para linhagens de células tumorais humanas (HCT116 – cólon humano, HL60 – leucemia, PC3 – carcinoma de próstata e SNB19 – glioblastoma);
c) Investigar as melhores condições de processo de extração para obtenção de compostos de interesse da própolis vermelha aplicando a SFE e utilizando CO_2 como fluido extrator;
d) Determinar a Curva Global de Extração (OEC – *Overall Extraction Curves*), S/F (massa de CO_2/massa de própolis), percentual de co-solvente (etanol) e isotermas de rendimento global (GYI – *Global Yield Isotherms*) em função das diferentes pressões e temperaturas;

e) Caracterizar os extratos obtidos nas diferentes condições em relação ao teor de compostos fenólicos totais, flavonoides totais, atividade antioxidante *in vitro* e quantificação de biomarcadores de interesse (formononetin, naringenin e kaempferol).

Capítulo 3. Revisão de Literatura

4.0 Extraction of propolis using supercritical carbon dioxide

Capítulo de livro (review) publicado em ***Green Sustainable Processes for Chemical and Environmental Engineering and Science*** – Supercritical Carbon Dioxide as Green Solvent, 1st Edition 2020.

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CHAPTER 9

Extraction of propolis using supercritical carbon dioxide

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Contents

1. Introduction

Propolis is a resinous substance collected by bees from the shoots and leaves of different trees and plants that is mixed with pollen and enzymes secreted by the bees [1–3]. Propolis has varied appearance and, categorized as yellow, green, brown, or red. The color and characteristics of propolis vary according to the plant source, collection season, seasonality, and geographical origin [4, 5]. It is considered a traditional folk remedy and has been the object of several pharmacological and chemical studies in recent years [6–8].

This matrix is widely used in cosmetic formulations, foodstuffs and pharmaceuticals, and thus is considered as one of the most useful products obtained from natural sources [9, 10]. Many compounds were identified in different samples of propolis [11–14], and new compounds are still being identified during the chemical characterization of the new samples. Phenolic compounds, particularly flavonoids, have been extensively related to various properties of this natural product [5]. Many studies on the biological properties of propolis and the differences in propolis compositions have been published [10, 15, 16].

New extraction methods are being investigated to replace the classic methods, such as solvent extraction, maceration, and vacuum distillation, for obtaining extracts from natural products [17]. One of the most promising methods is supercritical fluid extraction (SFE), specifically the method that uses carbon dioxide (CO_2) as the extraction fluid ($scCO_2$) [18–21]. This technology has proven effective for use in different processes,

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including chemical, petrochemical, pharmaceutical, environmental, and food processes, mainly because it is considered a clean technology. Particularly, $scCO_2$ has a lower critical temperature and pressure (31°C and 74 bar) than other supercritical solvents, which favors the extraction of thermosensitive compounds [18]. In addition, CO_2 is considered nonexplosive and nontoxic, is available in high purity, fully recoverable by the process, and has a low cost [22–24]. Another advantage of SFE is the ability to preserve the chemical properties (including antioxidant capacity) of the extracts obtained due to the use of low temperatures [17, 25–27].

It is important to highlight that one of the main aspects that should be considered in the SFE is the choice of operating conditions in the extraction process. Thus, for each sample the process parameters must be optimized with the objective of increasing the extraction of the compounds of interest. Aiming to effectively optimize the conditions that affect the process (temperature, pressure, time, types and percentages of modifiers or cosolvents, and sample particle size, among others), different approaches have been applied in studies on SFE [17, 21, 25]. Thus, the first step of SFE from natural matrices, such as propolis, is to optimize the temperature and pressure conditions to maximize the yield of the compounds of interest and to minimize the coextraction of undesirable compounds [28–32]. In addition, it is possible to extend and modify the selectivity and solubility of the compounds in $scCO_2$ through the addition of polar species as cosolvents. Thus, the extraction yields of propolis components can be increased due to the polar nature of phenolic compounds.

It should be noted that propolis extracts have been preferably obtained using conventional methods, and using low pressures, such as ethanol extraction, hydroalcoholic extraction, or oil extraction [13, 33, 34]. However, the application of SFE using supercritical CO_2 combined with a cosolvent for the extraction of propolis compounds is quite feasible, as already demonstrated in different studies. In this chapter, the supercritical extraction method for obtaining propolis extracts using $scCO_2$ and the biotechnological potential of this natural matrix is described. A survey and evaluation of the main scientific papers and patent documents involving the technology of interest are also presented.

2. Propolis: Geographical origin and biological properties

Propolis has been extensively studied since 1960s, with numerous studies revealing the potential of this matrix due mainly to the presence of several chemical compounds with proven biological activities [3, 35, 36]. The studies on propolis involve different lines of research. These studies mainly involve the chemical characterization and biological activity of the extracts; standardization and comparative analysis of the extracts obtained under different extraction conditions; evaluation of the composition of the extracts relative to the geographical origin, plant source, and season; and application of the extracts for product development.

More than 300 compounds (polyphenols, terpenoids, steroids, sugars, amino acids, and others) have been detected in raw propolis from different sources. The presence and concentration of these compounds are influenced by botanical and geographical factors, as well as by the sampling season [37–39]. Thus, the composition of the propolis varies according to the geographical origin that is influenced by the plant species that inhabit the region. As already observed during some studies, the chemical composition of propolis extracts, and consequently their biological activity, also varies according to the extraction methods employed [15, 33, 40].

The propolis varieties produced in different regions of the world have been categorized according to their botanical origin and predominant chemical composition. The poplar type (*Populus* spp.) originates mainly from Europe, the nontropical regions of Asia, New Zealand, and North America and is rich in esters of caffeic acid [41]. The birch type (*Betula verrucosa*) is rich in flavonols and flavones [34] and found mainly in Russia.

The green type, also called Brazilian propolis, originates from the plant species *Baccharis dracunculifolia* [42, 43]. An important phenolic acid present in samples of Brazilian green propolis that has already been reported in different studies is Artepillin C (3,5-diprenyl-4-hydroxycinnamic acid [4, 44–46]. Chen et al. [47] and Machado et al. [30] investigated the use of different organic solvents and supercritical CO_2 in the extraction processes to recover Artepillin C from Brazilian propolis. Some studies have pointed out important biological activities associated with Artepillin C, mainly its antitumor effects [48–51]. The inhibitory effects of Artepillin C have been reported on a variety of human and murine malignant solid tumor cell lines [52–54]. Thus, green propolis is currently the most valued worldwide and has been extensively studied in recent years due to its chemical composition that is rich in different compounds, such as diterpenes, lignans, and prenylated derivatives of p-coumaric acid, as well as different flavonoids.

Another important type of propolis that has attracted the attention of researchers around the world is the red propolis, which is derived from the plant species *Dalbergia ecastaphyllum* [38, 55, 56]. The main chemical components identified in samples of red propolis have been isoflavones, flavones, flavonoids, phenolic compounds, aurones, chalcones, pterocarpans, and xanthones [57, 58]. The biological activity of red propolis is mainly due to isoflavones, formononetin and kaempferol, which act in synergy with the other compounds. Formononetin is considered one of the biomarkers of red propolis, and several studies have confirmed its presence in extracts obtained from this matrix [38, 59], and kaempferol has a wide application in traditional Chinese medicine due to its antioxidant and free-radical scavenging properties. Red propolis is produced in Brazil, Mexico, and Cuba [55, 60].

Clusia propolis (*Clusia* spp.) is found in Cuba and Venezuela. It contains polyisoprenylated benzophenone [61]; Pacific propolis (*Macaranga tanarius*) originates in Indonesia, Taiwan, and Okinawa [62]; and the most recently identified Mediterranean propolis has

its main source plant species of the family as *Cupressaceae*, located in Greece, Sicily, and Malta, and is generally rich in diterpenes [63]. Brazil and China are the main propolis producing markets in the world. However, the propolis produced in these countries differs considerably due to the variety and diversity of the botanical sources. For the application of $scCO_2$ to obtain propolis extracts, Brazilian propolis, specifically the green type, has been the most investigated.

Several experimental and clinical investigations have shown the biological properties of propolis extracts obtained through classical processes (such as liquid-liquid extraction) and alternative processes (such as ultrasound and SFE). Due to the variety of chemical compounds, especially phenolic compounds, many biological activities such as antioxidant [37], antibacterial [60], fungicidal [64, 65], antiparasitic [40, 57], anticancer [66], antiviral [67], anticarious [68, 69], antiinflammatory [70], healing [6], and immunomodulatory [71] activities have been reported in recent years for propolis extracts of different types and origins. In addition to biological research, propolis extracts have been used as active ingredients for the development of food products, active packaging [72], new drugs [73], and vaccine adjuvant formulations [71, 74], among other uses.

Raw propolis cannot be used as a raw material and therefore must be purified. The process should remove the waxy material and preserve the polyphenol fraction. Thus, the industrial extraction method commonly used to obtain biocompounds from propolis is infusion, where the sample is submerged in an extraction solvent for days, weeks, or months, thus requiring a substantial amount of time on an industrial scale and still resulting in a low yield [56, 63, 75].

Due to the chemical characteristics of propolis, ethanol is currently the most used extracting solvent to obtain propolis extracts. Ethanol has interesting chemical characteristics, such as suitable polarity, to obtain the target compounds present in propolis, mainly flavonoids [28, 29]. Different studies indicate that propolis extracts can be obtained using classical or alternative methods, such as the application of ultrasound technology [76] or SFE [40, 47, 77].

3. SFE using CO_2

SFE has very interesting attributes, mainly due to its high flexibility. It is possible to obtain products of excellent quality, principally when compared to classical techniques. Other advantages of SFE are the low use of polluting organic solvents, as well as the complete elimination of the solvent at the end of the process.

Several solvents, such as water [78], ammonia [79], benzene [80], ethanol [81], methanol [82], and toluene [83] have been used in SFE. It is emphasized that, any solvent can be used as a supercritical solvent. However, the temperature and pressure conditions to reach the critical properties, as well as their toxicity, price and their solvation are the main

characteristics that define the solvent most suitable for application in SFE. Due to the reasons previously discussed, CO_2 is the first-choice solvent and the solvent most used in SFE in different processes.

$scCO_2$ is considered a green technology for the extraction of biologically active compounds from different natural matrices [84]. This technique is very efficient in terms of selectivity and separation; however, depending on the polarity of the compounds of interest, it may be necessary to add small amounts of a modifier or cosolvent to the system to improve the extraction yield [37].

As evidenced in different studies, flavonoids are the main compounds present in propolis and are associated with the biological effects of this matrix. Among the classes of flavonoids found in propolis, the most important are: flavones, flavonols, flavanones, flavanonols, chalcones and dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavans, and neoflavonoids [34, 85].

Most of these compounds present in propolis samples are not extractable in pure CO_2, due to the chemical characteristics. However, the extraction can be improved when a mixture of CO_2 with ethanol or ethanol and water is used, allowing its separation, and consequently the extraction, based on the molecular weight of each compound and polarity [28, 30, 77].

Given the great relevance to $scCO_2$ extraction as an alternative extraction process, which mainly aims to replace the use of organic solvents in the extraction of materials at the scientific and industrial scales, and considering the important and promising biological effects of propolis, some studies have been published in the last decades on the application of this technology to obtain propolis extracts and evaluate their biological effects. All the 29 scientific articles identified on the use of SFE as extraction method to obtain propolis extracts performed that CO_2 was used as the extraction fluid, with or without different cosolvents.

The identified studies focus mainly on the determination of the ideal operating conditions for the extraction of propolis extracts rich in specific components [29, 86], with or without other techniques [87], on the biological evaluation of the extracts obtained by $scCO_2$ [88–90], and on comparative analyses of the extracts obtained by $scCO_2$ with those obtained by conventional methods [28, 40, 90]. Table 1 presents the main studies on the use of $scCO_2$ as an extraction medium for obtaining propolis extracts of different types from geographical origins.

One of the first studies describing the application of $scCO_2$ for obtaining propolis extracts was by Stahl et al. [96]. The cleaning of raw propolis to remove the wax and obtain extracts rich in flavonoids was performed using $scCO_2$ at 600 bar and a temperature of 39.8°C. Later, Kimoto et al. [97] carried out a study on a commercial propolis extract (Japan) obtained by $scCO_2$ and identified its potent effect against mammary carcinogenesis in rats.

Table 1 Main studies performed using $scCO_2$ to obtain propolis extracts of different types and geographical origins

Sample type and source	Compounds of interest	Parameters used	References
New Zealand—Propolis tincture	Flavonoids and cinnamic acid derivatives	CO_2, ethanol as cosolvent, 300 bar, 60°C	Catchpole et al. [91]
Italy	Flavonoids	CO_2, 82.3–317.7 bar, 31.6–41.4°C, 30 min	Zordi et al. [29]
Southeast Brazil—green propolis	Artepillin C, p-coumaric acid and kaempferide	CO_2, ethanol + water as cosolvent, 250 bar, 50°C	Monroy et al. [92]
Southern Brazil—green propolis	Phenolic compounds and flavonoids	CO_2, ethanol as cosolvent (2%, 5%, and 7%), 100–250 bar, 30°C, 40°C, and 50°C	Biscaia et al. [28]
Southern Brazil—green	Artepillin C and p-coumaric acid	CO_2, ethanol as cosolvent (1% and 2%), 250, 350 and 400 bar and 40°C and 50°C	Machado et al. [30]
Brazil—green, red, and brown	Phenolic compounds	CO_2, ethanol as cosolvent (1%), 350 bar and 50°C	Silva et al. [40]
Brazil—green	Cinnamic acid derivatives	CO_2 ethanol as cosolvent, 207 bar, 40°C, 50°C, and 60°C	Chen et al. [47]
Brazil—dry green propolis extract	Artepillin C, p-coumaric acid and kaempferide	CO_2, 150 and 350 bar, 60°C	Paviani et al. [93]
Brazil	Phenolic compounds	CO_2, 50, 100, 150, and 200 bar and 50°C	Wang et al. [94]
Brazil—green, red, and brown	Artepillin C, p-coumaric acid and kaempferide	CO_2, ethanol as cosolvent (1%), 350 bar and 50°C	Machado et al. [90]
Brazil—green	Artepillin C	CO_2, ethanol as cosolvent, 50 bar, 50°C	Lee et al. [32]
China	Flavonoids	CO_2, ethanol as cosolvent, 276–345 bar, 45°C	You et al. [95]

The influences of the different parameters on the extraction of $scCO_2$ from Italian propolis were studied by Zordi et al. [29], focusing on the extraction yields and chemical compositions of the fractions obtained. Thus, the authors showed that pressure and time have significant linear effects on the yield and chemical profile of the extract obtained

under the conditions employed. A comparative analysis with other methods (ultrasound-assisted extraction and conventional ethanol extraction) indicated that it is possible to use $scCO_2$ as a pretreatment of propolis and thus to enable more efficient extraction of the antioxidant compounds with ethanol. In addition, another option for $scCO_2$ would be to obtain compounds of interest in propolis with polar characteristics.

Some studies on the extraction of Artepillin C (the main compound of interest of green propolis) using organic solvents have been published [44, 71]; however, the selectivity demonstrated by SFE has motivated the study of the process parameters for obtaining and fractionating Artepillin C-rich extracts. Lee et al. [32] used $scCO_2$ modified with ethyl acetate to extract Artepillin C from Brazilian propolis and obtained a highly pure compound using $scCO_2$ extraction. A positive relationship between the purity of the Artepillin C in the extracts obtained by $scCO_2$ and the antioxidant capacity was also demonstrated.

According to Machado et al. [30], who specifically evaluated the process conditions for obtaining p-coumaric acid and Artepillin C from green propolis (Brazil), the presence of ethanol as a cosolvent (1%) increased the extraction capacity of these compounds on using $scCO_2$. In addition, high p-coumaric acid and Artepillin C contents were obtained at a pressure of 350 bar and temperature of 50°C. It was observed that extraction of these compounds was difficult at high temperatures and pressures, as a high degree of solubility required [98]. In the study, a cross of isotherms between 360 and 380 bar pressures was identified. In the crossover region, it was observed that the effect of temperature exerted greater influence on the increase of vapor pressure, in relation to the decrease of the density of the solvent. Paviani et al. [93] evaluated the selectivity of the process in the fractionation of dry green propolis extract to obtain some specific compounds, including Artepillin C. The supercritical process showed that the CO_2 selectivity was higher at lower pressures (150 bar) but with a lower extraction yield.

A comparative evaluation of extracts obtained by $scCO_2$ and by other conventional methods was performed. Green propolis extracts were obtained by different conventional methods. When applied to SFE, CO_2, ethanol, and water (and mixtures) were used as process solvents or as cosolvents of CO_2 [92]. In spite of the advantages of SFE, high overall yields were obtained with conventional ethanol extraction (80%) or with a two-step extraction procedure involving $scCO_2$ extraction followed by conventional ethanol extraction, resulting in higher phenolic and total flavonoid contents. The results indicated that ethanol was more selective to obtain compounds present in green propolis, such as Artepillin C and p-coumaric acid.

Biscaia et al. [28] investigated the yields of propolis extracts obtained under different conditions, SFE in one step, using only CO_2 or CO_2 with ethanol as cosolvent, and SFE in two steps, as well as other low-pressure extraction methods using different liquid organic solvents. The highest yield was obtained by Soxhlet extraction using chloroform, while for $scCO_2$, the maximum yield was obtained using 5% ethanol as a cosolvent. It has

already validated in the literature that the use of ethanol as a cosolvent in the process activated the extraction yield of propolis compounds of interest [31, 86, 93].

As previously discussed, the chemical composition of propolis depends on various factors including the botanical and geographical origins, as well as the sampling time; in addition, the extraction method used directly influences the compounds of interest to be obtained [13, 33, 45]. Thus, propolis extracts of different types and origins may have different biological effects. Thus, the problem of the standardization of the chemical composition and concentrations of the compounds limits the uses of propolis.

Machado et al. [90], evaluated samples of green, red, and brown varieties of propolis of different geographical origins and the extracts obtained by $scCO_2$ were compared with those obtained by liquid ethanol extraction at atmospheric pressure, it was observed that the concentrations of specific phenolic compounds (Artepillin C, p-coumaric acid, kaempferol, and formononetin) in the extracts obtained by $scCO_2$ were much higher. However, the higher amount of extracted components of interest, the extraction yield was lower, which may indicate a greater selectivity of the extraction process [18]. Similar results were obtained by Wang et al. [94] for propolis extracts obtained by $scCO_2$, and these extracts exhibited biological effects superior to those of extracts obtained with organic solvents.

Silva et al. [40] investigated the antioxidant, antimicrobial, antiparasitic, and cytotoxic activities of several Brazilian propolis extracts (green, red, and brown) obtained by $scCO_2$ (using ethanol as a cosolvent) and by conventional ethanol extraction. It was evidenced that the propolis extracts obtained by the two techniques studied showed high antioxidant activity that varied according to the concentration of the extracted compounds; however, the propolis extracts obtained by conventional ethanolic extraction showed highest antioxidant activities and biological activities. In the most recent study involving the application of SFE, Devequi-Nunes et al. [99] investigated propolis extracts (green, red and brown) obtained by $scCO_2$ and ethanol extraction. Extracts with high antioxidant capacity were obtained; however, the extracts obtained with $scCO_2$ showed higher IC_{50} values in relation to extracts obtained with conventional ethanol extraction (infusion), which showed lower antioxidant activity. It is noteworthy that Silva et al. [40] and Devequi-Nunes et al. [99] used process parameters adopted in previous studies [28, 30] (the same pressure conditions, temperature, and type and percentage of cosolvent) to obtain extracts by $scCO_2$, which may lead to lower process efficiencies since other types of samples were used.

To obtain extracts rich in specific components, some studies have evaluated the application of different concentrations and types of cosolvents in the system [28, 30, 100] or the application of CO_2 as an antisolvent [100–102]. For example, Monroy et al. [92] obtained extracts of green propolis rich in phenolic compounds using $scCO_2$ as antisolvent. In this process, a total of four separators were used in series to selectively fractionate the ethanolic and hydroalcoholic extracts of the green propolis by precipitation.

Catchpole et al. [91] developed a process using $scCO_2$ as an antisolvent for the fractionation of a propolis tincture. The proposed method was efficient in obtaining flavonoids as well as essential oil fractions and removing high-molecular-weight components by precipitation.

$scCO_2$ has also been used in some studies to obtain propolis microparticles for application in different pharmaceutical and food products. Wu et al. [101] and Yang et al. [103] used an antisolvent process to obtain micronization and encapsulation of propolis using polyethylene glycol as the encapsulating agent. More recently, Di-Capua et al. [87] successfully encapsulated propolis through $scCO_2$-assisted atomization. Two carriers were used in the process (hydroxypropyl-β-cyclodextrin and polyvinylpyrrolidone) with the aim of increasing the shelf life of the compounds extracted from propolis, and consequently improving their bioavailability.

In general, when evaluating only the total content of phenolic compounds and flavonoids (by spectrophotometric methods), the ethanolic extracts obtained by conventional techniques were more efficient than those obtained with SFE. However, the application of $scCO_2$ becomes extremely viable for the extraction of target compounds from propolis samples, as confirmed by the chromatographic analysis of the extracts. In this case, studies of the extraction process for determining the best conditions of pressure, temperature, and type and concentration of cosolvent, among other factors, should be carried out to maximize the extraction of the compound(s) of interest. Better biological activities of propolis extracts obtained by $scCO_2$ have also been demonstrated in some studies because of the greater concentrations of the active compounds of interest. Additional advantages include the reduced use of organic solvents and preservation of the bioactivity of some compounds due to the low temperature of the extraction.

4. Patents

A survey of patent documents was conducted at Espacenet (European Patent Office), which includes applications for patents filed in more than 90 countries worldwide. A total of 27 patent documents referring to the application of $scCO_2$ for obtaining specific propolis compounds as well as extracts or applications in food, cosmetic, or pharmaceutical products were identified.

The first patent document identified was from 1993 (JPH06276966), filed in Japan, and referred to a process of cleaning the waxy and lipophilic material from raw propolis using $scCO_2$, with a flow rate of 5 mL/min at 40°C and 250 bar for 6 h. The patent documents identified had their countries of origin as China (17), Japan (4), Taiwan (4), Korea (1), and Brazil (1).

All identified documents applying SFE in propolis used CO_2 as the extraction fluid. The use of a cosolvent was also reported in some documents, with the goal of the extraction and purification of target compounds from propolis. For example, propolis extracts

obtained by $scCO_2$ and ethanol as cosolvent of the process, improved the extraction efficiency of biologically active compounds present in the raw sample, including Artepillin C and p-coumaric acid.

5. Conclusion

From the results found during numerous studies involving SFE, extracts obtained by this technology showed a differentiated profile when compared to extracts obtained by classical techniques, such as liquid-liquid extraction. For propolis, the majority of the studies showed that extracts obtained by $scCO_2$ (using ethanol as cosolvent) have higher biological potential. In view of the different types and the diverse chemical composition of propolis, studying the process conditions to obtain compounds of interest is extremely feasible. For example, optimized parameters for the extraction of specific compounds present in green propolis may not be efficient for obtaining red propolis markers due to the differentiated characteristics of these compounds. Thus, $scCO_2$ proved to be very efficient in obtaining specific components of propolis samples of different types. As regard the application of $scCO_2$ to obtain propolis extracts, Brazilian propolis, specifically green propolis, has been the most investigated. Studies applying SFE with optimized conditions to obtain compounds from red propolis are necessary, considering the biotechnological potential of this new type of propolis.

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Capítulo 4. Artigo Experimental 1

5.0 Evaluation of the antioxidant profile and cytotoxic activity of red propolis extracts from different regions of northeastern Brazil obtained by conventional and ultrasound assisted extraction

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RESEARCH ARTICLE

Evaluation of the antioxidant profile and cytotoxic activity of red propolis extracts from different regions of northeastern Brazil obtained by conventional and ultrasound-assisted extraction

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Abstract

Propolis is a complex mixture of resinous and balsamic material collected from the exudates of plants, shoots, and leaves by bees. This study evaluated red propolis extracts obtained by conventional (ethanolic) extraction and ultrasound-assisted extraction of six samples from different regions of northeastern Brazil. The total phenolic compounds and flavonoids, *in vitro* antioxidant activity, concentration of formononetin and kaempferol and the cytotoxicity against four human tumor cell lines were determined for all twelve obtained extracts. Significant variations in the levels of the investigated compounds were identified in the red propolis extracts, confirming that the chemical composition varied according to the sampling region. The extraction method used also influenced the resulting propolis compounds. The highest concentration of the compounds of interest and the highest *in vitro* antioxidant activity were exhibited by the extracts obtained from samples from state of Alagoas. Formononetin and kaempferol were identified in all samples. The highest formononetin concentrations were identified in extracts obtained by ultrasound, thus indicating a greater selectivity for the extraction of this compound by this method. Regarding cytotoxic activity, for the HCT-116 line, all of the extracts showed an inhibition of greater than 90%, whereas for the HL-60 and PC3 lines, the minimum identified was 80%. In general, there was no significant difference ($p>0.05$) in the antiproliferative potential when comparing the extraction methods. The results showed that the composition of Brazilian red propolis varies significantly depending on the geographical origin and that the method used influences the resulting compounds that are present in propolis. However, regardless of the geographical origin and the extraction method used, all the red propolis samples studied presented great biological potential and high antioxidant activity. Furthermore, the ultrasound-assisted method can be efficiently

applied to obtain extracts of red propolis more quickly and with high concentration of biomarkers of interest.

Introduction

Propolis is a complex mixture formed by resinous and balsamic material originating from various parts of plants, such as shoots, exudates, branches, and leaves, that is collected by different bee species [1–3]. It is used by bees to protect the beehive against insects and to prevent the proliferation of invading microorganisms, thus functioning as a protective barrier [4–6]. In general, propolis is composed of around 50% resins and plant balsams, 30% wax, 10% essential oils, 5% pollen and 5% of other substances and materials, including organic compounds [7–9]. More than 300 chemical compounds of interest have already been identified in propolis samples of different geographical origins, [10,11] and the major constituents of propolis are phenolic compounds, which have been extensively studied to date as antioxidants present in natural products [12–14]. Therefore, different kinds of propolis are present all over the world and each propolis is chemically different and has specific properties and applications [15,16].

Brazilian red propolis is primarily found in the coastal region of northeastern Brazil, and its chemical composition is highly variable and directly related to the compounds found in its main botanical origin, *Dalbergia ecastaphyllum* (L) Taub. [2,17–21]; however, a second plant species likely participates as one of the main sources of resins for red propolis [11]. Presently, it is the second most produced and traded type of Brazilian propolis, being produced mainly on the littoral of the state of Alagoas (northeast Brazil) [22]. The biological activity of red propolis is mainly due to isoflavones, which act in synergy with the other compounds. Formononetin is the main isoflavone present in red propolis samples [23,24]. Other compounds identified in the fractions and extracts from Brazilian red propolis, such as vestitol, neovestitol, biochanin A and liquiritigenina, are also considered important markers and have been associated with different biological effects [25,26].

Different studies have demonstrated a wide variety of biological activities for red propolis extracts, such as antioxidant [2,19], antimicrobial [27,28], antitumor [1,29,30], anti-inflammatory [31,32], antiparasitic [33–35], and anti-nociceptive activities [32]. Red propolis is currently recognized as the most promising type of propolis because of its biotechnological potential. The phenolic compounds, including the flavonoids, have been considered the main biologically active constituents of this resin, together with the cinnamic acid derivatives, esters, and some terpenes [3,25].

The chemical composition of propolis, and consequently its biological activity, varies according to its geographical origin, botanical source, race of bees, sampling season and climate conditions of the region [36–40]. As a result, different studies have investigated the influence of different factors on the chemical composition of propolis [41–46]. However, few available studies have compared red propolis samples collected in different regions of Brazil regarding their antioxidant composition and cytotoxic activity against different tumor cell lines. Studies evaluating the biological activities of red propolis performed by Machado et al. [27], Silva et al. [1], and Teles et al. [47] found differences in antimicrobial and antitumor capacity, antioxidant and antiparasitic capacity, and hypertension and renal damage attenuation capacity, respectively, for samples obtained from different sources. Regueira-Neto et al. [28] investigated the effect of seasonality on the antibacterial activity and chemical composition of a Brazilian red propolis sample and found an important variation in the concentrations

of the investigated compounds and, consequently, in the antibacterial activity of the extracts according to the sampling period (dry vs. rainy season).

Several methods are used worldwide to extract the propolis components, and extraction using ethanol as a solvent is the most commonly used method [48]. Ethanolic extracts have been more commonly used due to of their content in phenolic acids and flavonoids [49]. Different studies describe different chemical compositions and biological activities for propolis extracts depending on the extraction method employed, demonstrating that the extraction conditions, as well as the extraction solvent used, directly influence the yield and selectivity of some compounds [50–55] and, consequently, the biotechnological potential of the extracts obtained. Thus, although ethanol extraction is the method most commonly used by the industry to obtain different types of propolis extracts, this method has the disadvantages of low selectivity and low yield in the extraction of some compounds of interest in addition to long extraction periods [51], thus increasing the extraction costs. Therefore, other methods have been used to increase the efficiency of the extraction of the bioactive components of propolis, such as ultrasound and microwave-assisted extraction and supercritical fluid extraction [48,56–58].

In this context, ultrasound-assisted extraction represents a reliable alternative to traditional extraction methods and has been widely applied in the extraction of compounds from different natural matrices [59–62]. The study by Tan et al. [63] demonstrated greater avocado oil extraction efficiency with ultrasound-assisted extraction and supercritical fluid extraction when compared to conventional methods. Figueiredo et al. [64] demonstrated the higher efficiency of ultrasound-assisted extraction to obtain phytosterols in vegetable oils.

Despite the advantages of ultrasound technology to obtain compounds of interest in a shorter time with higher yields and lower solvent consumption when compared to the conventional extraction methods, few studies have investigated the extraction of propolis extracts using this technology [65–67]. Thus, the aim of this study was to evaluate the antioxidant profile and *in vitro* cytotoxic activity of extracts obtained by conventional extraction and ultrasound-assisted extraction of six red propolis samples collected in different regions of northeastern Brazil.

Materials and methods

Materials

Ethanol, methanol, acetic acid, aluminum chloride, DMSO (dimethyl sulfoxide), Folin-Ciocalteu reagent and the standards kaempferol (CAS number 520-18-3), rutin hydrate (CAS Number 207671-50-9), formononetin (CAS number 485-72-3), gallic acid (CAS number 149-91-7), quercetin (CAS number 117-39-5), p-coumaric acid (CAS number 501-98-4), epicatechin (CAS number 490-46-0), caffeic acid (CAS number 331-39-5), catechin (CAS number 7295-85-4), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAS number 1898-66-4), and trans-ferulic acid (CAS number 537-98-4) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A 0.45-μm regenerated cellulose membrane filter (SLCR025NS, Millipore Corporation Co., Bedford, Mass., USA) was used.

Obtaining and processing raw red propolis from northeastern Brazil

Approximately 800 g of each red propolis sample were obtained from six different apiaries located in northeastern Brazil (Fig 1), more specifically in the states of Alagoas (samples A and B), Bahia (samples C and D), Rio Grande do Norte (sample E) and Sergipe (sample F), as shown in Table 1. The different samples were donated by the companies Apis Jordans (Vitória da Conquista—Bahia—Brazil), Apis Nativa Produtos Naturais (Prodapys—Santa Catarina—

Fig 1. Approximate geographical location of the samples of the red propolis evaluated (A and B—Alagoas; C and D—Bahia; E—Rio Grande do Norte; and, F—Sergipe).

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Brazil) and Bee Product Natural (Alagoas—Brazil). The samples were ground in an electric mill (Cadence—Brazil) and sieved through a 52–92 μm aluminum sieve for uniformity of particle size and to increase the surface area. The samples were stored in an ultra-freezer at -20˚C and were protected from light in an inert atmosphere (N_2) to avoid degradation of the material.

Obtaining the extracts

The extracts from the six red propolis samples were obtained by two methods: conventional extraction (A1, B1, C1, D1, E1, and F1) and ultrasound-assisted extraction (A2, B2, C2, D2, E2, and F2), totaling 12 extracts (Table 1).

For the ultrasound-assisted extraction, the methodology of Chen et al. [68] was used with modifications. Thus, 2 g of each propolis sample was homogenized with 25 mL of ethanol: water (80:20 v/v) in an Erlenmeyer flask and placed in an ultrasonic bath (RMS, Quimis,

Table 1. Identification, extraction method used and geographic location of red propolis samples from different regions of northeastern Brazil.

Sample identification	State of Brazil	Extraction method	Geographic location
A1	Alagoas	Conventional	9°41'59.7"S, 36°20'10.7"W
A2	Alagoas	Ultrasound-assisted	9°41'59.7"S, 36°20'10.7"W
B1	Alagoas	Conventional	9°46'10.9"S 35°50'52.4"W
B2	Alagoas	Ultrasound-assisted	9°46'10.9"S 35°50'52.4"W
C1	Bahia	Conventional	12°53'13.9"S, 40°56'38.5"W
C2	Bahia	Ultrasound-assisted	12°53'13.9"S, 40°56'38.5"W
D1	Bahia	Conventional	15°40'24.4"S 38°56'42.8"W
D2	Bahia	Ultrasound-assisted	15°40'24.4"S 38°56'42.8"W
E1	Rio Grande do Norte	Conventional	5°39'52.8"S, 36°15'35.8"W
E2	Rio Grande do Norte	Ultrasound-assisted	5°39'52.8"S, 36°15'35.8"W
F1	Sergipe	Conventional	10°28'10.5"S, 37°17'47.3"W
F2	Sergipe	Ultrasound-assisted	10°28'10.5"S, 37°17'47.3"W

In this study we used six samples of red propolis collected in different regions of Brazil and two extraction methods were used to obtain the extracts, totaling 12 extracts. Number 1 after the letter means the conventional method of extraction and the number 2 after the letter means the method with the application of the ultrasound. A1, B1, C1, D1, E1 and F1—Extracts obtained by conventional extraction; A2, B2, C2, D2, E2 and F2—Extracts obtained by ultrasound-assisted extraction.

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Brazil) with a power of 200 W, frequency of 60 kHz, and a temperature of 50˚C for 50 minutes. Conventional extraction was carried out in a similar way to the industrial process used for propolis extracts in Brazil. The same amount of each red propolis sample (2 g) was infused with ethanol:water (80:20 v/v) and allowed to stand for seven days with periodic shaking (25˚C). During the extraction process (conventional or ultrasound-assisted) all samples were kept protected from light.

Next, the extracts obtained by the two methods were centrifuged in a refrigerated centrifuge (Routine 380R, Hettich, Germany) at 20,000 RPM for 10 minutes at 4˚C, and the resulting supernatant was filtered on qualitative filter paper (80 g). Finally, the extracts were dried at 40˚C in a forced-air oven (Thermo Scientific, Massachusetts, USA) until reaching constant weight.

Identification and quantification of compounds by HPLC

The quantification and identification of ten phenolic compounds (caffeic acid, gallic acid, formononetin, kaempferol, trans-ferulic acid, *p*-coumaric acid, catechin, epicatechin, quercetin, and rutin hydrate) in the red propolis extracts was performed by high-performance liquid chromatography (HPLC). Initially, solutions of 10 $mg.mL^{-1}$ were prepared and dissolved in methanol and then placed in an ultrasonic bath (TECNAL, São Paulo, Brazil) for 30 minutes. Methanol solutions of the red propolis extracts were prepared at 1 $mg.mL^{-1}$ with the two methods adopted in this study. The samples were filtered with a 0.45 μm cellulose membrane filter (Millipore) for subsequent injection into an HPLC system (Shimadzu, LC-20AT, Japan) equipped with an automatic injector and diode array detector (DAD) (Shimadzu, SPD-M20, Japan). The chromatographic separation was performed according to the methodology proposed by Castro et al. [69] and Cabral et al. [70]. A NUCLEODUR 100–5 C18 EC column (150 x 4 mm ID, 5-μm particle size) was used in conjunction with a ZORBAX Eclipse Plus C18 4.6 x 12.5 mm precolumn (Agilent, USA).

Gradient elution, with a mobile phase of 5% acetic acid and methanol at different ratios and with a total analysis time of 42 minutes (from 0 to 35 minutes (0–92% B), 35 to 40 minutes (92–0% B), and 40 to 42 minutes (0% B)) was used as the analysis condition. The injection

Table 2. HPLC identification and quantification parameters of phenolic compounds from six red propolis samples obtained by conventional and ultrasound-assisted extraction.

Standards	t_R (min)	Λ (nm)	Working range ($mg.L^{-1}$)	LD ($mg.L^{-1}$)	LQ ($mg.L^{-1}$)
Gallic acid	2.26	280	1.0–12.5	0.92	3.05
Caffeic acid	8.13	30	1.0–15.0	0.82	2.73
Trans-ferulic acid	11.38	320	0.5–12.5	0.28	0.92
p-Coumaric acid	10.36	300	1.0–15.0	0.82	2.72
Catechin	6.42	280	1.0–15.0	0.81	2.68
Epicatechin	8.44	280	0.5–15.0	0.28	0.93
Formononetin	19.46	300	0.5–12.5	0.31	1.02
Kaempferol	17.53	320	0.5–12.5	0.12	0.41
Quercetin	15.30	320	0.5–12.5	0.21	0.71
Rutin hydrate	11.00	320	0.5–12.5	0.27	0.91

TR = retention time, Λ = wavelength, LD = limit of detection and LQ = limit of quantification of the ten phenolic compounds investigated in the samples studied by HPLC.

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volume was 20 μL, and the flow rate was 1 $mL.min^{-1}$. The machine was operated at a temperature of 25±2˚C. The detection wavelengths were set at 300 and 320 nm, and the DAD was operated within a wavelength range of 190 to 800 nm. For the identification of the compounds, comparisons of retention time and ultraviolet spectrum were performed between samples and standards. This analysis was performed according to the parameters of limits of detection, and limits of quantification [71,72] (Table 2).

Content of total phenolic compounds by spectrophotometry

The content of total phenolic compounds in the red propolis extracts obtained by the two extraction methods was determined using the methodology of Singleton et al. [73] and Singleton et al. [74], which are based on the reaction with the Folin-Ciocalteau reagent. First, the reaction was prepared with a 0.5-mL aliquot of each propolis extract dissolved in 95% ethanol to a final concentration of 500 $\mu g.mL^{-1}$, 10% aqueous Folin-Ciocalteu solution (2.5 mL) and 7.5% sodium carbonate (2.0 mL). The vials containing the obtained mixture were heated in a temperature-controlled bath at 50˚C for 5 minutes (Marconi, M127, Brazil), after which the absorbance was read in a UV/Vis spectrophotometer (PerkinElmer, LAMBDA 25 UV/Vis Systems, WA, USA) at 765 nm using a quartz cuvette with a 10 mm optical path and a 3.5 mL volume. The amount of total phenolic compounds was expressed as Gallic acid equivalents per gram of sample ($mgGAE.g^{-1}$) by calculating a calibration curve ($y = 0.0096x–0.0311$, $R^2 = 0.9994$) using Gallic acid standard solutions (12 to 200 $\mu g.mL^{-1}$) under the same conditions.

Content of total flavonoid compounds by spectrophotometry

The content of total flavonoid compounds was determined using the method proposed by Meda et al. [75] with adaptations. First, 2.0 mL of each extract (0.5 $mg.mL^{-1}$) was added into test tubes along with 2.0 mL of a 2% methanol solution of aluminum chloride ($AlCl_3$). The samples were then homogenized on a vortex shaker (IKA Lab Dancer, Germany) and placed in the dark for 30 minutes, after which the absorbance was read in a UV/Vis spectrophotometer (PerkinElmer, LAMBDA 25 UV/Vis System) at the wavelength of 415 nm. A quercetin standard curve (5 to 105 $\mu g.mL^{-1}$) was obtained under the same conditions ($y = 0.0271x–0.014$, $R^2 = 0.9994$), and the amount of total flavonoids in the extracts was expressed as quercetin equivalents per gram of sample ($mgQE.g^{-1}$).

DPPH (2,2-diphenyl-1-picrylhydrazine): *In vitro* antioxidant activity

To determine antioxidant capacity, the 2,2-diphenyl-1-picrylhydrazine reactive (DPPH) method was used according to the methodologies proposed by Brand-Williams et al. [76] and Molyneux et al. [77] with adaptations. First, six dilutions of each extract were prepared at concentrations of 10 to 85 $\mu g.mL^{-1}$ (in triplicate). Next, a 1-mL aliquot of each dilution was transferred to test tubes containing 3.0 mL of ethanol solution (99%) of the DPPH• radical (0.004%). The DPPH free radical reduction was determined by reading the absorbance at a wavelength of 517 nm (calibration curve y = 0.897x–4.5, R2 = 0.9955) with a UV/Vis spectrophotometer (PerkinElmer, LAMBDA 25 UV/Vis System) after 30 minutes of incubation in the dark at 25˚C.

The free radical scavenging capacity was expressed as the percentage inhibition of the radical oxidation and calculated according to Eq 1. A similar procedure was used for the blank, where the extract sample was replaced with ethanol. The EC_{50} value (effective concentration of extract required to scavenge DPPH• radical by 50%) was obtained and was based on the line equation for the extract concentrations and respective percentages of DPPH• radical scavenging.

$$\% \text{ scavenging} = 100 - [(\text{final absorbance of sample x } 100)/\text{absorbance of the blank}]. \quad (1)$$

In vitro cytotoxic activity

The human tumor cell lines HL-60 (leukemia), PC3 (prostate carcinoma), SNB19 (glioblastoma), and HCT-116 (colon carcinoma) were kindly provided by the National Cancer Institute (USA) and used for the analysis of *in vitro* cytotoxicity of the different Brazilian red propolis extracts. All cell lines were cultured in RPMI 1640 complete medium (Gibco, Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin antibiotic solution and were incubated in an incubator (Thermo Scientific, 3425, Massachusetts, USA) at 37˚C with 5% CO_2. Trypsin (0.25%) was used to detach the cells from the walls of the culture flasks.

The MTT [3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl -2H- tetrazolium bromide] (Sigma Aldrich, Missouri, USA) assay was used for determining the cytotoxic (antitumor) potential of the extracts against the cell lines [78,79]. The samples were distributed in 96-well plates ($100\ \mu L.well^{-1}$) at a final concentration of $0.1x10^6$ $cells.mL^{-1}$. After 24 hours, the extracts were dissolved in 0.001% DMSO and added to the wells to a final concentration of 50 $\mu g.mL^{-1}$. The experiment was performed three independent times (in triplicate), with 0.25 $\mu g.mL^{-1}$ doxorubicin and 0.001% DMSO as positive and negative controls, respectively (incubation for 72 hours in an incubator with 5% CO_2, at 37˚C). At the end of the incubation, the plates were centrifuged (15 g/15 min) at 4˚C and the supernatants were discarded. Subsequently, 150 μL of the MTT solution ($0.5\ \mu g.mL^{-1}$) was added, and the plates were incubated for 3 hours. After this period, the plates were centrifuged again ($3\ g.min^{-1}$) at 4˚C, the supernatants were discarded, and the precipitates were resuspended in 150 μL of sterile pure DMSO. For the quantification of formazan produced by viable cells, the absorbance was read using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Packard, ON, Canada) at a wavelength of 595 nm. All values were expressed as the 100% inhibitory concentration (IC100).

Statistical analysis

The results of this study were expressed as the mean ± standard error of mean (SEM) (n = 3). The statistical analysis of the results was performed using the Statistica 6.0 software from

StatSoft (Tulsa, USA). A one-way ANOVA and the Tukey test (95% confidence level) were used to identify the differences between the concentrations of phenolic compounds, flavonoids, concentration of compounds by HPLC, and antioxidant and cytotoxic activity in the extracts obtained through the two extraction methods and for the different propolis samples. In all statistical procedures, the level of significance was set at $p<0.05$.

Results and discussion

Antioxidant profile of red propolis extracts

Table 3 and Fig 2 show the results for the total phenolic compounds, flavonoids, and antioxidant activity of the ethanol extracts of the different red propolis samples obtained by the two extraction methods (conventional and ultrasound-assisted).

In general, a significant variation ($p>0.05$) was observed for phenolic compounds, flavonoids, and antioxidant activity among the extracts obtained for red propolis samples from different origins. The content of phenolic compounds ranged from 277.8±1.32 (D1) to 398.3±11.15 mgGAE.g^{-1} (B1), the flavonoid content from 42.0±0.75 (E1) to 108.0±0.18 mgQ.g^{-1} (A2), and the antioxidant activity from 102.94±5.94 (E1) to 47.42±4.28 (IC_{50}) (F2) (Fig 2 and Table 2). The variations observed between the samples ($p>0.05$) were expected considering that the propolis obtained from different geographical regions exhibited different chemical profiles [4,27,28,45]. Samples of the same specific type of propolis (red color) show variation in the content of antioxidant compounds when collected in different geographic regions. Thus, the results found in this study confirm the effect of the origin of the raw material on the composition of the extracts.

The red propolis samples collected in the northeastern region of Brazil had high amounts of phenolic compounds and flavonoids, as well as a high antioxidant capacity, as previously demonstrated by Machado et al. [27] and Andrade et al. [66]. The phenolic compounds, specifically the flavonoids, are the main components responsible for the biological activity of propolis [33,80].

Table 3. Determination of the content of total phenolic compounds (mgGAE.g^{-1}), flavonoids (mgQE.g^{-1}) and antioxidant activity (DPPH—IC_{50} μg.mL^{-1}) of the extracts from Brazilian red propolis obtained by conventional (1) and ultrasound-assisted extraction (2) (mean ± standard error of mean).

Samples	Phenolic compounds (mgGAE.g^{-1})	Flavonoids (mgQE.g^{-1})	DPPH (IC_{50}) (μg.mL^{-1})
A1	307.63±0.92^{c,d}	81.42±4.45^{b,c}	57.27±0.73^{d,e}
A2	337.72±13.08^{b,c}	108.02±0.18^{a}	48.00±2.45^{e}
B1	398.31±11.15^{a}	62.01±0.51^{e}	70.41±3.22^{c,d}
B2	380.73±13.60^{a,b}	61.17±1.18^{e}	72.02±2.79^{c,d}
C1	308.49±6.91^{c,d}	82.87±0.35^{b,c}	76.58±4.17^{b,c}
C2	314.75±14.00^{c,d}	90.38±3.36^{b}	72.70±3.01^{c,d}
D1	277.81±1.32^{d}	57.07±2.20^{e}	103.85±1.23^{a}
D2	283.74±5.17^{c,d}	65.34±0.85^{d,e}	94.28±1.82^{a}
E1	332.74±11.68^{b,c}	42.00±0.75^{d,f}	102.94±5.94^{a}
E2	335.16±12.55^{b,c}	43.64±1.90^{f}	90.61±2.98^{a,b}
F1	333.06±9.39^{b,c}	75.89±2.50^{c,d}	65.96±0.10^{c,d}
F2	334.89±15.34^{b,c}	79.67±2.10^{b,c}	47.42±4.28^{e}

The results of the quantitative analysis of phenolic compounds, flavonoids and DPPH in the extracts obtained by the two extraction methods from different Brazilian red propolis samples are presented. The samples A1, B1, C1, D1, E1 and F1 are the extracts obtained by conventional extraction and the samples A2, B2, C2, D2, E2 and F2 are the extracts obtained by ultrasound-assisted extraction. Lower values of IC_{50} indicate higher activity of radical elimination (DPPH results).
Statistical analysis: Values showing the same letter in the same column do not show significant difference ($p>0.05$) through the Tukey test at a 95% confidence level.

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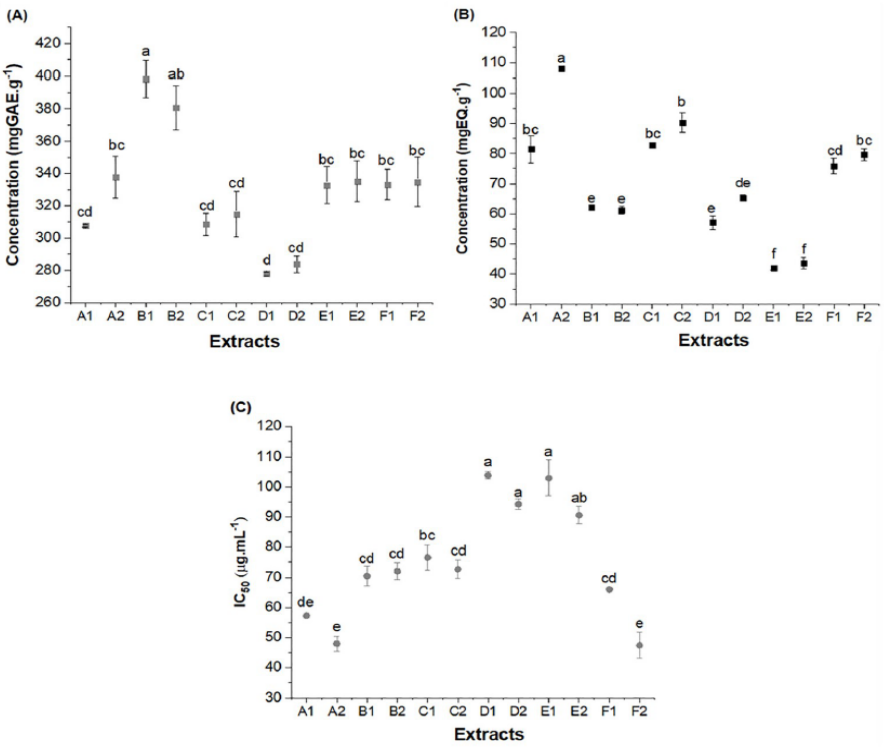

Fig 2. Total phenolic compound content expressed in mgGAE.g^{-1} (A); flavonoids expressed in mg mgQE.g^{-1} (B); and DPPH expressed as IC_{50} –µg.mL^{-1} (C) of the extracts of different samples of Brazilian red propolis (mean ± standard error of mean). IC_{50}: Lower values of IC_{50} indicate higher activity of radical elimination. A1, B1, C1, D1, E1 and F1—Extracts obtained by conventional extraction; A2, B2, C2, D2, E2 and F2—Extracts obtained by ultrasound-assisted extraction. IC_{50}: Lower values of IC_{50} indicate higher activity of radical elimination. Statistical analysis: Values showing the same letter in the same analysis do not show significant differences ($p>0.05$) based on the Tukey test at a 95% confidence level.

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The highest amount of phenolic compounds was identified in sample B (extracts B1 and B2), while the highest flavonoid content and highest antioxidant activity were observed in sample A (extracts A1 and A2), both from the state of Alagoas. Notably, that the Brazilian red propolis produced in Alagoas is the only propolis in the country that has a certificate of origin (geographical indication) due to the scientific recognition of its differentiated chemical composition [81,82].

When comparing the results from other studies that also evaluated the antioxidant profile of Brazilian propolis, the current study found higher phenolic concentrations (approximately 4-fold, sample B) than those reported in the studies by Cottica al. [83] and Mello et al. [84], which found values ranging from 48 to 87 mgGAE.g^{-1} and 49 to 100 mgGAE.g^{-1}, respectively.

In regards to the flavonoid content, the values obtained are in agreement with the literature for red propolis [18,85,86]. Righi et al. [2] reported a variation of between 27 and 43 mgQE.g^{-1}, while Alencar et al. [19], Hatano et al. [87] and Wang et al. [88] obtained flavonoid concentrations ranging from 43 to 55 mgQE.g^{-1} when they evaluated different types of propolis.

Red propolis, regardless of its origin, has a high antioxidant potential, as has been demonstrated in previous studies [31,32,84,89]. Andrade et al. [66] showed a higher antioxidant

capacity of red propolis when comparing samples of different types of propolis (green, red, and brown) from the Brazilian northeast.

In this study, sample A (from Alagoas) had the highest antioxidant activity, which was represented by the lower IC_{50} (extracts A1 and A2). These results indicate that the chemical nature of the phenolic compounds, and perhaps the presence of other compounds, contributes to the total antioxidant capacity of the extracts [51]. Similar results for antioxidant activity were identified by Alencar et al. [90] (57.0±3.2%) when evaluating the ethanol extract of red propolis from Alagoas. Frozza et al. [91] found an IC_{50} of 270.13±24.77 $\mu g.mL^{-1}$ for ethanol extracts of red propolis from Sergipe state (Brazil). Machado et al. [27] observed IC_{50} values of between 31 and 183 $\mu g.mL^{-1}$ in Brazilian red propolis extracts (Sergipe and Alagoas), while Christov et al. [92] found values of between 65 and 79% inhibition by DPPH for the ethanol extract of propolis from Canada at 210 $\mu g.mL^{-1}$.

When evaluating the extracts obtained by the different methods (conventional and ultrasound-assisted) from the same sample, in general, no significant differences were identified ($p>0.05$) (Table 3 and Fig 2). However, according to Dent et al. [93] ultrasound-assisted extraction is the rapid extraction technique, which in comparison to conventional extraction, offers high reproducibility in a short time with simplified manipulation, reduced solvent consumption and lower energy. The achieved results have shown how ultrasound-assisted extraction resulted in shorter extraction time [94,95].

Ethanol extraction has been described as the most suitable medium for the extraction of biologically active phenolic components from propolis [96–99]. In addition, the industrial extraction method commonly used to obtain biocompounds from propolis is conventional extraction (ethanolic or hydroethanolic extraction), where the sample can be submerged in a solvent for days, weeks, or months, which requires an enormous amount of time when extracting on an industrial scale (usually at room temperature) [1,100,101]. Thus, the findings of this study show that the use of ultrasound technology as a treatment during the extraction process is a viable alternative for obtaining antioxidant compounds from propolis in a short period of time when compared with the applied conventional extraction (ethanolic extraction for 7 days) that is usually employed by industry. Furthermore, the ultrasound-assisted method can be efficiently applied to reduce extraction time and energy consumption which is reflected in the lowering of the final cost.

It is important to emphasize that there is little literature on the application of ultrasound technology for obtaining propolis extracts, despite the advantages already mentioned in different studies using other types of matrices [59,68,102–104]. In the study by Taddeo et al. [53], a higher (28% higher) amount of biocompounds was obtained in Italian propolis extracts when using ultrasound exposure combined with conventional solvent extraction. Therefore, the application of ultrasound technology may be useful to increase the extraction of antioxidant compounds in propolis samples when applied in conjunction with the conventional method (ethanolic extraction), or it may reduce extraction time, as shown in this study. Ultrasound-assisted extraction has been confirmed as one of the most economic and efficient extraction methods for recovery of valuable compounds, especially for extraction purposes [94,95].

Quantification of compounds by HPLC

Analysis by HPLC is an important and efficient technique for the identification of compounds in complex mixtures such as propolis [97,105,106] and enables the quantification of compounds of chemical and biotechnological interest. As previously reported and evidenced in different studies, the chemical composition of propolis depends on its geographical location, and as such, its biological activity is closely related to the native vegetation of the collection site

[19,27,107,108]. However, Brazilian red propolis presents a composition similar to that of the Cuban red propolis produced in the province of Pinar Del Rio, without benzophenones, but with several isoflavones, such as medicarpin, homopterocarpin, and formononetin [11,18,109].

Previous studies have shown that formononetin is one of the main components, is an important marker of Brazilian red propolis [32,110,111], and is also present in its botanical origin, *Dalbergia ecastaphyllum (L)Taub* [10,112]. Cavendish et al. [32] demonstrated some biological activities of the hydroalcoholic extract of the red propolis due to the presence of formononetin, as it was antinociceptive and anti-inflammatory in experimental models. Formononetin has also been associated with the reduced action of IL-1β and nuclear factor κB (NF-κB) *in vitro* [113]. In addition, the anti-inflammatory and antioxidant activities of formononetin promoted neural and pulmonary protective effects *in vivo*, decreasing TNF-α and IL-6 levels [114,115] and improving the activity of superoxidase dismutase [116]. These studies evidenced the importance of formononetin in red propolis extracts; therefore, the identification of an efficient method to obtain this important compound is of great relevance.

In the current study, of the ten phenolic compounds investigated (Table 2), only formononetin and kaempferol were found to be above the limits of detection and quantification in the extracts. Quercetin and hydrate rutin were present in the chromatograms obtained, however, below the limits of quantification or detection (S1 Table). Neves et al. [23] investigated ethanol extracts of Brazilian red propolis (two samples from Pernambuco) and found formononetin as the main component (2.86 and 1.71 $\mu g.mg^{-1}$). The isoflavones rutin (0.21 and 0.02 $\mu g.mg^{-1}$) and quercetin (0.37 and 0.39 $\mu g.mg^{-1}$) were present at very low concentrations. Ruffato et al. [117] investigated fractions ethanol extract of the red propolis from Brazil (Alagoas) and also determined formononetin as one of the main biomarkers, in addition to flavonoids biochanin A and liquiritigenin. Similar results have also been demonstrated by Ruffato et al. [6].

The results of the quantitative analysis of formononetin and kaempferol in the extracts obtained by the two extraction methods from different Brazilian red propolis samples are presented in Table 4 and Fig 3. The chemical structures of the biomarkers formononetin and kaempferol are shown in Fig 4. The formononetin content ranged from 5.22±0.01 (D1) to 13.64±0.04 $mg.g^{-1}$ (B2), whereas the kaempferol content ranged from 0.43±0.01 (A2) to 3.72±0.05 $mg.g^{-1}$ (B1) among the extracts.

The extracts from sample B (Alagoas) presented the highest contents of the analyzed compounds, being the sample that also exhibited the highest content of total phenolic compounds (Fig 2 and Table 3). As expected, formononetin was present in significant amounts in all extracts, regardless of the geographical origin of the sample or the extraction method employed. Lopez et al. [11] investigated red propolis samples of different origins to identify the main chemical markers by mass spectrometry. In that study, formononetin was present at significant concentrations in 10 of the 14 investigated samples and was considered as the main marker of this type of propolis.

According to the results in Table 4 and Fig 3, significant differences ($p>0.05$) were observed for the levels of formononetin and kaempferol when comparing the two extraction methods applied for the same sample, and when comparing the extracts obtained by the same method for samples of different origins. Thus, this study also proves that the extraction method [50,118–120] and geographical origin [121,122] influence the content of specific compounds in propolis extracts. The achieved results and statistical analysis have shown how ultrasound-assisted extraction resulted in shorter extraction time, and increased extraction capacity of biomarkers with high antioxidant activity from Brazilian red propolis.

In this study, ethanol extraction combined with ultrasound was more efficient for extracting the formononetin compound (Table 4 and Fig 3) ($p>0.05$). Thus, the ultrasound

Table 4. Content of formononetin and kaempferol of extracts from different samples of Brazilian red propolis obtained by conventional (1) and ultrasound-assisted extraction (2) (mean ± standard error of mean).

Sample	Formononetin (mg.g^{-1})	Kaempferol (mg.g^{-1})
A1	6.54±0.01^{i}	0.65±0.01$^{e.f}$
A2	6.15±0.01^{j}	0.43±0.01^{g}
B1	12.67±0.01^{c}	3.72±0.05^{a}
B2	13.64±0.04^{a}	3.02±0.01^{b}
C1	8.68±0.01^{e}	0.88±0.00^{d}
C2	8.40±0.01^{g}	0.51±0.00$^{f.g}$
D1	5.22±0.01^{m}	---------
D2	8.49±0.02^{f}	0.69±0.00^{e}
E1	11.39±0.01^{d}	1.87±0.00^{c}
E2	12.88±0.03^{b}	2.94±0.05^{b}
F1	5.63±0.01^{l}	1.76±0.02^{c}
F2	7.17±0.01^{h}	1.95±0.04^{c}

The results of the quantitative analysis of formononetin and kaempferol in the extracts obtained by the two extraction methods from different Brazilian red propolis samples are presented. The samples A1, B1, C1, D1, E1 and F1 are the extracts obtained by conventional extraction and the samples A2, B2, C2, D2, E2 and F2 are the extracts obtained by ultrasound-assisted extraction.

Statistical analysis: Values showing the same letter in the same column do not show significant difference ($p>0.05$) through the Tukey test at a 95% confidence level.

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application was extremely efficient for the extraction of formononetin from extracts of red propolis. Ultrasound has been applied in different studies for intensification of bioactive compounds extraction [123–125].

For the other investigated compound, kaempferol, it was not possible to determine which method was the most efficient, since there was a variation depending on the sample analyzed. For example, for samples A, B, and C, conventional extraction was superior, whereas for samples D and E, the application of ultrasound had a very significant effect on kaempferol extraction ($p>0.05$). However, from the results found in this study, it can be stated that the

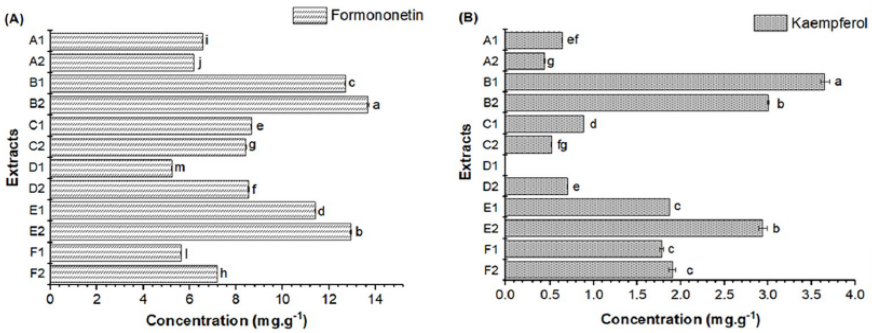

Fig 3. Concentration of formononetin (A) and kaempferol (B) in Brazilian red propolis extracts from different geographical sources obtained by conventional extraction (1) and ultrasound-assisted extraction (2) (mean ± standard error of mean). A1, B1, C1, D1, E1 and F1—Extracts obtained by conventional extraction; A2, B2, C2, D2, E2 and F2—Extracts obtained by ultrasound-assisted extraction. Statistical analysis: Values showing the same letter in the same analysis do not show significant difference ($p>0.05$) through the Tukey test at a 95% confidence level.

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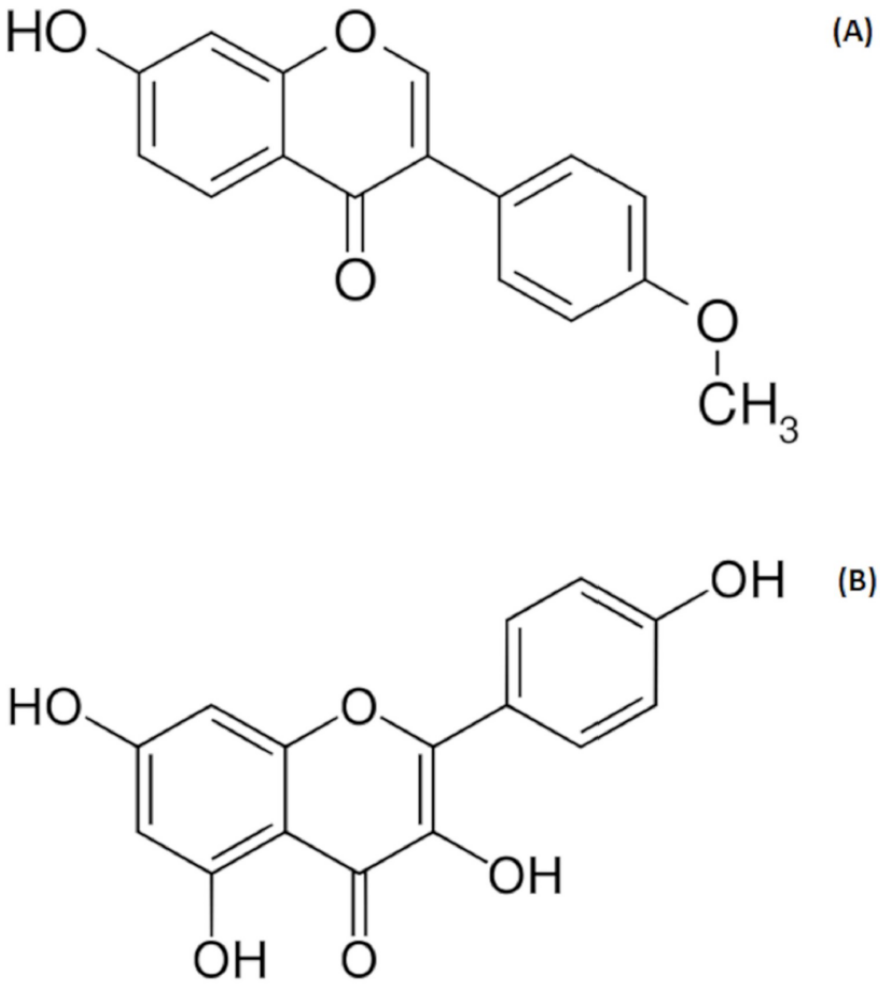

Fig 4. Chemical structures of the biomarkers formononetin (A) and kaempferol (B).

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application of ultrasound is efficient to obtain extracts with high content of antioxidant compounds, such as the formononetin and kaempferol, and as a faster extractive method, compared to conventional extraction.

Andrade et al. [66], Machado et al. [27], Szliszka et al. [126], and Jansen-Alves et al. [127] found kaempferol in samples of Brazilian green propolis, and it was considered to be one of the main constituents of this type of propolis. In the current study, significant amounts of kaempferol were identified in the red propolis extracts from northeastern Brazil (Fig 3). These results may suggest that other plant species [11,18,128,129] in addition to *Dalbergia ecastaphyllum (L)Taub* are important sources of resins for red propolis in northeastern Brazil. Similar results were obtained by Andrade et al. [65] and Andrade et al. [66], who identified the presence of kaempferol in ethanol extracts of red propolis from the Brazilian states of Sergipe and Alagoas, respectively.

Important biological effects have been reported for kaempferol in recent studies [127,130,131]. In addition to propolis, kaempferol is a flavonoid found in botanical products

that are commonly used in traditional medicines, such as *Ginkgo biloba* [132,133] and *Sophora japonica* [134–136]. Kaempferol and some of its glycosides have different pharmacological activities, including antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, and anti-osteoarthritis activity [137–140].

Filomeni et al. [141] demonstrated the neuroprotective effect of kaempferol on SH-SY5Y cells and primary neurons from rotenone toxicity, such as a reduction in caspase cleavage and apoptotic nuclei. Kaempferol has also been associated with a protective effect in the brains of rats with induced ischemic injury [142].

As showed in this study, ultrasound-assisted extraction can also provide the opportunity for enhanced extraction of specific bioactive components at lower processing time [143], and is more effective than conventional ethanolic extraction for obtaining many compounds from natural matrices using between 15–60 minutes of extraction [93,144].

Based on the results of the chromatographic analysis, red propolis extracts from northeastern Brazil obtained by ultrasound-assisted extraction are important sources of formononetin and kaempferol, which are described in the literature as having a high biotechnological potential given their demonstrated pharmacological effects. Therefore, considering the antioxidant potential of the extracts, they can be considered as important candidates for use in new functional foods or new drugs.

Determination of antitumoral activity *in vitro*

The present study also investigated the cytotoxicity of the extracts from the six red propolis samples obtained by conventional extraction and ultrasound-assisted extraction against four tumor cell lines: HCT116 (human colon), HL60 (leukemia), PC3 (prostate carcinoma), and SNB19 (glioblastoma), with the aim of evaluating antiproliferative effects, as shown in Fig 5 (percentage inhibition).

Fig 5 shows that red propolis extracts (tested at a concentration of 50 $\mu g.mL^{-1}$) altered the viability of the investigated cell lines (Fig 5A–5D), with a significant reduction ($p<0.05$) at the final cell concentration (except for the D2 extract against the HL60 line). Franchi et al. [145] comparatively evaluated propolis extracts of different types and geographical origins and found a higher antiproliferative activity for the extracts obtained from the red propolis samples, evidencing the high biological potential of this matrix due mainly to its differentiated composition. Machado et al. [146] evaluated the chemical composition and biological activity of yellow, green, brown, and red Brazilian propolis and found the highest selectivity against all tumor cells was shown by red propolis especially against HL60.

Awale et al. [29] found similar cytotoxic effects when comparing Brazilian red propolis extracts and antitumor drugs, such as 5-fluorouracil and doxorubicin, in six tumor cell lines (including HCT-116), thus evidencing the biological potential of this natural matrix. Frozza et al. [91] also showed the *in vitro* antiproliferative effect of Brazilian red propolis against human laryngeal squamous cell carcinoma (Hep-2), human cervical adenocarcinoma cells (HeLa), and normal human embryonic kidney cells (Hek-293).

For the HCT-116 cell line (colon carcinoma) (Fig 5A), regardless of the geographical origin of the sample or the extraction conditions employed, all extracts had a percent inhibition greater than 90%. In general, few significant differences ($p>0.05$) were observed for the percent inhibition for the HL-60 (leukemia) (Fig 5B) and PC3 (prostate carcinoma) cell lines (Fig 5C) when evaluating the different extracts (two extraction methods and six samples from different sources). For these lines, all of the extracts had a percent inhibition above 80% (except for extract D2, which showed no inhibition against HL-60). In general, for these three tumor lines, the extraction method used did not influence the cytotoxic response (Fig 5A, 5B and 5C).

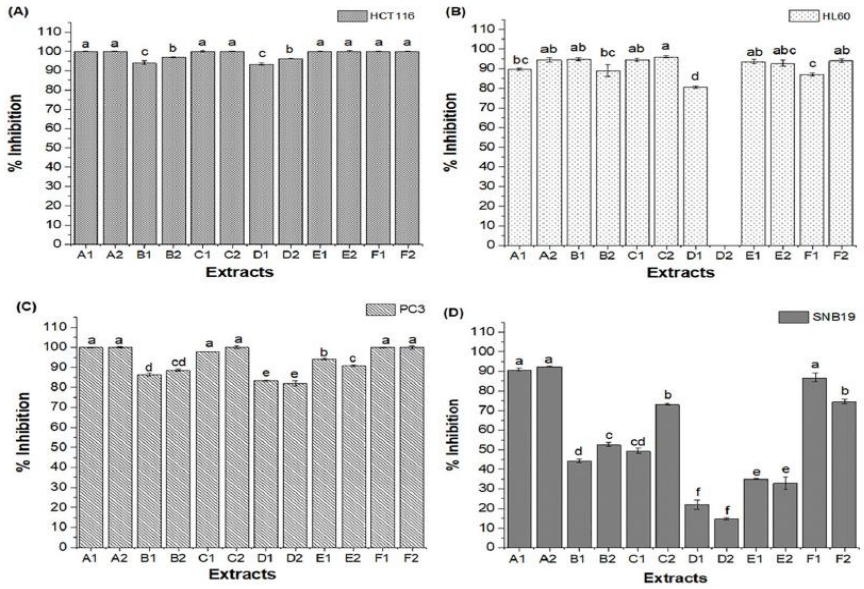

Fig 5. Percentage growth inhibition of tumor cell lines by propolis extracts obtained by conventional extraction (1) and ultrasound-assisted extraction (2): (A) HCT-116 (colon carcinoma), (B) HL-60 (leukemia), (C) PC3 (prostate carcinoma), and (D) SNB19 (glioblastoma). A1, B1, C1, D1, E1 and F1—Extracts obtained by conventional extraction; A2, B2, C2, D2, E2 and F2—Extracts obtained by ultrasound-assisted extraction. Statistical analysis: Values showing the same letter for the same analysis do not show significant differences ($p>0.05$) through the Tukey test at a 95% confidence level.

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However, significant differences ($p>0.05$) in percent inhibition were observed between the extracts against SNB19 (glioblastoma) (Fig 5D), and thus, the cytotoxic response was related to the origin of the sample. Overall, a lower percent inhibition was obtained against this line, with only four of the twelve evaluated extracts showing a percent inhibition greater than 80%. In addition, it was found that the geographical origin of the sample significantly influenced ($p>0.05$) the inhibition potential of the SNB19 line (Fig 5D). The extracts obtained from samples A (Alagoas) and F (Sergipe) showed the best results for the antiproliferative activity against SNB19, and these extracts were the only ones that were able to inhibit more than 80% of cell growth for all cancer cell lines investigated in this study. However, comparing the extracts from the same sample (collection source) obtained by the two different methods, in general, no influence of the method on the cytotoxic response was also observed for the SNB19 cell line, as shown in Fig 5D (with the exception of samples B2 and C2 –extracts obtained by ultrasound-assisted extraction were more efficient).

Thus, the results found in this study suggest that in some cases the extraction with ultrasound can positively influence the biological activity against the tumor lines tested. In addition, because it is a faster extraction method, when compared to conventional ethanol extraction, it may be considered as the method of choice to obtain extracts of red propolis from northeastern Brazil (regardless of geographic origin).

Mendonça et al. [147] and Silva et al. [1] also investigated cytotoxic activity against different cell lines (including HCT-116 and SF295) for red propolis extracts from northeastern Brazil and identified the cell proliferation inhibition potential of the extracts. Banzato et al. [148] also found cytotoxic activity of the crude extract and fractions of Brazilian red propolis against

seven tumor cell lines that included PC3 (prostate), OVCAR-3 (ovary), K-562 (leukemia), and U251 (glioma). Brazilian red propolis induced cell death and decreased the migration potential of bladder cancer cells, suggesting a potential source for the development of new drugs and/or herbal medicines for the treatment of this type of cancer [149]. Thus, red propolis extracts present high levels of cytotoxicity against different tumor cell lines, as was previously demonstrated in other studies [91,128,150–152].

Although different studies have shown the potent antiproliferative effect of formononetin [114,115,153] and kaempferol [154–156], in this study, it was not possible to establish a direct correlation of the effect of these components on the efficiency of the growth inhibition of the tested cell lines, considering the high percentage inhibition exhibited by the extracts, regardless of the concentration of these compounds (Fig 3 and Table 4).

These results may indicate that the presence of 5.22±0.02 $mg.g^{-1}$ and 0.43±0.01 $mg.g^{-1}$ of formononetin and kaempferol in the extracts, respectively, can be sufficient to achieve a percent inhibition greater than 80% (HCT-116, HL-60, and PC3 lines). In addition, another indicator would be the synergistic action of other phenolic compounds [128,131,157], which were not evaluated in the study but would be present in significant concentrations in the extracts based on the complexity of the chromatograms obtained (S1 Fig). The chemical nature of phenolic compounds and, perhaps, the presence of other compounds contribute to the cytotoxic capacity of the extracts [51]. In addition to formononetin and kaempferol (studied and identified—Tables 2 and 4), biochanin A [18,91,158], daidzein [90] and xanthochymol [159]may be some of the compounds present in extracts of red propolis with synergistic action on cytotoxic activity. According to Hernandez et al. [160], studies investigating the chemical composition of propolis samples can help establishing criteria for the quality control of this matrix, mainly due to its use worldwide and demonstrated differences in relation to geographic origin and extraction method.

For example, the cytotoxic activity of eleven different flavonoids isolated from propolis against colon cancer (HCT-116) and breast cancer (MDA-MB-231) cell lines were investigated by Vukovic et al. [131], who found six flavonoids with potential cytotoxic effects. In the study by Li et al. [128], the cytotoxic activity of 42 compounds isolated from red propolis against six different tumor cell lines was investigated. Although formononetin showed good results, the authors found that the compounds (2S)-7-hydroxy-6-methoxyflavanone and (3S)-mucronulatol presented the best antiproliferative effects against the studied lines (26-L5, B16-BL6, LLC, A549, HeLa, HT-1080), suggesting that these flavonoids could be good candidates for the development of anticancer drugs.

Based on the results found in this study and the findings in the literature, ethanol extracts of red propolis from northeastern Brazil (treated or not with ultrasound) present high antiproliferative capacities against different tumor cell lines. However, the application of ultrasound was efficient for obtaining red propolis extracts in a shorter time when compared to the conventional method and resulted in extracts with important cytotoxic effects *in vitro*. Because of this, we suggested that assisted-ultrasound extraction may be considered as a more efficient technology for the extraction of red propolis from northeastern Brazil. Future studies are needed to demonstrate the safety of using red propolis extracts *in vivo* [161], given its wide application in food, pharmaceuticals, and cosmetics.

Conclusions

In this study, the levels of flavonoids, phenolic compounds, antioxidant activity, and cytotoxicity against different tumor cell lines were determined for red propolis extracts from different geographical origins and obtained by two extraction methods. The results showed an effect of

the origin and of the extraction method in the chemical profile and biological activity of these extracts. We suggested that propolis extracts showed a high in vitro antioxidant activity. The application of ultrasound technology to obtain extracts rich in active compounds proved to be efficient, mainly due to the shorter time needed to obtain the extracts, thus enabling production on an industrial scale.

Therefore, our results demonstrated that extracts from Brazilian red propolis obtained by conventional extraction or assisted-ultrasound extraction may act in a selective way against tumor cells and show potential antitumor activity. Propolis has been a subject of intensive research, especially in the area of cancer. Future studies are needed to evaluate the biological potential of these extracts with *in vivo* models.

Supporting information

S1 Fig. Chromatogram obtained from extract C1 (sample of red propolis from Bahia).
(DOCX)

S1 Table. Evaluation by HPLC-DAD of the biochemical content of extracts of red propolis obtained by conventional (1) and ultrasound-assisted extraction (2) (mean ± standard error of mean).
(DOCX)

S2 Table. Raw data from formonometin analysis (HPLC) (mean ± standard deviation).
(DOCX)

S3 Table. Raw data from kaempferol analysis (HPLC) (mean ± standard deviation).
(DOCX)

S4 Table. Standards and parameters used for the analysis of the phenolic compounds in the different extracts by HPLC.
(DOCX)

S5 Table. Raw data from the analysis of antioxidant compounds phenolic compounds (mgGAE.g^{-1}), flavonoids (mgQE.g^{-1}) and DPPH (IC50) (μg.mL^{-1}) (mean ± standard derivation).
(DOCX)

S6 Table. Raw data from the cytotoxic analysis (mean ± standard deviation).
(DOCX)

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Writing – original draft: João Henrique de Oliveira Reis, Francine Ferreira Padilha, Janice Izabel Druzian, Bruna Aparecida Souza Machado.

Writing – review & editing: João Henrique de Oliveira Reis.

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Capítulo 5. Artigo Experimental 2

6.0 Supercritical Extraction of Red Propolis: Operational Conditions and Chemical Characterization

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Supercritical Extraction of Red Propolis: Operational Conditions and Chemical Characterization

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Abstract: The objective of this study was to determine the best operational conditions for obtaining red propolis extract with high antioxidant potential through supercritical fluid extraction (SFE) technology, using carbon dioxide (CO_2) as the supercritical fluid and ethanol as the cosolvent. The following parameters were studied: overall extraction curve, S/F (mass of CO_2/mass of sample), cosolvent percentage (0, 1, 2 and 4%) and global yield isotherms as a function of different pressures (250, 350 and 450 bar) and temperatures (31.7, 40 and 50 °C). Within the investigated parameters, the best conditions found were an S/F of 131 and the use of ethanol at the highest concentration (4% *w*/*w*), which resulted in higher extract yields and higher content of antioxidant compounds. Formononetin, the main biomarker of red propolis, was the compound found at the highest amounts in the extracts. As expected, the temperature and pressure conditions also influenced the process yield, with 350 bar and 40 °C being the best conditions for obtaining bioactive compounds from a sample of red propolis. The novel results for red propolis found in this study show that it is possible to obtain extracts with high antioxidant potential using a clean technology under the defined conditions.

Keywords: red propolis; supercritical fluids; carbon dioxide; ethanol; phenolic compounds; antioxidants

1. Introduction

Propolis is a natural compound defined as a complex resin matrix produced by bees from a mixture of exudates from different plants, wax and salivary secretions; it has major applications in the food, pharmaceutical and cosmetic industries [1,2]. Numerous studies have demonstrated its antioxidant [3,4], antimicrobial [5,6], anti-inflammatory [7,8] and antitumor [7,8] activities, among others [9–12]. These activities are attributed to the bioactive chemical compounds in propolis, such as phenolic acids, flavonoids, terpenes and sesquiterpenes [13–15]. Given this context, research has intensified on different extracts from natural matrices that have a wide variety of compounds with beneficial effects on health, such as propolis [16–19]. Among the various types of propolis, classified according to their physicochemical properties and geographical location, red propolis has gained prominence due to its composition and pharmacological properties [20–22]. Originating in Northeast Brazil, red propolis has biologically active flavonoids as its major compounds and may also contain cinnamic acid derivatives, esters and some terpenes [14,22,23]. Flavonoids are the most common and most varied group among

the phenolic compounds of red propolis, play a role in different physiological processes and perform different functions, including beehive protection [13,14,22].

In natural matrices, the extraction method plays a critical role in the types of components obtained and extraction yield, and methods that involve the use of organic solvents such as ethanol, ethyl ether, methanol and chloroform are the most commonly employed. Much of the work involving propolis is performed using the conventional extraction process (hydroalcoholic) to obtain its extracts [20,24,25]. However, despite being commonly employed, conventional extraction has some disadvantages, mainly because it is a slow process that presents solvent residues in the final product. Due to increasingly restrictive environmental regulations and new trends related to "green chemistry principles", extraction with supercritical fluids (SFE) has gained special interest among extraction techniques and is also considered a differentiated route for the extraction of compounds/substances from natural matrices [26]. This technology has been disseminated in multiple industrial areas due to its unique advantages and to the characteristics of supercritical fluids, such as diffusivity and viscosity close to those of a gas, a density similar to that of a liquid, low extraction temperatures, selective extraction, simplicity and recovery of the solvent-free product, and generating products with high added value at the end of the process [21,27]. In addition, SFE presents good yields and preserves the physico-chemical characteristics of the components to be extracted, presenting numerous advantages over conventional extractions, mainly considering the greater selectivity of the process [27–29]. Important aspects that should be considered in SFE are the choice of operational conditions in the extraction process for each studied matrix [27,29,30], i.e., the use of optimized values for the different conditions (pressure, temperature, solvent flow rate and volume, cosolvent type and concentration, among others) can significantly improve the yield and recovery of the compound or class of compounds of interest [31,32].

Despite being considered a promising alternative, few studies have used SFE to obtain propolis extracts, especially with regard to the evaluation of process conditions [20,33,34]. A previous study by our group determined the process conditions for the extraction of artepillin C and *p*-coumaric acid from green propolis [33]. Fachri et al. [34] also demonstrated the influence of pressure, temperature and CO_2 mass flow rate on the extraction yield of Indonesian propolis, while Biscaia et al. [35] performed a comparative study of different extraction methods for green propolis. In addition, different studies and patents have shown that SFE has a high potential to improve the quality of the propolis extract and to increase the aggregate value of this matrix [36–40]. This novel study is an investigation of the best process conditions for obtaining compounds of interest from red propolis by SFE. It is important to highlight that red propolis possesses distinct biological properties and chemical compositions from the other types of propolis. For example, red propolis is mainly composed of active flavonoids and may also contain cinnamic acid derivatives, esters and some terpenes [23], while green propolis is well characterized by the presence of prenylated phenylpropanoids (e.g., artepillin C) and caffeoylquinic acids [41]. This characterizes each propolis as two different samples and, therefore, each one should present a different extraction profile and should be analyzed differently, once the molecular structure of the compound contributes to the difference in extraction behavior in SFE [42].

Given this context, the objective of this study was to determine the best extraction conditions for obtaining red propolis extract with high antioxidant potential using carbon dioxide (CO_2) as a supercritical fluid and ethanol as a cosolvent. For this purpose, the overall extraction yield (X_0) and extraction kinetics, determined from the overall extraction curves (OECs), were obtained, different cosolvent percentages were investigated and the global yield isotherms (GYIs) were obtained for total phenolic compounds, antioxidant activity and for the main compounds of interest present in red propolis (formononetin, naringenin and kaempferol).

2. Results and Discussion

2.1. Extraction Kinetic Curve and S/F

The first study step involved the determination of the supercritical extraction kinetic curve of propolis using CO_2 as a solvent for the purpose of determining the amount of CO_2 required for the diffusion period of the process to be reached and for the process time to be estimated. The literature shows that extraction kinetic curves can be divided into three phases. The first is related to the constant extraction rate (CER) phase, when the solute is easily accessible on the surface of matrix particles; the second represents the falling extraction rate (FER) phase, when the mass transfer rate decreases rapidly as a result of the decrease in the effective mass transfer area; and last, the diffusional period (DP), characterized by the absence of an easily accessible solute in the third phase [43]. Thus, and as evidenced in different studies, obtaining the diffusional period is of great importance to ensure that the global yield tests present results close to the real exhaustion of the extraction bed [44].

To obtain the diffusional period in the present study, the bed was filled with 7.5 g of red propolis sample, and the extraction was performed under temperature and pressure conditions that were milder than those studied (40 °C and 100 bar, solvent density of 1.8204 kg/m^3). A total of 13 experiments were obtained for each assay used. Table 1 shows the results obtained for each experiment in relation to the S/F ratio, the mass yield of extract, the accumulated yield percentage and the mass of extract (g), and the yield for the content of total phenolic compounds and antioxidant activity. The total yield under the conditions used was 5.89% after a period of 5 h and 42 min of extraction using 0.81 m^3 CO_2. With the increase in the dynamic extraction time at constant pressure and temperature, an increase in the efficiency of the whole process was observed, and therefore, an increase in the yield of extracted compounds was expected [45].

Table 1. Determination of pilot extraction kinetics for red propolis using supercritical fluid extraction by CO_2 with the results for S/F; mass yield of extract ± standard deviation (SD); mass yield of accumulated extract ± SD; total phenolic compounds in mg/GAE/g ± SD; and antioxidant activity (%) ± SD (parameters: 7.5 g of sample; 40 °C; 100 bar; CO_2 flow rate 6.0 g/min).

Experiment Number	S/F	Mass Yield of Extract (g)	Accumulated Yield (%) and Extract Mass (g)	Phenolic Compounds (mg GAE/g)	Antioxidant Activity (%)
1	7.28	0.04 ± 0.01	1 (0.04 ± 0.01)	170 ± 20	20 ± 4
2	14.56	0.04 ± 0.01	1 (0.09 ± 0.01)	200 ± 10	30 ± 2
3	21.84	0.04 ± 0.01	2 (0.1 ± 0.01)	210 ± 4	20 ± 1
4	29.13	0.03 ± 0.01	2 (0.2 ± 0.01)	200 ± 20	20 ± 1
5	43.69	0.03 ± 0.01	3 (0.2 ± 0.01)	200 ± 10	30 ± 1
6	58.25	0.04 ± 0.01	3 (0.2 ± 0.01)	200 ± 20	30 ± 4
7	72.82	0.04 ± 0.01	4 (0.3 ± 0.01)	180 ± 20	30 ± 0.2
8	87.38	0.04 ± 0.01	4 (0.3 ± 0.01)	220 ± 10	30 ± 2
9	109.22	0.03 ± 0.01	4 (0.3 ± 0.01)	220 ± 5	30 ± 2
10	131.07	0.03 ± 0.01	5 (0.4 ± 0.01)	230 ± 21	30 ± 1
11	152.91	0.03 ± 0.01	5 (0.4 ± 0.01)	190 ± 20	20 ± 1
12	174.76	0.02 ± 0.01	6 (0.4 ± 0.01)	160 ± 1	20 ± 4
13	196.60	0.02 ± 0.01	6 (0.4 ± 0.01)	210 ± 30	20 ± 2

S/F = (mass of CO_2/mass of sample).

In studies on the extraction of compounds from the herbs *Plantago major* and *Plantago lanceolata*, Mazzuti et al. [44] obtained fully developed kinetic curves, reaching the diffusional period at 240 min, using 15 g of crude extract from the samples defined for a pressure condition of 240 bar and a temperature of 50 °C. The same kinetic curve profile was found by Teixeira et al. [46] when analyzing the impact of temperature and pressure on SFE tests using 15 g of pracaxi seed oil (*Pentaclethra macroloba*), reaching the diffusional period at 240 min under the conditions of 40 °C and 250 bar.

Figure 1 shows the kinetic curve obtained for the mean accumulated extract mass versus the total extraction time of the propolis sample. According to different studies, extraction kinetics experimental data are fundamental for scaling up studies of the supercritical technology applied to obtain extracts of solid matrices [44,46,47]. Using the SFE technology in natural matrices, different studies have shown that the kinetic curve is not a linear function of time, and its shape is an indicator that different mechanisms control the mass transfer in the different extraction stages [44,48,49]. In the curve obtained for red propolis (Figure 1), a classical pattern that occurs in SFE was observed, that is, the presence of a period of constant extraction rate in the first hours. This is related to the extraction of substrates that are easily accessible to supercritical CO_2 (process solvent). Subsequently, there is a progressive reduction in the extraction rate over time, which represents the extraction of substrates that are difficult to access by the supercritical solvent [42,50].

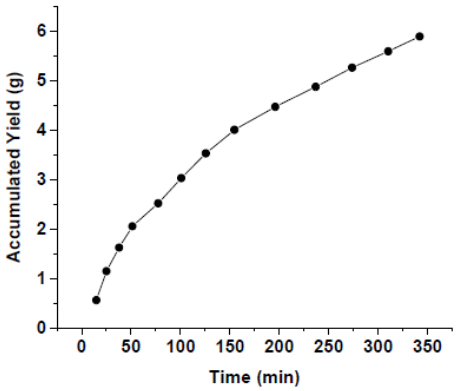

Figure 1. Kinetic curve obtained for the mean accumulated extract mass versus the total extraction time of the propolis sample.

The extraction kinetics of red propolis using CO_2 as a supercritical fluid are also represented by the overall extraction curves (Figure 2). Figure 2a shows the yield of accumulated extract (accumulated mass) during the process as a function of S/F, while Figure 2b shows the global yield of phenolic compounds and antioxidant activity versus S/F considering the total extraction time.

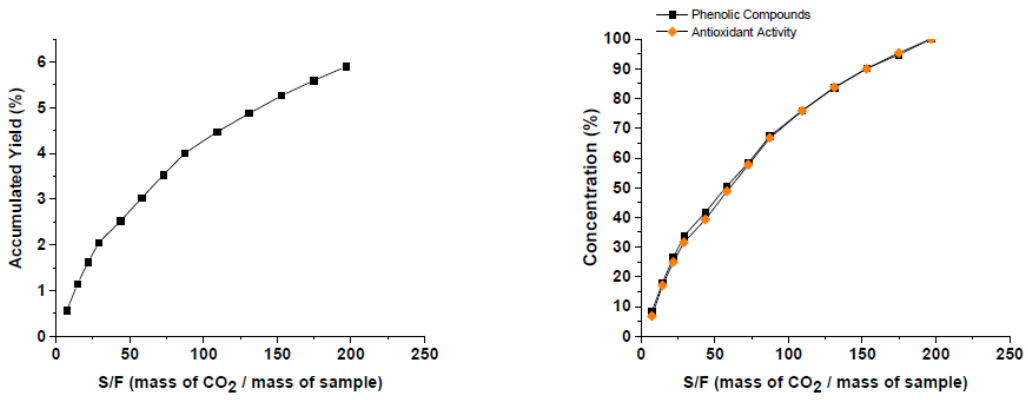

Figure 2. Results obtained for the overall extraction curves for (**a**) yield in mass (g) of accumulated extract versus S/F and (**b**) yield of phenolic compounds (%) and antioxidant activity (%) versus S/F (mass of CO_2/mass of sample) (cumulative values).

Based on the results presented for study step 1 and after analyzing the extraction results and behavior using CO_2 as a supercritical fluid under the extraction conditions defined, an S/F of 131 was determined because more than 80% of the phenolic compounds were extracted, representing 63%

of the global yield in mass of the extraction. At this point of the relationship between the solvent mass and sample mass, an accumulated yield of 4.88% (63% of the total mass extracted) was obtained, where a total CO_2 volume of 0.540 m^3 with an approximate extraction time of 240 min was obtained. Albuquerque et al. [43] obtained extracts rich in tocotrienols and defatted bixin-rich seeds from annatto and determined an S/F ratio of 35. Similar behavior was observed by Machado et al. (2012) [33], who determined the extraction kinetics curve for green propolis with a total extraction time of 150 min and an S/F ratio of 110. In the study by Krakowska-Sieprawska et al. [26], the highest yields were obtained at 60 and 120 min of the process and reached 6.42% for extracts from yerba mate and 9.60% for extracts from yellow lupine, respectively.

The low mass yield of propolis extract by SFE has been demonstrated in other studies, which may be related to the fact that propolis is not very soluble in supercritical CO_2 but can be much more soluble in a mixture containing CO_2 and ethanol [51–53]. SFE is considered a very efficient technique in terms of selectivity for the extraction of target compounds, as well as for the separation and fractionation of different classes of compounds; however, depending on the polarity of these compounds, it is necessary to add small amounts of a modifier or cosolvent to the system to improve process yield [54]. For this reason, the influence of ethanol as a cosolvent at different concentrations in obtaining red propolis extracts by SFE with high antioxidant capacity was also evaluated in this study.

2.2. Determination of the Cosolvent Concentration

Although CO_2 is the most commonly used solvent in SFE, one of the main limitations of its use is the reduced capacity to dissolve polar molecules, as is the case for the compounds present in red propolis, even at high densities [55]. Phenolic compounds and flavonoids are the bioactive components of propolis of major importance from a biological significance standpoint due to their pharmacological activities and their applicability in different areas [56]. It is well documented that the total content of phenolic compounds present in propolis varies according to several factors, including the extraction method and conditions used [57]. Regarding red propolis, previously studies have reported the presence of more than 300 components, which are representatives of terpenes, flavonoids, aromatic acids and fatty acids [22]. Flavonoids, are the most common and widely distributed group of phenolics in red propolis, being the most active compound in this natural matrix, with formononetin as the most relevant chemical marker of this type of propolis [58].

Due to the hydrophilic properties of phenolic compounds, to increase the extraction capacity of these compounds using SFE, soluble polar solvent (e.g., methanol, ethanol, and water) can be added to supercritical CO_2 to modify its properties during extraction. In the present study, the SFE conditions using ethanol as a cosolvent were also evaluated. Table 2 presents the results for the yields of phenolic compounds, flavonoids, antioxidant activity (IC_{50}-DPPH•), and concentrations of the formononetin and kaempferol compounds using 1, 2 and 4% ethanol (cosolvent) in relation to the mass of CO_2 (*w*/*w*) and without the presence of a cosolvent.

Based on these data, it was observed that the best extraction conditions to obtain a higher yield of the compounds of interest—formononetin and kaempferol—as well as the highest content of total phenolics and antioxidant activity (represented by the lowest IC_{50}) were achieved when 4% cosolvent was used (highest concentration investigated in this study). The extraction yields of total phenolics from red propolis can be increased by up to 57% with the presence of ethanol in the system as a cosolvent to supercritical CO_2. Furthermore, the antioxidant capacity of the extracts was increased by 70%, thus demonstrating that it is possible to increase the solubility of antioxidant compounds in supercritical CO_2 by adding ethanol. This may be related to the increased polarity of the solvent together with the ability of ethanol to improve the extraction surface area in a natural solid matrix [59]. The expansion of the material, generated by the interactions between solids and solvents, and the affinity between the liquid solvent and the associative compounds in the raw material leads to greater miscibility of the gas in the sample, resulting in greater extraction efficiency, as reported in previous studies [59,60].

Table 2. Results of the yields of total phenolic compounds (mg GAE/g), flavonoids (mg QE/g), IC_{50} (DPPH.) (μg/g), and the biomarkers formononetin (mg/g) and kaempferol (mg/g) using 1, 2 and 4% ethanol (cosolvent) in relation to the mass of CO_2 (*w*/*w*) and without the presence of cosolvent (mean ± SD) (50 °C; 250 bar; CO_2 flow rate of 6 g/min; S/F 131).

	Analyses				
Cosolvent Parameters (% *w*/*w*)	Total Phenolic Compounds (mgGAE/g)	Flavonoids (mgQE/g)	IC_{50} (DPPH•) (μg/g)	Formononetin (mg/g)	Kaempferol (mg/g)
0	440 ± 40 [a]	1 ± 0.03 [c]	200 ± 2 [b]	1 ± 0.2 [b]	1 ± 0.1 [b]
1	460 ± 70 [a]	6 ± 0.20 [b]	140 ± 2 [a]	2 ± 1 [b]	1 ± 1 [b]
2	510 ± 80 [a]	9 ± 0.1 [a]	190 ± 12 [b]	1 ± 1 [b]	1 ± 0.1 [b]
4	690 ± 200 [a]	6 ± 2 [b]	140 ± 14 [a]	7 ± 2 [a]	2 ± 0.3 [a]

Values followed by the same letter in the column are not significantly different ($p < 0.05$) according to Tukey's test at the 95% confidence level.

Previous studies with various types of propolis have also shown that ethanol is an important system modifier [35,61]. In a comparative study of extraction methods to obtain extracts from green propolis by SFE, Monroy et al. [36] found that when using 32% of the solution containing 80% ethanol and water as a cosolvent, a 43% increase in yield was obtained compared to SFE without the cosolvent, in addition to 220 mg GAE/g of phenolic compounds (extraction conditions 50 °C and 250 bar). Paviani et al. [53], when adding 15% ethanol as a cosolvent to obtain extracts of green propolis (extraction conditions 50 °C and 250 bar), obtained an increase of 43.7% compared to the same process without the use of a cosolvent. The influence of the addition and concentration of cosolvent on supercritical CO_2 extraction was also shown for other natural matrices [62], demonstrating the importance of these modifiers to improve the extraction capacity of polar compounds. Cruz et al. [63] showed that the presence of hydrated ethanol (1/1, *v*/*v*) significantly increased the extraction yield of yacon leaves, obtaining extracts with higher antioxidant activity compared to extraction without the use of cosolvent, leading to higher yields. In a recent study, Guedes et al. [60] showed that as the concentration of ethanol added to the process was increased (0.5/1, 1.1/1 and 1.5/1, *w*/*w*), the yield and efficiency of SFE extraction in samples of *Synadenium grantii* increased.

The compound mainly found in the red propolis extract analyzed in this study was formononetin in the concentration range of 1 ± 0.2 mg/g (control 0%) to 7 ± 2 mg/g (extract with 4% of cosolvent), followed by kaempferol (from 1 ± 0.1 to 2 ± 0.3) (Figure 3). A 518% increase in the extraction capacity of formononetin was observed in the presence of 4% cosolvent in the system. For kaempferol an increase of 122% was observed.

Previous studies have also reported higher concentrations of phenolic compounds, such as formononetin, in extracts of red propolis, which is identified as its major component [4,15,22]. López et al. [64], when evaluating samples of red propolis from different regions by mass spectrometry, identified the presence of formononetin (*m*/*z* 267.06, retention time, rt 4.5 min) in all analyzed samples. Hanski et al. [65] demonstrated the antimicrobial effect of formononetin on *Chlamydia pneumoniae*, while Li et al. [66] demonstrated the protective effect of formononetin in vitro, decreasing the levels of TNF-α and IL-6. Thus, the pharmacological activities of red propolis may be closely related to their phenolic compounds due to their antioxidant, anti-inflammatory and microorganism proliferation inhibition capacity. Batista et al. [67] also associated the antioxidant and anti-inflammatory effects of red propolis with the involvement of polyphenols in the photoprotective activity observed in their study. These studies show the importance of obtaining extracts rich in formononetin for a greater biological capacity of the obtained product.

Despite the knowledge that the chemical composition of propolis depends on the biodiversity, type and geographical location of the beehives [22] and that the extraction method influences the extract composition [20,33], the identification of ideal conditions, especially regarding the use of clean technologies with the presence of adequate concentrations of system modifiers, given the polar nature

of the compounds present in red propolis, is of great importance to obtain extracts with high antioxidant capacity, especially with high levels of formononetin. Formononetin has been associated with the antioxidant [68], antitumor [66], antimicrobial [22] and anti-inflammatory [8] effects of red propolis extracts. Lower contents of formononetin (6.15 and 6.54 mg/g) and kaempferol (0.43 and 0.65 mg/g) were identified by Reis et al. [23] in ethanolic extracts (ultrasound-assisted or not) of red propolis from the same geographical origin (Barra de Sto. Antonio, Porto Calvo, Alagoas, Brazil). Bueno-Silva et al. [69] evaluated the effect of season on chemical composition and found lower formononetin contents (78.76 to 112.78 µg/g) than those found in this study for ethanolic extract of red propolis of the same origin. This may demonstrate that SFE (CO_2 as the solvent and ethanol as the cosolvent) may be an important and promising technology for obtaining red propolis extracts with potential for nutraceutical and cosmetic applications [70]. In the present study, it was possible to demonstrate that ethanol (as a cosolvent) played a role by causing an increase in the polarity and eluting power of supercritical CO_2, maintaining the same process parameters without significantly changing selectivity.

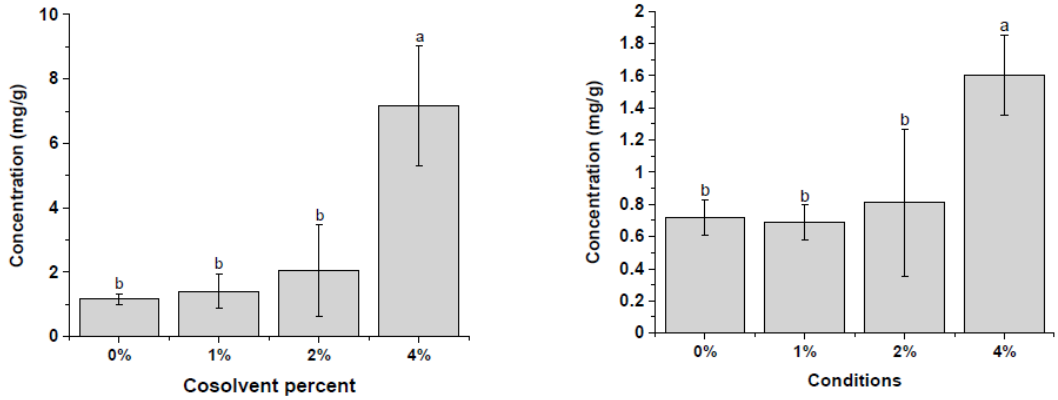

Figure 3. Determination of the levels of (**a**) Formononetin (mg/g); and (**b**) Kaempferol (mg/g) by HPLC-DAD in the extracts of red propolis obtained with the presence of different cosolvent percentages and using CO_2 as a supercritical fluid (50 °C; 250 bar; CO_2 flow rate of 6 g/min; S/F 131). Bars with the same letter are not significantly different ($p < 0.05$) according to Tukey's test at the 95% confidence level.

2.3. Global Yield Isotherms (GYI)

Figure 4 shows the global yield isotherms for total yield, total phenolic compound content, flavonoid content and antioxidant activity (IC_{50}) determined for the red propolis extracts obtained under the different temperature (31.7, 40 and 50 °C) and pressure (250, 350 and 450 bar) conditions used (S/F 131-step 1; 4% ethanol-step 2).

Regarding yield, a higher extraction percentage (63.46%) was obtained at a pressure of 450 bar at 40 °C, while the highest content of phenolic compounds (380 mgEAG/g) and flavonoids (10 mg EQ/g) and higher antioxidant capacity (110 µg/mL) were obtained under 450 bar and 50 °C. Figure 4a shows that at constant pressures of 350 and 450 bar the increase in temperature significantly favors the extraction capacity, and consequently, the global yield of the process.

In general, the solubility of solutes in supercritical fluids increases with temperature at constant pressure [71]. When keeping the temperature constant at 31.7 and 40 °C, increasing the pressure from 350 to 450 bar also favors the process yield. The lowest yields at constant pressure were observed at the highest temperature (50 °C). It is known that the effect of temperature on SFE is complex due to the increase in the solute vapor pressure and reduction in the density of the supercritical solvent [72]. Increased temperature increases the vapor pressure of the solute, promoting an increase in its solubility in supercritical CO_2; however, the temperature increase also promotes a reduction in the density of supercritical CO_2, thus reducing the solubility of the solute in the solvent [73]. In this case, the effect of reduced supercritical CO_2 density was favored in relation to the increased solvent density (Figure 4a).

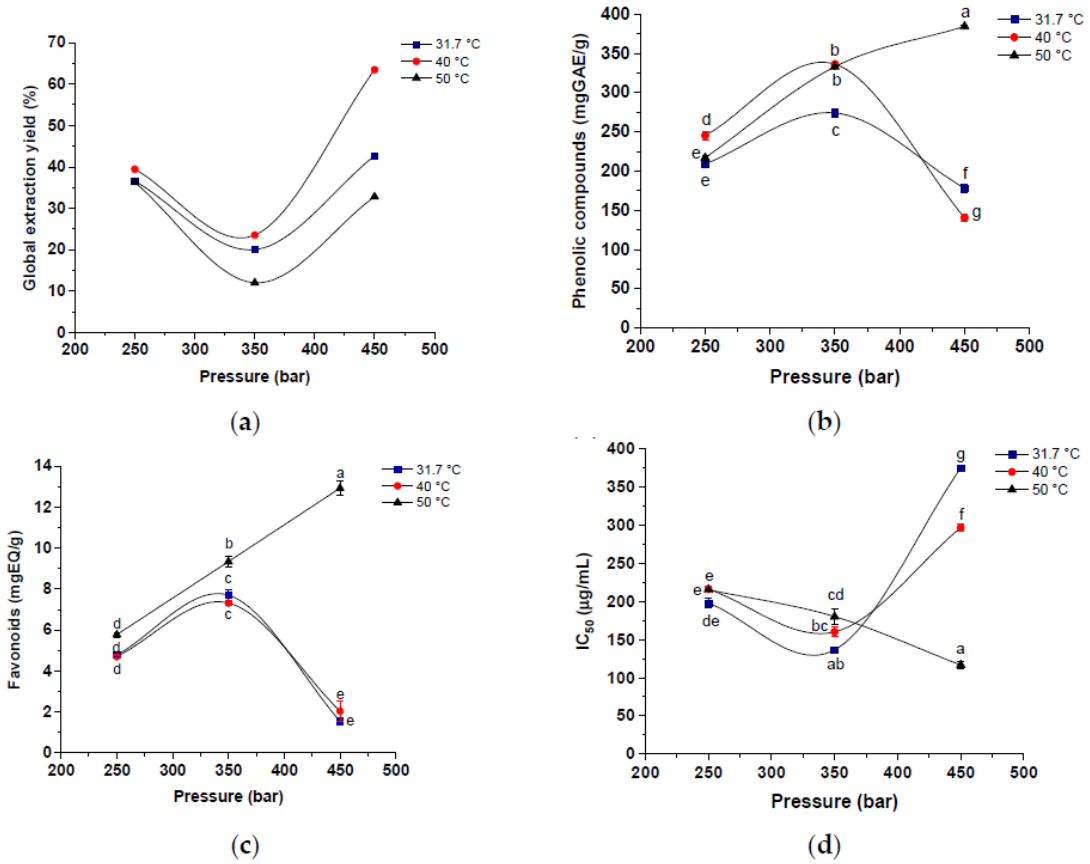

Figure 4. Global yield isotherms for (**a**) total yield percentage (%); (**b**) content of total phenolic compounds (mg EGA/g); (**c**) content of total flavonoids (mg EGA/g); and (**d**) antioxidant activity (IC50) (µg/mL) for the red propolis extracts using CO_2 as supercritical fluid, ethanol as cosolvent (4% *w*/*w*) at temperatures of 31.7, 40 and 50 °C and pressures of 250, 350 and 450 bar (CO_2 flow rate of 6 g/min and S/F of 131). Points with the same letter are not significantly different ($p < 0.05$) according to Tukey's test at the 95% confidence level.

In general, at a constant pressure of 250 bar, temperature had no effect on the extraction of phenolic compounds and total flavonoids or on the antioxidant activity (without significant differences). At the intermediate pressure studied (350 bar), variations in temperature promoted an increase in the extraction of these compounds in a sample of red propolis. For example, for phenols (40 and 50 °C), total flavonoids (50 °C) and antioxidant activity (50 °C), higher temperatures contributed to a higher extraction yield of these compounds. The positive effect of the temperature increase at a constant pressure of 450 bar (highest studied pressure) was clearly evident. Thus, the effect of temperature increase had a positive influence on the vapor pressure of the solutes [74] and, therefore, the temperature of 50 °C was the most efficient for obtaining extracts with high antioxidant capacity.

It is noteworthy that at constant pressure, the influence of temperature cannot be treated in such a simple way [75]. When evaluating the behavior of pressure in the 31.7 and 40 °C isotherms, it was observed that the increase in pressure from 250 to 350 bar contributes significantly to the increase in the extraction of phenolic compounds, flavonoids and antioxidant activity. However, a reduction in the levels of phenolic compounds and flavonoids, and consequently antioxidant capacity, was observed when the pressure increased from 350 to 450 bar in the 31.7 and 40 °C isotherms. Thus, at these temperatures, intermediate pressures (350 bar) may be more efficient for the extraction of phenolic compounds from red propolis.

The best phenolic and flavonoid yields, as well as antioxidant activity, were observed in the 50 °C isotherm at a pressure of 450 bar. In the 50 °C isotherm, the increase in pressure significantly

accelerated the mass transfer in the supercritical extractor bed and contributed to increasing the extraction yield of phenolic compounds from red propolis. As demonstrated by Barroso et al. [76] and Fujii et al. [77], the density of supercritical CO_2 is dependent on the pressure used in the extraction process; therefore, at higher pressures, supercritical CO_2 will have a higher density, and consequently, its solvation power will be higher.

It is important to note that the isotherms crossed between 400 and 450 bar for total phenols (Figure 4b) and between 350 and 400 bar for antioxidant activity (Figure 4d). Below this pressure level, known as the crossover pressure, the solubility of the antioxidant compounds of red propolis decreases with increasing temperature. A crossover is observed near the critical region [78,79]; in these regions, the effect of temperature on the increase in vapor pressure compensates for the effect of temperature on decreasing solvent density [27]. Due to the existence of this point, the lowest and highest concentrations of phenolic compounds occurred in the same isotherm (50 °C). Azevedo et al. [80] evaluated the extraction of caffeine using SFE and found that near the critical point, below the crossover pressure, small increases in temperature result in a drastic decrease in the density of the solvent and, consequently, in its extraction capacity. For the extraction of artemisinin from *Artemisia annua* L., Rodrigues et al. [81] found a crossover pressure of 200 bar, whereas Cadena-Carrera et al. [82] evaluated the bioactive properties of the extracts from guayusa leaves (*Ilex guayusa* Loes.) and found a crossover pressure close to 200 bar.

Figure 5 shows the global yield isotherms for the three compounds identified and quantified by HPLC-DAD ((a) formononetin; (b) naringenin; and (d) kaempferol)) in the red propolis extracts under the different temperature (31.7, 40 and 50 °C) and pressure (250, 350 and 450 bar) conditions employed (S/F 131-step 1; 4% ethanol-step 2) in this study. Figure 6 shows the chemical structure of each phenolic compound analyzed in this study.

Formononetin (Figure 5a) was obtained at higher concentrations (13 mg/g) at a pressure of 350 bar and a temperature of 40 °C (intermediate conditions). It is possible to observe that at constant pressures of 250 and 350 bar, there is an increase in the concentration of formononetin extracted when the temperature increases from 31.7 °C (2 mg/g and 6 mg/g) to 40 °C (5 mg/g and 10 mg/g), but there was a reduction at 50 °C (3 mg/g and 9 mg/g). In this case, the increase in temperature increased the vapor pressure of the solute, positively favoring the extraction process. However, at high temperatures, the effect of solvent density reduction may favor both the degradation of formononetin and the decrease in extraction efficiency for this compound [83,84]. In addition to solubility, the effect of temperature on the conversion or degradation of formononetin should also be taken into consideration. At a constant pressure of 450 bar, the highest concentration was observed at the highest and lowest temperatures employed, with concentrations of 9 mg/g and 7 mg/g, respectively. Thus, the effect of the supercritical CO_2 solubility at 450 bar should be more strongly considered than the effect of conversion or degradation of formononetin in terms of extraction yield.

In addition, a crossover pressure close to 400 bar was also observed. When evaluating the pressure behavior at a constant temperature of 40 °C, an extraction profile similar to the extraction of total phenolic compounds, flavonoids and antioxidant activity was observed (Figure 5): when increasing the pressure from 250 to 350 bar, there is an increase in extraction, but when increasing the pressure from 350 to 450, extraction is reduced. Theoretically, the higher the pressure, the greater the density and solubility of the supercritical fluid, increasing the extraction efficiency [85]. However, the concentration of some of the target compounds may be reduced because at high pressures, other compounds can also be extracted, and thus, there is a reduction in specificity [86]. In general, high pressures may not be efficient for the extraction of formononetin because the effect of decreased diffusivity overlaps the effect of increased density, thus decreasing the extraction yield of this compound at 450 bar. A similar effect was found by Saito et al. [61] when obtaining phenolic compounds from green and red propolis, with higher yields (14%) observed at 60 °C and 200 bar and the lower yields (3.6%) under 40 °C and 300 bar.

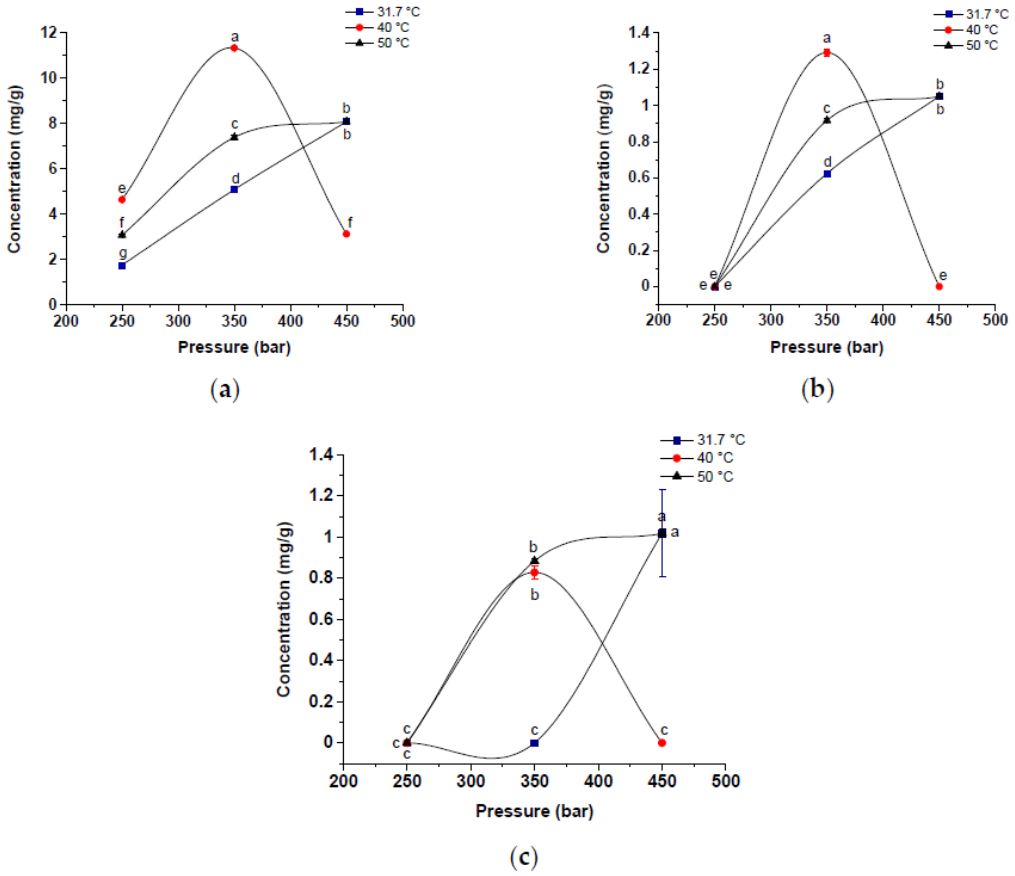

Figure 5. Isotherms for the concentration of: (**a**) formononetin; (**b**) naringenin; and (**c**) kaempferol for the extraction of red propolis using CO_2 as the supercritical fluid, ethanol as the cosolvent (4%, *w*/*w*) at temperatures of 31.7, 40 and 50 °C and pressures of 250, 350 and 450 bar (CO_2 flow rate of 6 g/min and S/F 131). Values that show the same letter in the same graph are not significantly different ($p < 0.05$) by the Tukey test at a confidence level of 95%.

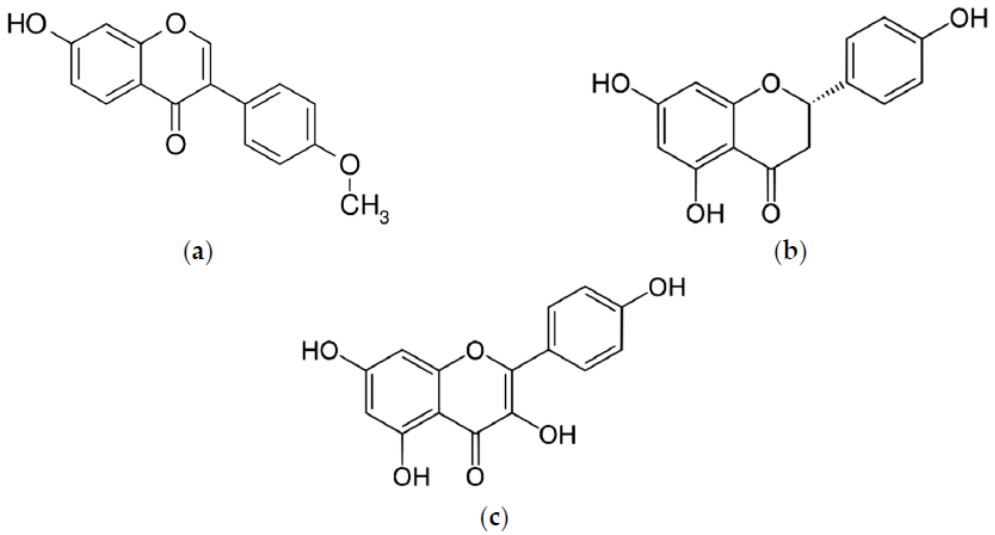

Figure 6. Chemical structures of phenolic compounds (**a**) Formononetin; (**b**) Narigenin; and (**c**) Kaempferol.

Regarding the compound naringenin (Figure 5b), when analyzing the behavior of the isotherms at a constant pressure of 250 bar, it was observed that the temperature did not influence the extraction

process. However, when the pressure increased to 350 bar, the effect of temperature is evident because by raising the temperature from 31.7 to 40 °C, an increase of 114% in extraction is obtained, and when raising the temperature from 40 to 50 °C, a reduction of 27.3% is observed. For this compound, crossing of the temperature curves was also observed at a pressure close to 400 bar, and a higher concentration (2 mg/g) at 40 °C and 350 bar. This behavior is in agreement with that observed for total phenolic compounds, flavonoids and antioxidant activity (Figure 5). When analyzing the behavior of pressure at constant temperature, it is noted that for the temperatures of 31.7 and 50 °C, the pressure has a positive effect on the extraction process. Under these conditions, the increase in pressure increases the density of the supercritical fluid and the solvation power of the solute, consequently increasing the extraction yield. At a temperature of 40 °C, extraction was only significant at a pressure of 350 bar. Majdoub et al. [87] found similar effects when raising the pressure from 100 to 300 bar at a constant temperature of 50 °C for obtaining extracts of *Daucus carota*.

Similar to what occurred for the compound naringenin, the temperature variation also did not influence the kaempferol extraction process (Figure 5c) at a constant pressure of 250 bar, and this compound may have its extraction reduced or become nonextractable under these conditions. However, at the pressure of 350 bar, there is an increase in extraction when the temperature increases from 31.7 to 40 and 50 °C (1 mg/g and 1.078 mg/g, respectively), but with no significant differences between the concentrations obtained at the higher temperatures. The isotherms cross at approximately 400 bar, and the influence of the crossover pressure on the extraction profile was evident because up to 400 bar pressure, the extraction is more effective at the lowest and highest temperatures studied, with no significant difference, and below this value, the extraction is more effective at 40 °C. Higher concentrations were obtained at a pressure of 450 bar and 50 °C (1 mg/g). Zordi et al. [51], in a study on SFE to obtain propolis extracts, also observed that the chemical composition of an extract is significantly influenced by pressure and temperature in both a linear and quadratic way, obtaining optimal operating conditions of highest yield (14.3%) in the isotherms of 317 bar and 45 °C.

Therefore, the present study shows that the conditions of global yield isotherms in SFE affect the composition of the extract obtained and should be analyzed and defined according to the extraction objective. In general, the best yields of bioactive compounds isolated from red propolis were observed at the intermediate isotherms of 40 °C and pressure of 350 bar.

3. Materials and Methods

3.1. Materials and Reagents

DMSO (dimethyl sulfoxide) and methanol were from Sigma-Aldrich Chemical Co., St. Louis, MO, USA, and acetic acid (standard HPLC) and ethyl alcohol (standard HPLC) were from EMSURE®, Merck, Darmstadt, Germany. A regenerated cellulose membrane filter, 0.45 µm (SLCR025NS, Millipore Co., Bedford, MA, USA) was used. Rutin hydrate (CAS number 207671-50-9), kaempferol (CAS number 520-18-3), formononetin (CAS number 485-72-3), gallic acid (CAS number 149-91-7), quercetin (CAS number 117-39-5), *p*-coumaric acid (CAS number 501-98-4), epicatechin (CAS number 490-46-0), caffeic acid (CAS number 331-39-5), catechin (CAS number 7295-85-4-4) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (CAS number 1898-66-4) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and *trans*-ferulic acid (CAS number 537-98-4) was purchased from Fluka (St. Louis, MO, USA).

3.2. Study Sample

The sample of red propolis used in this study was kindly donated by the company Bee Product Natural (Barra de Sto. Antonio, Porto Calvo, Alagoas, Brazil) with "Mangroves of Alagoas" denomination of origin (IG201101) [88]. Approximately 1 kg of red propolis was ground (Cadence-Brazil) and sieved (270–325 µm diameter) to allow the homogenization of the sample in the

extraction bed. Samples of 200 g of red propolis were kept at −30 °C in vacuum-sealed packaging away from light.

3.3. Process Parameters for Red Propolis Extraction by SFE

The process parameters used to obtain the extracts of red propolis was performed as previously described by Machado et al. [33] with modifications and using a SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc., Newark, NJ, USA) under the different conditions used in the study. The CO_2 flow rate in the system was 6.0 g/min in all experiments (Figure 7).

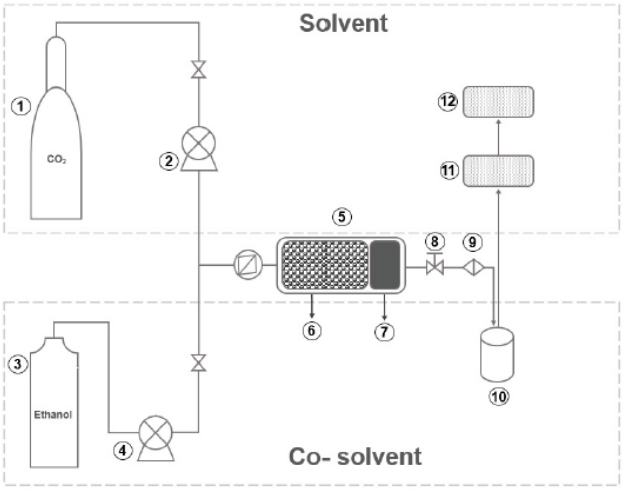

Figure 7. Schematic of the supercritical extraction system used in this study. 1—CO_2 cylinder with dip tube; 2—CO_2 pump; 3—Cosolvent cylinder; 4—Cosolvent pump; 5—Extraction cell; 6—Glass beads; 7—Sample (raw material); 8—Dynamic/static valve; 9—Restrictor valve; 10—Sample collection vial; 11—Flow meter; 12—Totalizer.

The extraction bed was packed to avoid the formation of preferential paths by the solvent (CO_2), and for this purpose, glass wool and beads were used to fully fill the bed (Figure 8). In this work, it 7.5 g of red propolis was used, as reported in previous optimization studies [33,53], mainly to avoid very long extraction times. The best extraction operational condition was determined in three steps. Figure 8 presents an illustrative summary of the process used with all parameters studied at each step, as well as the number of experiments and analyses performed. The results for the parameters evaluated and determined in this study were expressed as the mean ± standard deviation (n = 3), and all analyses were performed in triplicate.

3.3.1. First Step: Overall Extraction Curve and S/F (Mass of CO_2/Mass of Sample)

In the present study, to obtain the overall extraction curve, a temperature and pressure of 100 bar and 40 °C were used, respectively, with the objective of guaranteeing the worst extraction scenario (under mild extraction conditions), a sample of 7.5 g propolis and 6 g/min CO_2 flow rate [33,49]. The experiment was performed as follows: the extracts were collected at predetermined periods in vials of previously known weight. The *S*/*F* value was calculated according to Equation (1).

$$\frac{S}{F} = \frac{MCO_2}{M_{sample}} \quad (1)$$

where:

MCO_2 = total mass (g) of CO_2 used in the system at each extraction point (considering the volume and density of CO_2 in the system)

M_{sample} = total mass (g) of propolis used to feed the system

The overall yield (X_0) was calculated in accordance with Equation (2).

$$X_0 = \frac{M_{extract}}{M_{sample}} \quad (2)$$

where:

$M_{extract}$ is the mass (g) of extract obtained in each extraction

M_{sample} is the mass (g) of propolis used to compose the extraction bed

For the extracts obtained at the predetermined times (in each collection vial), the yield, total phenolic content and antioxidant activity were determined. A total of 13 experiments were obtained for each assay.

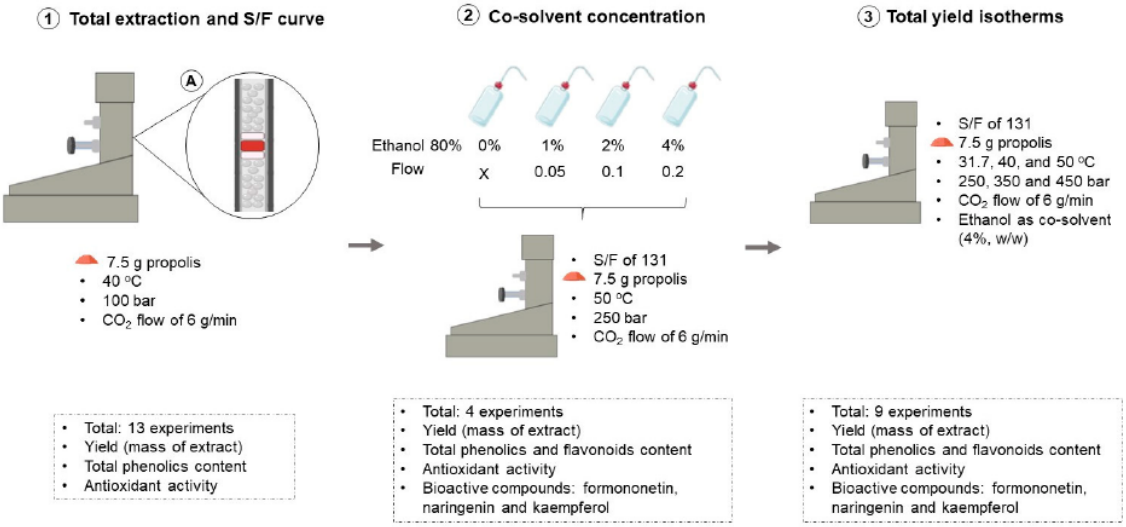

Figure 8. Illustrative summary of the steps, parameters used, total experiments and analyses performed at each step of the present study to obtain red propolis extract with high antioxidant capacity by SFE. **1**—First step: determination of the overall extraction curve and S/F (mass of CO_2/mass of sample); **2**—Second step: influence of the cosolvent percentage; **3**—Third step: determination of the global yield isotherms. In A, the packing of the extraction bed to avoid the formation of preferential CO_2 paths is shown.

3.3.2. Second Step: Influence of Cosolvent Percentage

In this step, 80% ethanol was used as a cosolvent to obtain the extracts in relation to the yield (in mass), content of total phenolic compounds, antioxidant activity and concentration of the compounds of interest (formononetin, naringenin and kaempferol). The extracts were obtained under the following conditions: S/F of 131 (calculated in the previous step), temperature of 50 °C and pressure of 250 bar (CO_2 flow rate of 6 g/min) [33]. The extractions were performed using 0, 1, 2 and 4% of the cosolvent, calculated in relation to the mass of CO_2 used (S/F), totaling 4 experiments for this step. Ethanol (80%) was diffused in the system using a cosolvent pump with a flow rate of 0.05 (1%), 0.1 (2%) and 0.2 (4%) mL/min for a total time of approximately 140 min of extraction (considering S/F = 131).

3.3.3. Third Step: Global Yield Isotherms

The yield isotherms were studied using three temperatures (31.7, 40 and 50 °C) and three pressures (250, 350 and 450 bar). The *S/F* value used was obtained in the first step (131), and the percentage of cosolvent (80% ethanol) was determined in the previous step (4% *w*/*w*) (CO_2 flow rate of 6 g/min). The extracts obtained under the different conditions were evaluated for yield (mass), content of total phenolics, flavonoids, antioxidant activity (EC_{50}) and concentration of the compounds of interest

(formononetin, naringenin and kaempferol). For this step, a total of 9 experiments were obtained for each test performed.

3.4. Total Phenolic Compounds, Flavonoids Content and Antioxidant Activity by DPPH• (2,2-Diphenyl-1-picrylhydrazyl)

The content of total phenolic compounds of the red propolis extracts was determined from the reaction with the Folin–Ciocalteu method [89,90]. The reaction was prepared as previously described by Devequi-Nunes et al. [20]. The results are expressed as milligram of gallic acid equivalent (GAE) per gram of sample (mg GAE/g). For this, a calibration curve ($y = 0.0104x + 0.0688$, $R^2 = 0.9976$) was determined using standard solutions of the gallic acid (concentrations 0 and from 10 to 200 µg/mL).

The content of flavonoids of the extracts was determined using the method proposed by Meda et al. [91] with adaptations, as previously described by Machado et al. [21]. The same procedure was performed using standard solutions of quercetin (0 and from 1 to 75 µg/mL) to obtain a standard curve ($y = 0.0311x + 0.0259$, $R^2 = 0.9987$). The content of total flavonoids was expressed as milligram of quercetin equivalent (QE) per gram of sample (mg QE/g).

To evaluate the antioxidant activity, the 2,2-diphenyl-1-picrylryrazine-reactive method (DPPH•) was applied [92,93], as previous described by Reis et al. [23]. The extracts were diluted to five concentrations (50, 100, 150, 200, 250 and 300 µg/mL) in triplicates. The free radical sequestration capacity was expressed as the percentage of radical oxidation inhibition (Equation (3)) (extracts obtained in the first and second study steps).

$$AA\ \% = 100 - \left(\frac{Ab_{sample} \times 100}{Ab_{blank}}\right) \quad (3)$$

where:

$AA\ \%$ = antioxidant activity in percentage

Ab_{sample} = absorbance of the extract sample

Ab_{blank} = absorbance of the blank (without sample)

The IC_{50} value (effective concentration of the extract to sequester 50% of the DPPH• radical) was calculated based on the linear equation obtained from the extract concentrations and respective DPPH• radical sequestration percentages. For the extracts obtained in the third study step, the results are expressed as IC_{50}.

3.5. High-Performance Liquid Chromatography (HPLC): Identification and Quantification of Phenolic Compounds

The red propolis extracts obtained by extraction with supercritical CO_2 in the second (cosolvent influence) and third (yield isotherms) study steps were evaluated by high-performance liquid chromatography (HPLC—Shimadzu, LC-20AT, Japan equipped with an automatic injector and diode array detector—DAD, Shimadzu, SPD-M20, Kyoto, Japan) as previously described by Reis et al. [23], Salgueiro and Castro [94] and Cabral et al. [95]. For this, an analytical standard curve formed by 13 phenolic standards was obtained, and the presence of these compounds in the extracts was investigated. A NUCLEODUR 100-5 C18 ec column (150 × 4 mm internal diameter; 5 µm particle size) was used in conjunction with a ZORBAX Eclipse Plus C18 precolumn (4.6 × 12.5 mm) (Agilent, Folsom, CA, USA). The chromatographic analysis conditions were tested with an elution gradient with a mobile phase of 5% acetic acid (Phase A) and methanol (Phase B) at different proportions and with a total analysis time of 42 min (from 0 to 35 min [0–92% B]; 35 to 40 min [92–0% B]; 40 to 42 min [0% B]). The injection volume was 20 µL, and the flow rate was 1 $mL.min^{-1}$. The device was operated at a temperature of 40 ± 2 °C. The DAD reading was adjusted in the range of 190 to 800 nm, and chromatographic acquisition was defined between 280 and 370 nm [23].

The compounds were identified by comparing the retention time (RT) and the ultraviolet spectrum between samples and standards. The working range for all investigated compounds was 0.5 to 15 mg/g.

The wave length (λ) ranged from 280 to 370. The RT ranged from 2.31 to 19.27 min, the detection limit (DL) ranged from 0.19 to 0.47 mg/g and the quantification limit (QL) ranged from 0.64 to 1.58 mg/g, and all these parameters were dependent on each compound. Figure 9 shows the chromatogram obtained for the construction of the analytical curve with the studied standards.

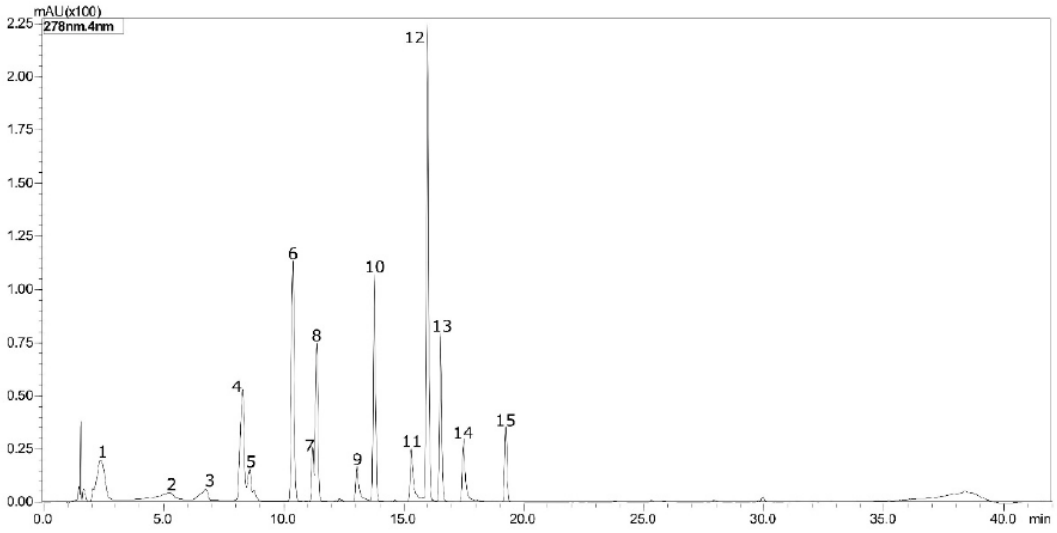

Figure 9. Chromatogram with the phenolic standards evaluated in this study by HPLC-DAD for the identification and quantification of the compounds present in the red propolis extracts obtained under different conditions (wave length = λ/retention time = RT/detection limit = DL/quantification limit = QL). 1—Gallic acid (RT 2.31 min, λ 280 nm, DL 0.47 mg/g, QL 1.58 mg/g); 2—*O*-dianiside (RT 4.92, λ 280, DL 0.62 mg/g, QL 2.50 mg/g); 3—Catechin (RT 6.51 min, λ 280 nm, DL 0.31 mg/g, QL 1.04 mg/g); 4—Caffeic acid (RT 8.15 min, λ 300 nm, DL 0.25 mg/g, QL 0.82 mg/g); 5—Epicatechin (RT 8.46 min, λ 280 nm, DL 0.20 mg/g, QL 0.68 mg/g); 6—*p*-coumaric acid (RT 10.32 min, λ 300 nm, DL 0.20 mg/g, QL 0.66 mg/g); 7—Rutin hydrate (RT 11.17 min, λ 320 nm, DL 0.35 mg/g, QL 1.15 mg/g); 8—*trans*-ferulic acid (RT 11.35 min, λ 320 nm, DL 0.26 mg/g, QL 0.86 mg/g); 9—Myricetin (RT 13 min, λ 370 nm, DL 0.30 mg/g, QL 1.00 mg/g); 10—Resveratrol (RT 13.77 min, λ 300 nm, DL 0.21 mg/g, QL 0.70 mg/g); 11—Quercetin (RT 15.36 min, λ 280 nm, DL 0.20 mg/g, QL 1.30 mg/g); 12—*trans*-Cinnamic (RT 15.99 min, λ 280 nm, DL 0.18 mg/g, QL 0.61 mg/g); 13—Naringenin (RT 16.53 min, λ 280 nm, DL 0.20 mg/g, QL 0.67 mg/g); 14—Kaempferol (RT 17.53 min, λ 320 nm, DL 0.24 mg/g, QL 0.82 mg/g); 15—Formononetin (RT 19.27 min, λ 300 nm, DL 0.19 mg/g, QL 0.64 mg/g).

3.6. Statistical Analysis

The results were statistically analyzed using StatSoft 6.0 (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) and Tukey's test at the 95% confidence level were performed to identify significant differences between the results obtained for each test ($p < 0.05$).

4. Conclusions

This novel study showed the feasibility of applying SFE to obtain red propolis extracts with high antioxidant potential and that it is important to consider aspects related to process parameters, such as the volume of CO_2 applied, addition of cosolvents and total yield isotherms. In the present study, the addition of ethanol as a cosolvent improved the extraction of bioactive compounds present in red propolis, including formononetin, the major compound of interest from this type of propolis. The best yield was obtained under the conditions of 40 °C and 450 bar using 4% ethanol as the cosolvent, a CO_2 flow rate of 6 g/min and S/F of 131. The best conditions to obtain extracts rich in phenolic compounds, flavonoids and antioxidant activity (represented by a low IC_{50}) were at a temperature of 50 °C and pressure of 450 bar. The intermediate conditions (40 °C and 350 bar) showed the greatest potential for obtaining high concentrations of formononetin and naringenin, as well as extracts with high antioxidant

capacity. Formononetin is considered to be the compound with the greatest pharmacological interest in red propolis. In general, the presence of these compounds (formononetin, naringenin and kaempferol) at high concentrations in the extracts obtained in this study demonstrates, despite the low total yield, that SFE using CO_2 is a promising alternative to obtain red propolis extracts with high added value. However, to obtain the specific bioactive compounds investigated in the present study, it is necessary to evaluate the individual properties of each one.

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Sample Availability: Not available.

Capítulo 6. Conclusão

7.0 Conclusão

Neste estudo foi possível determinar que:

- Extratos de própolis vermelha do Nordeste do Brasil apresentam uma composição química diferenciada e alto potencial biotecnológico;
- A composição da própolis vermelha brasileira oriunda do Nordeste do Brasil varia significativamente a depender da região geográfica de coleta, bem como o tipo de método de extração empregado e as condições de processo influenciam na obtenção dos compostos presentes na matriz investigada, bem como na capacidade antioxidante e atividade biológica dos extratos;
- A aplicação da tecnologia de ultrassom para obtenção de extratos de própolis vermelha com elevado teor de compostos ativos (antioxidantes) se mostrou eficiente quando comparada com a extração convencional, principalmente pelo menor tempo de obtenção dos extratos, o que seria vantajoso para uma aplicação em escala industrial;
- Extratos da própolis vermelha brasileira obtidos por extração convencional ou extração assistida por ultrassom podem atuar de forma seletiva contra células tumorais e apresentar potencial atividade antitumoral;
- A própolis vermelha pode ser uma matriz importante para o desenvolvimento de produtos na área de oncologia, e o crescimento das pesquisas nessa área corroboram com a importância dos resultados aqui demostrados. Entretanto, apesar dos importantes resultados *in vitro* encontrados, estudos futuros são necessários para avaliar o potencial biológico desses extratos com modelos *in vivo*.
- A extração com fluido supercrítico utilizando CO2 como fluido extrator se mostrou como uma técnica viável e sustentável para a obtenção de extratos

de própolis vermelha com elevado teor de formonometina, naringenina e kaempferol;

- Na SFE, a adição de etanol como co-solvente melhorou a extração de compostos bioativos presentes na própolis vermelha, incluindo a formononetina, principal composto de interesse desse tipo de própolis;
- Na SFE, as condições intermediárias de temperatura e pressão (40°C e 350 bar) apresentaram o maior potencial para a obtenção de altas concentrações de formononetina e naringenina, além de extratos com alta capacidade antioxidante;
- Apesar do baixo rendimento total para os extratos obtidos por SFE, essa tecnologia é uma alternativa promissora para a obtenção de extratos de própolis vermelha de alto valor agregados;
- Estudos futuros são necessários para avaliar a viabilidade econômica da aplicação da SFE para obtenção de extrato de própolis vermelha e ainda, avaliar a atividade biológica *in vitro* e *in vivo* do extrato obtido nas condições definidas neste estudo.

Capítulo 7

8.0 Produção Técnica e Científica

Artigos

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