



# ***Melipona Scutellaris* Geopropolis: Composição Química e Bioatividade**

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## Lista de abreviaturas

ATCC - Coleção American Type Culture

ITIS- Sistema Integrado de Informação Taxonómica

DPPH- 2,2-difenil-1-picrilhidrazil

HPLC- Cromatografia líquida de alta eficiência

IC50 – Concentração inibitória média

SLB- Abelhas sem ferrão

ABTS- ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico)

ORAC- Capacidade de Absorção de Radicais de Oxigénio

ADN- Ácido desoxirribonucleico

## RESUMO

A geoprópolis tem sido usada na medicina tradicional há séculos. Neste estudo foram avaliadas a origem botânica, as características físico-químicas, o perfil fenólico e as atividades biológicas da geoprópolis de *Melipona scutellaris* colhida nos períodos chuvoso e seco. A análise palinológica permitiu identificar mais de 50 tipos polínicos, sendo *Schinus terebinthifolius* e *Cecropia* os tipos predominantes. A geoprópolis da estação chuvosa apresentou maior teor total de fenóis e flavonoides (determinado por métodos convencionais – 25,13% e 3,92%, respectivamente) em comparação à estação seca (19,30% e 2,09%); os principais picos (determinados por Cromatografia líquida de alta Eficiência (naringina, ácido gálico e catequina) foram semelhantes nas amostras. A capacidade antioxidante foi avaliada através de ensaios de DPPH, poder redutor e descoloração de  $\beta$ -caroteno/ácido linoleico. As amostras da estação chuvosa apresentaram atividade antioxidante superior independentemente do método utilizado. O efeito antimicrobiano foi avaliado por microdiluição, enquanto o impacto na enzima colinesterase foi quantificado pela acumulação do ácido 5-tio 2-nitrobenzóico. As atividades anti-inflamatórias e antimutagênicas foram quantificadas através da inibição da enzima hialuronidase e utilizando células *Saccharomyces cerevisiae* ATCC-20113. Ambas as amostras exibiram propriedades anti-inflamatórias e antimutagênicas. Foi também, observada uma inibição significativa da acetilcolinesterase, com valores de IC50 de 0,35  $\mu\text{g/mL}$  durante a estação chuvosa e 0,28  $\mu\text{g/mL}$  durante a estação seca. Adicionalmente, a geoprópolis apresentou atividade antimicrobiana, principalmente contra *Staphylococcus aureus*, ATCC-4330. Os nossos resultados sugerem o potencial terapêutico da geoprópolis de *M. scutellaris* no contexto de doenças inflamatórias, oxidativas e infecciosas.

**Palavras-chave:** produtos apícolas; geoprópolis; produtos naturais; *Melipona Scutellaris*.

## ABSTRACT

Geopropolis has been used in traditional medicine for centuries. This study evaluated the botanical origin, physicochemical characteristics, phenolic profile and biological activities of *Melipona scutellaris* geopropolis collected during the rainy and dry seasons. The palynological analysis identified more than 50 pollen species, with *Schinus terebinthifolius* and *Cecropia* being the most abundant. The geopropolis from the wet season had a higher total phenolic and flavonoid content (determined by conventional methods - 25.13% and 3.92%, respectively) compared to the dry season (19.30% and 2.09%); the main peaks (determined by high performance liquid chromatography (HPLC) (naringin, gallic acid and catechin) were similar in the samples. Antioxidant capacity was assessed using DPPH, reducing power and  $\beta$ -carotene/lyclic acid decolorization tests. Rainy season samples showed higher antioxidant activity regardless of the method used. The antimicrobial effect was assessed by microdilution, while the effect on the enzyme cholinesterase was quantified by the accumulation of 5-thio-2-nitrobenzoic acid. The anti-inflammatory and antimutagenic activities were quantified by inhibiting the enzyme hyaluronidase and using *Saccharomyces cerevisiae* ATCC-20113 cells. Both samples showed anti-inflammatory and antimutagenic properties. Significant inhibition of acetylcholinesterase was also observed, with IC<sub>50</sub> values of 0.35  $\mu$ g/mL during wet and 0.28  $\mu$ g/mL during dry seasons. In addition, geopropolis showed antimicrobial activity, mainly against *Staphylococcus aureus* ATCC-4330. Our results suggest the therapeutic potential of *M. scutellaris* geopropolis in the context of inflammatory, oxidative and infectious diseases.

**Keywords:** bee products; geopropolis; natural products; *Melipona scutellaris*.

# Capítulo 1

Introdução

## **1. Abelhas – abelhas sem ferrão**

As abelhas e as plantas com flores evoluíram juntas ao longo de milhões de anos, estabelecendo uma relação de mutualismo. Existem aproximadamente 20.000 espécies de abelhas conhecidas no mundo (ITIS 2010), sendo a maioria delas de hábitos isolados. Cerca de 1.000 espécies são sociais, vivendo em colônias complexas (Imperatriz-Fonseca et al., 2010).

As abelhas desempenham um papel fundamental no meio ambiente, facilitando a polinização de plantas em áreas naturais e agrícolas. Essa atividade tem um impacto direto na produção de frutos e sementes, essencial para a manutenção dos ecossistemas e para a agricultura (Da Silva et al., 2020).

## **2. Geoprópolis**

As abelhas sociais nativas, conhecidas como meliponíneos ou abelhas sem ferrão, possuem características únicas que as distinguem de outras espécies. São denominadas "sem ferrão" devido à atrofia desta estrutura, o que as impossibilita de ferocar, mas a sua singularidade vai muito além deste detalhe anatômico. Tradicionalmente manuseadas por povos indígenas, também são designadas por "abelhas indígenas". Estas abelhas têm a capacidade de construir ninhos em locais variados, como troncos de árvores, fendas em pedras, no solo ou pendurados em ramos. Os favos das colônias são organizados em camadas horizontais sobrepostas, e possuem o hábito peculiar de misturar pólen ao mel. Outro aspecto distintivo é o destino dos machos: após fecundarem a rainha, são frequentemente expulsos ou perdem a sua função dentro da colônia.

Entre os inúmeros insetos polinizadores das regiões tropicais, as abelhas sem ferrão destacam-se pela sua elevada sociabilidade e pela intensa atividade das suas colônias. Com um hábito alimentar generalista, estas abelhas desempenham um papel crucial na manutenção de ecossistemas naturais e agrícolas. A sua contribuição para a polinização é impressionante, abrangendo entre 30% e 90% da flora nativa, evidenciando a sua importância ecológica e económica. (Santos et al., 2010)

Atualmente, foram descritas mais de 600 espécies de meliponíneos que estão distribuídas pelas regiões tropicais e subtropicais do planeta (América do Sul, América Central, Sul da América do Norte, África, Sudeste Asiático e Norte da Oceânia). No Brasil, existem mais de 200 espécies de meliponíneos, refletindo a diversidade e a importância ecológica destes polinizadores. De acordo com Pedro et al., 2014, aproximadamente 89 espécies de abelhas sem ferrão são endêmicas no Brasil, representando cerca de 20% do total de espécies neotropicais conhecidas.

Entre os géneros mais representativos destacam-se: *Plebeia*, *Trigona*, *Melipona*, *Scaptotrigona* e *Trigonisca* (Lavinias et al., 2019).

As abelhas meliponíneas são caracterizadas por possuírem um ferrão atrofiado, pelo que, não utilizam este órgão como arma defensiva. (da Silva Cruz et al., 2020).

A popularidade da apicultura com abelhas sem ferrão deve-se principalmente à segurança e à conveniência de instalar colmeias em diversos ambientes, como áreas naturais, agrícolas e urbanas. Algumas espécies de abelhas sem ferrão incorporam terra à resina das plantas, resultando na formação da geoprópolis. Este material é uma mistura complexa composta por resina e exsudatos vegetais provenientes de diversas fontes. Este produto resulta da combinação desses exsudatos com partículas adicionais, como cera, silte e areia (Bonsucesso et al., 2018).

A geoprópolis e o mel produzido por estas abelhas tem vindo a ser valorizado pelas suas propriedades biológicas. Estas, estão diretamente relacionadas à origem vegetal das resinas e são de grande interesse para as indústrias farmacêutica e alimentar, quer na formulação de medicamentos, quer como suplementos dietéticos (da Silva Cruz et al., 2020).

A geoprópolis tem sido usada empiricamente no tratamento de diversas condições de saúde, incluindo doenças respiratórias, distúrbios do trato digestivo, dermatoses e para o fortalecimento do sistema imunológico (Freitas et al., 2008; de Souza et al., 2018; Lavinias et al., 2019). A sua composição química e propriedades biológicas são influenciados por diversos fatores, entre os quais se destacam: localização geográfica, flora local, clima e espécies de abelhas envolvidas (Ferreira et al., 2019; da Silva Cruz et al., 2020;).

Os principais compostos isolados da geoprópolis incluem ácidos gordos, ácidos orgânicos, açúcares, álcoois e esteroides, além de polifenóis, triterpenos e saponinas (Dutra et al., 2019; Souza Júnior et al., 2019). Dentre estes, os polifenóis são particularmente importantes pela sua ação anti-inflamatória e pela sua capacidade de atuar como agentes anti carcinogénicos, bem como pela sua potencialidade na prevenção de doenças degenerativas e disfunções neurológicas (Ferreira et al., 2019).

Pelas razões enunciadas, a geoprópolis tem sido amplamente investigada quanto a uma variedade de propriedades farmacológicas, incluindo atividade antimicrobiana, citotóxica, anti tumoral, antioxidante, anti-inflamatória, imunomoduladora e gastro protetora (Santos et al., 2017; Oliveira et al., 2019; Souza Júnior et al., 2019).

Por outro lado, a análise polínica, em combinação com as análises físico-químicas, são uma abordagem robusta para a caracterização detalhada de amostras deste produto. A integração destas análises permite a avaliação de diversas características, como dureza, elasticidade, aroma e cor, facilitando uma compreensão abrangente das suas propriedades. Ao identificar e quantificar os

grãos de pólen presentes, contribui para a determinação da origem botânica e da diversidade floral que compõe este produto apícola. Juntas, estas análises para além de definirem a qualidade, fornecem também informações valiosas para a padronização e rotulagem dos produtos. Esta abordagem integrada é fundamental para garantir a autenticidade e a qualidade dos produtos, além de apoiar as atividades económicas associadas ao comércio da geoprópolis (Barros et al., 2013).

Os fatores que influenciam a preferência das abelhas na recolha de resina de fontes vegetais específicas ainda não são claros. No entanto, é reconhecido que as abelhas demonstram um comportamento seletivo em relação às fontes de resina que escolhem. Este comportamento pode ser influenciado por uma variedade de fatores, incluindo a disponibilidade de recursos, as suas características químicas e físicas e, a interação com outras fontes de alimento e materiais presentes na sua área de recolha (Salatino et al., 2005; Barros et al., 2013). Cerca de 5% do peso da geoprópolis é composto por grãos de pólen, que são frequentemente considerados contaminantes do produto. Estes grãos de pólen aderem à resina durante o transporte pelo vento e, são também provenientes das reservas alimentares das abelhas na colmeia (Barth et al., 1999; Barth & Luz, 2003). Adicionalmente, a presença de pólen na geoprópolis pode também servir como um importante indicador das fontes vegetais visitadas pelas abelhas durante a recolha da matéria-prima (Barros et al., 2013).

O aprofundamento dos estudos sobre a geoprópolis desta espécie específica é decisivo para entender melhor as suas propriedades e potencial terapêutico, especialmente considerando a importância associada à sua produção.

## **2.1. Origem botânica**

A palinologia é definida pela sua interdisciplinaridade e por dispor de várias aplicações, sendo considerada uma das ferramentas mais eficazes para a reconstituição de ambientes passados. O pólen é o principal recurso proteico usado pelas abelhas. Por esse motivo, estudos dos tipos polínicos recolhidos pelos meliponíneos são de extrema importância para determinar as suas relações com plantas encontradas numa determinada área.

O espetro polínico presente na geoprópolis contém quer grãos de pólen recolhidos pelas abelhas quer grãos de pólen anemófilos, transportados pelo vento e aderidos à resina. Assim, a análise polínica constitui uma ferramenta valiosa para a verificação e rotulagem de amostras deste produto apícola, uma vez que permite determinar a sua origem geográfica, identificando as diferentes regiões de produção e a estação do ano em que foram obtidas. (Barth 1998; da Silva et al., 2020). Este aspeto é também relevante quer para os incentivos dados ao desenvolvimento da meliponicultura, quer para a comercialização dos produtos produzidos por estes meliponídios,

pois estas características influenciam diretamente o valor deste produto no mercado (da Silva et al., 2020).

## 2.2. Propriedades Físico – Químicas

A geoprópolis, é uma substância natural extremamente complexa, composta por mais de 300 componentes que variam conforme a flora, o clima e a espécie de abelha presentes na região. Essa diversidade química confere à geoprópolis propriedades biológicas únicas, refletindo a adaptabilidade das abelhas (Sanches et al., 2017; Bonsucesso et al., 2018). Compreender a composição e as atividades biológicas da geoprópolis é essencial para estabelecer padrões de qualidade e reconhecer o seu potencial na saúde. A geoprópolis destaca-se, especialmente, pelas suas propriedades antimicrobianas, anti-inflamatórias e antioxidantes (Santos et al., 2017; Guzmán et al., 2018). Assim, a investigação não só enriquece o conhecimento acadêmico, mas também favorece aplicações práticas na saúde pública e na indústria farmacêutica (da Silva et al., 2023).

Na Tabela 1, apresentam-se as principais características físico-químicas da geoprópolis, incluindo cor, densidade, pH, teor de água, ácidos orgânicos e conteúdo de ceras. De acordo com os autores citados, estas propriedades conferem à geoprópolis características biológicas promissoras, as quais carecem ainda de investigação mais aprofundada. É fundamental realizar estudos adicionais para otimizar os métodos de análise, possibilitando a identificação de novas características que possam expandir a utilização da geoprópolis em diversos mercados. Estes estudos poderão contribuir ainda para o desenvolvimento de produtos mais adequados às exigências do consumidor, potencializando a sua aplicação em áreas como a saúde e alimentação.

**Tabela 1.** Propriedades físico-químicas da geoprópolis.

Propriedades	Método de Análise	Valores típicos	Referências
Cor	Método visual	Amarelo-esverdeado a negro	Souza et al., 2018 Oliveira et al., 2019
Densidade	Densitometria	1,1 - 1,3 g/cm <sup>3</sup>	Ferreira et al., 2019
pH	Medição de pH	3,5-5,0	Santos et al., 2017
Conteúdo de água	Secagem a 105°C e pesagem	10% - 20%	Dutra et al., 2019

<b>Ácidos orgânicos</b>	Cromatografia Líquida de Alta Eficiência (HPLC) Espectrofotometria	Varia conforme a origem	Souza et al., 2017 Ferreira et al., 2019
<b>Conteúdo de ceras</b>	Análise Gravimétrica	2% - 10%	Santos et al., 2017 Oliveira et al., 2019
<b>Conteúdo de pólen</b>	Análise Microscópica	Variável, dependendo da fonte	Santos et al., 2017 Oliveira et al., 2019

### 2.3. Composição Química

A composição química da geoprópolis é complexa e altamente diversificada. Vários estudos indicam a presença de diversos compostos polifenólicos, incluindo ácidos fenólicos, flavonoides e taninos (Silva et al., 2013; Souza et al., 2013; Dutra et al., 2014). Foram também encontrados terpenos (monoterpenos, sesquiterpenos, diterpenos e triterpenos), bem como ácidos gordos, esteroides e saponinas (Dutra et al., 2008; Cunha et al., 2009; Araújo et al., 2015). É relevante ressaltar que a composição química pode variar de acordo com a flora visitada pelas abelhas, bem como com a região geográfica e a época da colheita (Bankova, 2009; Ribeiro et al., 2013; Barth e Freitas, 2015). Esta variabilidade pode influenciar significativamente as propriedades e o potencial de utilização deste produto em diversas aplicações (Batista et al., 2016).

#### 2.3.1. Compostos fenólicos

Nos últimos anos, uma quantidade substancial de evidências tem indicado o papel chave dos radicais livres e outros oxidantes como grandes responsáveis pelo envelhecimento e pelas doenças degenerativas associadas ao mesmo, como o cancro, doenças cardiovasculares, cataratas, declínio do sistema imune e disfunções cerebrais (Sousa et al., 2007).

A resina, um dos principais constituintes presentes na colmeia, desempenha um papel multifuncional. É utilizada na construção e manutenção das estruturas da colmeia, atuando também como uma barreira de defesa contra invasores e microrganismos patogênicos. Além disso, a resina serve como uma importante fonte de compostos bioativos, que são aproveitados pelas abelhas no fabrico de diversos produtos, incluindo a geoprópolis (Leonhardt & Blüthgen, 2009; Souza et al., 2021; Rocha et al., 2023).

É importante destacar que a quantidade de resina produzida por uma planta não é o único fator que determina a sua seleção. Nem todas as espécies vegetais, mesmo aquelas que produzem grandes volumes de resina, atraem estes insetos. Segundo Lavinas et al., (2019) as abelhas são bastante seletivas, e fatores como a composição química da resina e a presença de compostos voláteis desempenham um papel crucial na escolha das plantas para forrageamento. De acordo com o reportado pelo mesmo autor, os terpenoides surgem como os principais constituintes voláteis envolvidos no processo de atração das abelhas (Rocha et al., 2023).

O conhecimento da origem botânica é fundamental, uma vez que existe uma correlação direta entre o perfil químico do produto e as espécies vegetais das quais as abelhas obtêm a resina. Sendo assim, compreender quais as plantas que são utilizadas permite, não apenas prever a sua composição química, mas também assegurar a qualidade e as propriedades terapêuticas do produto final (Daugsch et al., 2008; Rocha et al., 2023). Neste contexto, o estudo da origem botânica e dos fatores que influenciam a recolha de resina pelas abelhas é fundamental para compreender a variação química deste produto (Rocha et al., 2023).

Na Tabela 2, pode-se observar que, a geoprópolis apresenta compostos químicos (fenóis e flavonoides) que desempenham papéis essenciais na sua atividade biológica, particularmente nas capacidades antioxidante e antimicrobiana. Esses compostos são frequentemente estudados por diversos métodos analíticos, permitindo uma compreensão mais ampla das características e potenciais benefícios. Cada método de análise destaca aspetos diferentes dos compostos da geoprópolis, permitindo uma caracterização detalhada que orienta na sua aplicação prática e benefícios terapêuticos.

**Tabela 2.** Composição química e propriedades biológicas da geoprópolis.

<b>Propriedades</b>	<b>Método de Análise</b>	<b>Valores típicos</b>	<b>Referências</b>
<b>Teor fenóis totais</b>	Método de Folin-Ciocalteu e Cromatografia Líquida de Alta Eficiência (HPLC)	10% - 30% (ácido gálico)	Souza et al., 2017 Oliveira et al., 2019
<b>Conteúdo flavonoides</b>	Cromatografia Líquida de Alta Eficiência (HPLC) Método de AlCl <sub>3</sub>	2% - 5% (quercetina)	Souza et al., 2017 Oliveira et al., 2019
<b>Atividade Antioxidante</b>	Ensaio de DPPH, ABTS, ou ORAC	50% - 80% de inibição do DPPH	Oliveira et al., 2019 Ferreira et al., 2019
<b>Atividade Antimicrobiana</b>	Teste de Difusão em Agar ou Microdiluição	Inibição de crescimento de <i>Staphylococcus aureus</i>	Souza et al., 2018 Dutra et al., 2019

## 2.4. Propriedades Bioativas

A geoprópolis, tem destaque crescente na literatura devido às suas propriedades biológicas diferenciadas, resultantes da sua composição química singular, que combina resinas vegetais, cera e solo. Estas características conferem à geoprópolis um notável potencial terapêutico, com destaque para suas atividades antimicrobiana, antioxidante, anti-inflamatória entre outras. Devido à sua complexidade química e biológica, a geoprópolis emerge como um recurso natural promissor.

### 2.4.1. Atividades anti-inflamatória e antioxidante

Todos os organismos vivos, possuem mecanismos adaptativos para responder a estímulos no sentido de manter o equilíbrio homeostático. Nos vertebrados, esta resposta inclui uma série de alterações bioquímicas, fisiológicas e imunológicas coletivamente denominadas inflamação (Voltarelli et al., 1994).

Os neutrófilos têm um papel fundamental na defesa do organismo durante o processo inflamatório. O desenvolvimento de novos fármacos anti-inflamatórios que interfiram no movimento de neutrófilos no foco inflamatório tem despertado grande interesse entre os investigadores, e as estratégias procuradas são: inibição da transmigração e adesão dos

neutrófilos; bloqueio de moléculas envolvidas nesse processo; e estimulação de mediadores que inibem o processo (Franchin et al., 2013).

A acumulação excessiva de radicais livres no organismo, sem a presença de um sistema eficiente de neutralização pelos agentes antioxidantes, desencadeia o processo de stress oxidativo, que é responsável por causar danos a diversas biomoléculas celulares, como ácidos nucleicos, proteínas e ADN. Esse processo está frequentemente associado ao desenvolvimento de doenças neurodegenerativas, como Alzheimer e Parkinson, além de estar relacionado a patologias como o cancro, diabetes, aterosclerose e inflamação crónica (Santos et al., 2017).

Em condições fisiológicas normais, estes processos oxidativos e imunológicos atuam de maneira benéfica na resposta contra agentes patogénicos. No entanto, a ativação constante desses mecanismos podem aumentar o risco de doenças graves, como diabetes e obesidade. Neste contexto, os produtos naturais têm despertado grande interesse na pesquisa de novos fármacos com atividade biológica. Entre eles o extrato de geoprópolis de *Melipona scutellaris*, que apresentou ação anti-inflamatória significativa, além de reduzir a migração de neutrófilos em estudos *in vivo*. Outros estudos indicaram que a geoprópolis exerce atividade anti-inflamatória por meio da inibição da enzima hialuronidase, o que resulta na redução da degradação do ácido hialurónico. Este polissacarídeo desempenha funções biológicas cruciais, como a proliferação celular, diferenciação e reparação de tecidos (Santos et al., 2017). Dessa forma, a identificação de compostos com propriedades antioxidantes e antimutagénicas revela-se de grande importância terapêutica (Santos et al., 2017).

#### **2.4.2. Capacidade antimicrobiana**

A resistência antimicrobiana constitui um problema de saúde pública cada vez mais preocupante, especialmente em contextos hospitalares. Nos últimos anos, tem-se verificado uma redução significativa na eficácia dos antibióticos no tratamento de infeções comuns, o que tem favorecido o aparecimento de surtos causados por estirpes bacterianas multirresistentes. Neste cenário, os produtos naturais emergem como alternativas promissoras no combate a microrganismos resistentes, sobretudo devido aos efeitos sinérgicos resultantes da combinação dos seus compostos bioativos (Santos et al., 2017).

Em diversos países, a geoprópolis tem sido tradicionalmente utilizada pela população como agente cicatrizante, bem como no tratamento de gastrite e como antibacteriano. Estudos realizados com extratos etanólicos de geoprópolis produzidos pelas espécies *Melipona compressipes* e *Melipona quadrifasciata anthidioides* evidenciaram a sua significativa atividade antimicrobiana (Liberio et al., 2011). Do referido anteriormente, convém salientar que, as características

geográficas e as espécies de abelhas envolvidas influenciam de forma significativa a composição química e biológica deste produto (Acaroz et al., 2024).

Estudos sobre as atividades biológicas da geoprópolis, indicam que possui propriedades antivirais, anticancerígenas, anti-inflamatórias e antioxidantes idênticas às da própolis (Acaroz et al., 2024).

Velikova et al., 2000, descreveram a atividade antimicrobiana de amostras de geoprópolis brasileira contra *Staphylococcus aureus* e *Escherichia coli*, sugerindo que esta propriedade pode ser atribuída à presença de compostos não polares. Liberio et al., 2011 demonstraram que a geoprópolis do Maranhão, Brasil, recolhida por *Melipona fasciculata*, apresentou atividade antimicrobiana significativa contra *S. aureus* e *Candida albicans*, além de exibir efeitos bactericidas sobre biofilmes de *Streptococcus mutans*. No entanto, a atividade antimicrobiana foi observada principalmente nas amostras com maior concentração de flavonoides. Dado o perfil antimicrobiano promissor da geoprópolis recolhida por esta espécie de abelha, a elucidação detalhada das suas características biológicas e químicas torna-se essencial para explorar o potencial de utilização deste produto, como fonte para o desenvolvimento de medicamentos ou alimentos funcionais (da Cunha et al., 2013).

Noutro estudo, a geoprópolis revelou um efeito gastroprotetor significativo em ratos com lesões na mucosa gástrica, induzidas por etanol. Os resultados sugerem que este efeito protetor pode estar relacionado com mecanismos que envolvem a participação do óxido nítrico e das prostaglandinas, compostos fundamentais na preservação da integridade da mucosa gástrica (Ribeiro-Junior et al., 2015).

Na literatura ainda são reportadas outras capacidades da geoprópolis, em particular antivirais e anticancerígenas (Acaroz et al., 2024).

### **2.4.3. Propriedades antimutagénicas**

Os organismos vivos dispõem de mecanismos eficientes de reparação de mutações no ADN, contudo, esses sistemas nem sempre conseguem corrigir todas as alterações que ocorrem, o que pode deixar as células vulneráveis a danos genéticos e ao desenvolvimento de patologias associadas. Determinadas modificações no material genético estão fortemente associadas à origem e progressão de diversos tipos de cancro. Com efeito, compostos com atividade antimutagénica desempenham um papel crucial na prevenção desta doença, ao contribuírem para a proteção e estabilidade do genoma (Dos Santos et al., 2017).

O pólen de abelha, componente muito importante na geoprópolis, é considerado um alimento saudável com um vasto leque de propriedades terapêuticas (Bariliak et al., 1996; Viuda-Martos et al., 2008; Abdella et al., 2009; Fatrcová-Šramková et al., 2013). Para além disso, tem sido referido que este produto desencadeia efeitos benéficos na prevenção de problemas da próstata, arteriosclerose, gastroenterite, doenças respiratórias, melhoria dos sistemas cardiovascular e digestivo, imunidade do organismo e retardamento do envelhecimento (Estevinho et al., 2012). A promoção da reparação dos tecidos, que resulta da aceleração da taxa mitótica, também tem sido referenciada (Morais et al., 2011). Estes efeitos terapêuticos e protetores têm sido relacionados com o teor de polifenóis (Almeida-Muradian et al., 2005; Pascoal et al., 2014).

Recentemente, tem sido dada uma ênfase considerável à utilização de constituintes na dieta como medida químico-protetora para o controlo de doenças neoplásicas e genéticas (Abdella et al., 2009). Alguns estudos reportam as propriedades antimutagénicas de diversos produtos da apicultura contra a influência de alguns agentes mutagénicos químicos e físicos (Bariliak et al., 1996). A maioria dos agentes protetores atua diretamente sobre os compostos mutagénicos, neutralizando os radicais livres e as espécies reativas de oxigénio produzidas, ou inibindo o metabolismo mediado pelo citocromo P450. O mecanismo específico de proteção dos produtos da colmeia envolve a eliminação de eletrófilos e radicais livres potencialmente tóxicos e mutagénicos e a modificação das vias antioxidantes (Ohta et al., 2007; Pascoal et al., 2014).

#### **2.4.4. Inibição da acetilcolinestraxe**

Os processos neurodegenerativos são doenças do sistema nervoso central, que se caracterizam pela perda de estrutura e função neuronais. O aumento da esperança de vida agravou problemas de saúde como a doença de Alzheimer, a esclerose múltipla, a doença de Parkinson e a esclerose lateral amiotrófica, que são algumas das doenças neurodegenerativas que afetam principalmente a população idosa (Chen et al., 2017). O aparecimento destas doenças corresponde a uma incapacidade de resposta por parte dos neurónios de forma adaptativa ao aumento do stress oxidativo e neuro inflamatório relacionados com a idade, seguido da acumulação de proteínas danificadas, ADN e fragmentos de membrana (Patel et al., 2024)

A acetilcolina está envolvida na transferência de sinais nas sinapses e, após transferir os sinais, é normalmente hidrolisada, dando colina e acetato numa reação catalisada pela enzima acetilcolinesterase. Esta enzima associada à superfície da membrana extracelular desempenha um papel importante na proteção das células cerebrais (Mata et al., 2007).

Nas duas últimas décadas, a investigação tem-se centrado na descoberta e conceção de novos agentes neuroprotetores com elevada eficácia e baixo custo. Deste modo, substâncias neuroprotetoras naturais com potentes efeitos antioxidantes e anti-inflamatórios podem ser promissoras (Rios 2016, Solayman et al., 2017; Patel et al., 2024; Rios 2016).

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# Capítulo 2

**Objetivos**

## **2.1. Importância deste trabalho**

Apesar da correlação entre os compostos bioativos e as suas fontes vegetais, existe falta de informações sobre as plantas específicas utilizadas pelas abelhas para a produção de geoprópolis no Brasil. Além disso, não há estudos sobre o efeito da estação do ano na qualidade e bioatividade da geoprópolis. O objetivo principal deste trabalho foi avaliar o efeito das condições climáticas sobre o perfil polínico, características físico-químicas, perfil fenólico, atividades biológicas e atividade mutagénica.

## **2.2. Objetivo Geral**

Investigar as propriedades e o potencial terapêutico da geoprópolis de *Melipona scutellaris*, com ênfase na sua composição química e atividade biológica.

## **2.3. Objetivos Específicos**

1. Determinar a origem botânica;
2. Avaliar as características físico químicas;
3. Caracterizar o perfil fenólico;
4. Avaliar a capacidade antioxidante;
5. Analisar a atividade antimicrobiana;
6. Investigar as atividades anti-inflamatória e anti-mutagénica da geoprópolis;
7. Avaliar a inibição da enzima acetilcolinesterase.

# Capítulo 3

*Melipona scutellaris* Geopropolis:  
Chemical Composition and Bioactivity



## Article

# Melipona scutellaris Geopropolis: Chemical Composition and Bioactivity

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**Abstract:** Geopropolis has been used in traditional medicine for centuries. In this study, the botanical origin, physicochemical profile, and biological activities of geopropolis from *Melipona scutellaris* harvested during rainy and dry seasons were investigated. Palynological analysis identified over 50 pollen types, with *Schinus terebinthifolius* and *Cecropia* being the predominant types. The analytical results were in line with those reported in the literature. Rainy-season geopropolis exhibited higher total phenol and flavonoid content (determined using High Performance Liquid Chromatography—25.13% and 3.92%, respectively) compared to the dry season (19.30% and 2.09%); the major peaks (naringin, gallic acid, and catechin) were similar among samples. Antioxidant capacity was assessed via DPPH, reducing power, and  $\beta$ -carotene/linoleic acid discoloration assays. Rainy-season samples displayed superior antioxidant activity across methods. Antimicrobial effects were determined using microdilution, while the impact on the cholinesterase enzyme was quantified using 5-thio-2-nitrobenzoic acid accumulation. Anti-inflammatory and antimutagenic activities were assessed through hyaluronidase enzyme inhibition and by utilizing *Saccharomyces cerevisiae* ATCC-20113 cells. Both samples exhibited anti-inflammatory and antimutagenic properties. Moreover, a significant inhibition of acetylcholinesterase was observed, with IC<sub>50</sub> values of 0.35  $\mu\text{g}/\text{mL}$  during the rainy season and 0.28  $\mu\text{g}/\text{mL}$  during the dry season. Additionally, the geopropolis displayed antimicrobial activity, particularly against *Staphylococcus aureus*. These findings suggest the therapeutic potential of *M. scutellaris* geopropolis in the context of inflammatory, oxidative, and infectious diseases.

**Keywords:** beehive products; beehive products; geopropolis; natural products



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## 1. Introduction

In Brazil, geopropolis has been used as a traditional medicine for centuries, being used to treat a variety of ailments including respiratory and gastrointestinal illnesses. Propolis and geopropolis are both resins collected by bees for hive construction and protection. While they share similarities, they are distinct in composition. Propolis, produced by honeybees from the *Apis* family, is a mixture of tree resins, wax, enzymes, salivary secretions, and other organic ingredients. On the other hand, geopropolis is created by stingless bees of the *Melipona* family, which mix propolis with clay or soil. As a result, geopropolis has a powdery texture, an earthy aroma, and a higher mineral content [1]. The chemical composition of propolis and geopropolis is highly complex and influenced by various factors, including geographic origin, the available plant sources for the bees, bee species,

and soil type [1,2]. Brazil, being a megadiverse country with multiple geomorphological and climatic conditions, harbors more than 200 species, with around 20% of them being endemic [3]. According to Turco et al. [4], different *Meliponinis* species exhibit selective foraging behaviors, with *M. quadrifasciata* collecting resins from a wider range of plants and *Tetragonisca angustula* being more specific in their choice of vegetal sources. These variables contribute to the existence of numerous types of geopropolis and propolis, each with a chemical composition that is both qualitatively and quantitatively unique [4–6]. These substances typically contain phenylpropanoids, flavonoids, phenolic acids, hydrolysable tannins, triterpenes, saponins, alkaloids, lipids, and other compounds [1,2,4,6–8], which are associated with biological activities such as antioxidant [6–9], anti-inflammatory [6,9], antimicrobial [6,9] and anticancer [6,10] activities. Geopropolis is undoubtedly a great source of molecules with different biological properties, being used in traditional medicine of different peoples, and there are possibilities for new discoveries, with the search for phenolic compounds being the most promising [11].

Despite the correlation between bioactive compounds and their plant sources, there is a lack of information regarding the specific plants used by bees for geopropolis production in Brazil. Furthermore, there has been no study on the effect of the season on the quality and bioactivity of geopropolis. The main objective of this work was to evaluate the effect of climatic conditions on the pollen profile, physicochemical characteristics, phenolic profile, biological activities, and mutagenic activity.

## 2. Materials and Methods

In this study, several samples of geopropolis produced by *Melipona scutellaris* were collected, some in the rainy season (months between April and September) and others in the dry season (months between October and March). Geopropolis was collected at different times of the year, as these temporal variations can exert an influence on its attributes, thereby impacting the therapeutic qualities of geopropolis. The chemical compounds of geopropolis depend on the plant sources, geographical zone, season, and bee species. As the amount of geopropolis produced by stingless bees is very small, for the realization of the work, two composite samples—one consisting of the samples from the dry season and the other from the rainy season—were made. The samples were obtained from the meliponary of Fazenda São Lucas (12°01'786" S and 38°04'366" W), located in a region with predominance of secondary Atlantic Forest, in the municipality of Entre Rios. This municipality is located on the BR-110 highway between Salvador and Alagoinhas in the state of Bahia, Northeast Brazil. The state of Bahia has about 564,733.081 km<sup>2</sup>, with 417 municipalities, occupying approximately 36.4% of the Northeast region [12].

### 2.1. Reagents

Methanol and ethanol were obtained from Pronolab (Lisbon, Portugal). Culture media (Mueller Hinton) was obtained from Sigma-Aldrich (St. Louis, MO, USA). TTC solution (2,3,4-triphenyl-2H-tetrazolium chloride) was from Fluka (Buchs, Switzerland). Folin-Ciocalteu, DPPH, chloroform, sodium carbonate, calcium chloride, quercetin, gentamicin, amphotericin, linoleic acid, glacial acetic acid, hydrochloric acid (HCL), acetate buffer, and the other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The methanol used in the HPLC analysis was HiPerSolv CHROMANORM, 99.8% pure (VWR BDH Prolabo, Lutterworth, UK), and the formic acid came from Merck (Darmstadt, Germany). The standards for HPLC analysis, as the gallic acid (≥99%), protocatechuic acid (99.63%), (+)-catechin (≥98%), (–)-epicatechin (≥97%), vanillic acid (≥97%), resorcinic acid (≥97%), chlorogenic acid (>95%), caffeic acid (≥98%), sirinic acid (≥98%), p-coumaric acid (≥98%), ferulic acid (≥99%), sinapic acid (≥99%), rutin hydrate (≥94%), quercetin (95%) kaempferol (≥98%), naringin (≥95%), naringenin (98%), and cinnamic acid (≥99%), were purchased from Sigma-Aldrich (Steinheim, Germany). All enzymes were purchased from Sigma-Aldrich (Steinheim, Germany).

Ultra-pure water (18 MΩcm) was obtained in a Mili-Q purification system (Millipore, Bedford, MA, USA).

## 2.2. Palynological Analysis

The samples were processed according to the methodology described in [13]. For the palynological census, a minimum of 500 pollen grains per geopropolis sample was established. The identification of the botanical affinity of the pollen types was performed according to the indications of [14]. The number of samples in which a given pollen type was present (distribution frequency) was also observed. According to [15], samples were categorized into the following frequency classes: very frequent—>50%; frequent—20–50%; infrequent—10–20%, and rare—<10%. The pollen grains were identified taxonomically with the aid of the slides deposited in the palynoteca of the Laboratory of Plant Micromorphology (UEFS), in which all the prepared slides were also deposited. Catalogs and other works were also used to assist in the identification of the botanical origins of the pollen types. After completion of the analysis, the main pollen types were photomicrographed under an optical microscope.

## 2.3. Physico-Chemical Analysis

As reported by [16], the following parameters were evaluated: moisture, pH, conductivity, ash, and wax content. All analyses were performed in triplicate.

The determination of moisture was ascertained using the standard method (AOAC Official Method 934.01). Five grams of geopropolis were dried in a mechanical convection oven at 105° for 1 h. After this time, it was removed and let to cool at room temperature and weight back. The water content was determined using an equation in which A1 = weigh of sample and A2 = weigh of sample dried: Moisture (%) =  $100 \times (A1 - A2) / A1$ .

The pH of geopropolis was measured with a combined pH glass electrode connected to pH-meter Basic 20 in a solution prepared with 10 g of geopropolis in 75 mL of methanol (NP 1309/1976), using methanol as control. Calibration was performed with three standard buffer solutions.

Electrical conductivity of a geopropolis solution at 20% (*w/v*) (dry matter basis) in methanol was measured at 20 °C in a Crison 522 conductimeter. Results were expressed in milliSiemens per centimeter (mS/cm).

The method used in the experiments to determine the mineral content and other inorganic matter in geopropolis consisted of the desiccation of an amount of 5 g, for each geopropolis sample, in a platinum dish (AOAC Official Method 920.181). To do so, they were kept in the thermostat at 80 °C for 4 h, after which the samples underwent calcination at 550 °C in an electric laboratory furnace, SNOL 8.2/1100-1 (AB “Umega”, Utena, Lithuania), to constant mass. Total ash content, expressed as the percentage of residue left after dry oxidation by weight (%), was calculated from the following equation, where m1 is the mass of dish and ash, m2 is the mass of platinum dish prior to calcination, and m0 is the mass of the propolis taken: Ash (%) =  $(m1 - m2 / m0) \times 100$ .

For wax determination, we weighed 250 g of each sample and added 750 mL of methanol. The mixture was placed in a freezer overnight (−20 °C). Afterwards, the solution was filtered to obtain the wax. The wax was expressed in percentage (W%) using the sample weigh (SW) and the wax weigh (WW).

The equation used was  $W (\%) = WW / SW \times 100$ .

## 2.4. Preparation of Ethanolic Extracts

The geopropolis samples were frozen at −4 °C, crushed, and sieved to a homogeneous powder. Subsequently, 15 g of the samples was diluted in 240 mL of 70% ethanol. The mixture was stirred (200 rpm) for 24 h at room temperature, after which it was refrigerated for 12 h, filtered, rotoevaporated at 40 °C, and lyophilized [17]. The yields were 3.62 g (summer sample) and 4.05 g (winter sample).

## 2.5. Quantification of Total Phenols and Flavonoid Content

### (a) Determination of total phenols

Total phenolic compounds were determined according to the Folin–Ciocalteu colorimetric method [16]. A volume of 0.5 mL of the geopropolis extract (100 µg/mL) was mixed with 2.5 mL of Folin–Ciocalteu reagent and 2.0 mL of 14% sodium carbonate solution (Na<sub>2</sub>CO<sub>3</sub>). The mixture was incubated for 2 h in the dark at room temperature. After this period, the absorbance was read at 760 nm. The calibration curve was made using gallic acid (0.3–12 µg/mL) as standard. The results, obtained in triplicate, were expressed in percentage format (%).

### (b) Determination of total flavonoid content

The evaluation of total flavonoid content was performed as described by Castro et al. [18] with slight modifications. A quantity of 0.5 mL of extract (from 0.1 to 200 µg/mL) was diluted with 2.5 mL of ethanol solution of aluminium chloride hexahydrate 2% (AlCl<sub>3</sub>·6H<sub>2</sub>O). Then, after incubation for 1 h at room temperature, the absorbance of the mixture was read at 420 nm. Quercetin (0.3–18 µg/mL) was used as standard. The flavonoid content results, obtained in triplicate, were expressed in %.

## 2.6. Antioxidant Activity

The huge diversity of antioxidant compounds present in natural products makes it difficult to separate and study them individually. Indeed, individual antioxidant compounds do not necessarily reflect the total antioxidant capacity of the product, as there may be synergistic or antagonistic interactions between the different compounds present. The methods often used to determine *in vitro* antioxidant capacity can be broadly divided into two categories: hydrogen atom transfer (HAT) reaction-based assays and electron transfer (ET) reaction-based assays. In our study we used three methodologies to evaluate the antioxidant capacity of geopropolis extracts, two based on TE (DPPH radical scavenging activity assay and reducing power) and one based on HAT (β-carotene decolorization inhibition assay) [17,19].

### (a) Scavenging of DPPH radicals

In the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity (RSA) assays, the methodology described by Moreira et al. [20] was followed. An aliquot of 0.3 mL of geopropolis extract (concentrations ranged from 0.1 to 175 µg/mL) was mixed with 2.7 mL of DPPH reagent (2.0 × 10<sup>-4</sup> M). The mixture was left to stand in the dark for 60 min. After this time, the absorbance of the solutions, read at 517 nm using ascorbic acid, was used as the standard to establish the calibration curve. The IC<sub>50</sub> was calculated as the percentage of decolorization of the DPPH solution, according to the following equation:

$$\% \text{ IC}_{50} = [(A_{517} \text{ S} - A_{517} \text{ DPPH}) / A_{517} \text{ DPPH}] \times 100$$

Here, A<sub>517</sub> S-corresponds to the absorbance of the DPPH solution in the presence of different concentrations of extract and A<sub>517</sub> DPPH to the absorbance of the blank (DPPH solution plus extraction solvent instead of extract solution).

### (b) Reducing power

This methodology was based on the protocol reported by Moreira et al. [20] (slightly modified). A volume of 0.25 mL (1 mg/mL) of the sample extract was mixed with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide. The mixture was placed in a bain Marie at 50 °C for 20 min. Then, after 1.25 mL of 10% trichloroacetic acid was added to this solution, it was centrifuged at 3000 rpm for 10 min. Subsequently, from the supernatant, 1.25 mL was removed and added to 1.25 mL of deionised water and 0.25 mL of 0.1% FeCl<sub>3</sub>. The absorbance was read at 700 nm and the results were noted. The concentration of extract that induced a 50% inhibition percentage (EC<sub>50</sub>) was calculated

from the plot of absorbance (700 nm) as a function of extract concentration in the solution. BHA (Butylated hydroxyanisole) was used as a control.

### (c) Discoloration of $\beta$ -carotene/linoleic acid

This assay was performed according to the method described by Ahn et al. [21] with some modifications.

Before starting the test, the chloroform solution of  $\beta$ -carotene was prepared (1 mg of  $\beta$ -carotene dissolved in 5 mL of chloroform). Then, 1 mL of this solution was mixed with 20 mg of linoleic acid and 200 mg of Tween 40. After extracting the chloroform using a rotary evaporator at 40 °C, 50 mL of oxygenated distilled water was slowly added to the mixture and the flask was shaken vigorously to form an emulsion. For this test, 250  $\mu$ L of the emulsion was added to 30  $\mu$ L of the geopropolis extract in a microplate. Absorbance measurements were taken at 492 nm immediately after adding the emulsion to the extract, as well as again after 120 min. The microplate was placed on a shaker at 50 °C. As a control, 30  $\mu$ L of the solvent (80% ethanol) was incubated with 250  $\mu$ L of the emulsion. Independent experiments were carried out in triplicate.

The inhibition of  $\beta$ -carotene decolorization was calculated using the following equation:

$$\% \text{ Discoloration} = [(Abs_{470} \text{ after 2 h} / Abs_{470} \text{ initial}) \times 100]$$

### 2.7. Anti-Inflammatory Activity

Anti-inflammatory activity was assessed via inhibition of hyaluronidase enzyme, using the method described by Sahasrabudhe et al. [22]. A volume of 150  $\mu$ L of bovine hyaluronidase enzyme (7900 units/mL) was dissolved in acetate buffer (pH 3.6). Subsequently, 25 mL of geopropolis extract (0.2–100 mg/mL) was added and the mixture was incubated for 30 min at 37 °C. Then, 50  $\mu$ L of calcium chloride (12.5 mM) was added and incubated again under the same conditions. After this period, 250  $\mu$ L of sodium hyaluronate (1.2 mg/mL) was added and the mixture was incubated again at 37 °C for 1.5 h. After this period, 50 mL of 0.4 M sodium hydroxide and 100 mL of 0.6 M sodium borate were added and placed in a water bath for 3 min. Finally, it was cooled on ice and 1.5 mL of PDMAB (p-dimethylaminobenzaldehyde) was added (4 g PDMAB dissolved in 50 mL of 10 M HCl and 350 mL of glacial acetic acid). The absorbance was measured at 585 nm (spectrophotometer: UV-VIS 3100PC). The assay was performed in triplicate. Epigallocatechin was used as control. The percentage of hyaluronidase enzyme inhibition was calculated according to the following equation:

$$\text{Inhibition of the enzyme hyaluronidase (\%)} = ((Abs_{600} \text{ control} - Abs_{600} \text{ sample}) / Abs_{600} \text{ control}) \times 100$$

### 2.8. Antimicrobial Activity

The biological material used in this work comprised strains of *Staphylococcus aureus* ATCC provided by the American Type Culture Collection 43300 (Gram-positive bacteria) and *Proteus mirabilis* ATCC 10231 (Gram-negative bacteria) and *Candida albicans* (yeast) ATCC 25933 from the collection (LGC Standards SLU, Barcelona) ATCC. Clinical isolates from Centro Hospitalar do Nordeste (Portugal), identified at Escola Superior Agrária do Instituto Politécnico de Bragança using molecular biology techniques, were also used. *S. aureus* ESA 321 was isolated from purulent exudates, *P. mirabilis* ESA 229 was isolated from an algalia (Foley probe), and *C. albicans* ESA 115 was isolated from sputum.

Mueller Hinton Broth (MHB, Sigma) and Mammalian Cell Culture Media (RPMI, Sigma) were employed to determine the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC). The MIC value represents the lowest concentration of geopropolis extract capable of inhibiting microbial growth (including bacteria and yeast), and MBC, the lowest concentration of the geopropolis extract that prevents microbial growth and reduces initial viability by at least 99.9%. The methodology used closely followed that outlined in the work of Moaris et al. [19], with certain modifications. Geopropolis extract samples were dissolved in 50% DMSO and Mueller–Hinton medium

for bacterial testing, and in 50% DMSO and Mammalian Cell Culture Media for yeast testing, resulting in a final concentration of 20 mg/mL.

In each well of the microplate, 100  $\mu$ L of MHB and RPMI medium were placed for bacteria and yeasts, respectively. Then, 50  $\mu$ L of the different concentrations of extract to be tested and 50  $\mu$ L of cell suspensions ( $1 \times 10^5$  for bacteria and  $1 \times 10^3$  for yeasts, grown in appropriate medium overnight) were introduced into the various wells. After being covered with sterile semi-permeable film, the microplates were incubated at 37 °C for 24 h. At the end of this time, the optical density was determined at 600 nm and 540 nm, respectively for yeasts and bacteria.

To determine the MIC, 20  $\mu$ L samples from both the last well where color change was observed as well as from all wells that remained unchanged were inoculated onto plates with MHB for bacteria or RPMI for yeast and incubated at 37 °C for 24 or 48 h. The lowest concentration that did not result in growth after this subculture was considered the MIC. All assays were performed in triplicate.

### 2.9. Antimutagenic Activity

The antimutagenic capacity of geopropolis extracts was determined using yeast cells—D7 diploid strain of *Saccharomyces cerevisiae* (American Type Culture Collection, ATCC 201137) [23]. Before starting the tests with geopropolis, the *Saccharomyces cerevisiae* colonies were evaluated for the frequency of spontaneous gene conversions at the tryptophan locus and reverse mutations at the isoleucine locus. Cultured cells with low spontaneous gene conversion and low reverse mutation frequency were inoculated into liquid medium at 28 °C until they reached stationary growth phase. The cultures were centrifuged and resuspended in sterile potassium phosphate buffer (0.1 M), pH 7.4, until a cell concentration of  $2 \times 10^8$  cells/mL was obtained. Subsequently, the mutagen ethyl methanesulphonate (EMS) (5 mg/mL) and the extract, evaluated at concentrations of 2.5 and 5.0 mg/mL, were added.

The mixture was incubated with stirring for 2 h at 37 °C. After this period, the cells were placed in a complete and selective incubator to determine yeast survival, tryptophan converters, and isoleucine reversers. The experiments were carried out in triplicate.

### 2.10. Inhibition of Acetylcholinesterase

The enzyme acetylcholinesterase converts acetylcholine into acetic acid and thiocoline. The latter compound reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) with formation of 5-thio-2-nitrobenzoic acid. The accumulation of 5-thio-2-nitrobenzoic acid was assessed at 405 nm. The acetylcholinesterase inhibition was determined spectrophotometrically, as described by Mata et al. [24]. The 5,5-dithiobis-(2-nitrobenzoic acid) (15 mM, DTNB, Sigma Chemical, St. Louis, MO, USA) and the electroreflective acetylcholinesterase substrate (electric eel Type-VI-S, Sigma Chemical, St. Louis, MO, USA) were used. Acetylcholinesterase hydrolysis was assessed through the formation of 2-nitro-5-thiobenzoate anion (yellow) at 412 nm for 15 min. To determine the percentage of inhibition, the reaction speeds of the samples were compared with those of the blank (ethanol in 0.2 M of phosphate buffer, pH = 8), using the expression  $((E - S)/E) \times 100$ , where E is the enzyme activity without the extract and S is the activity of the sample. The IC<sub>50</sub> values were estimated from the graph of the inhibition percentage as a function of the sample concentration. Eserine was used as a control.

### 2.11. Identification and Quantification of Compounds

Identification and quantification of phenolic compounds were performed according to the method previously described by Nastić et al. [25]. The detection of the phenolic profile of the extracts was obtained via high performance liquid chromatography with UV detection (HPLC/UV). The analyses were carried out at the Laboratory of the Materials Center of the University of Porto/CEMUP. Geopropolis samples were solubilized in HPLC grade methanol to a final concentration of 10 mg/mL, filtered through 0.2  $\mu$ m Millipore nylon filters, and stored in 2.5 mL amber glass vials.

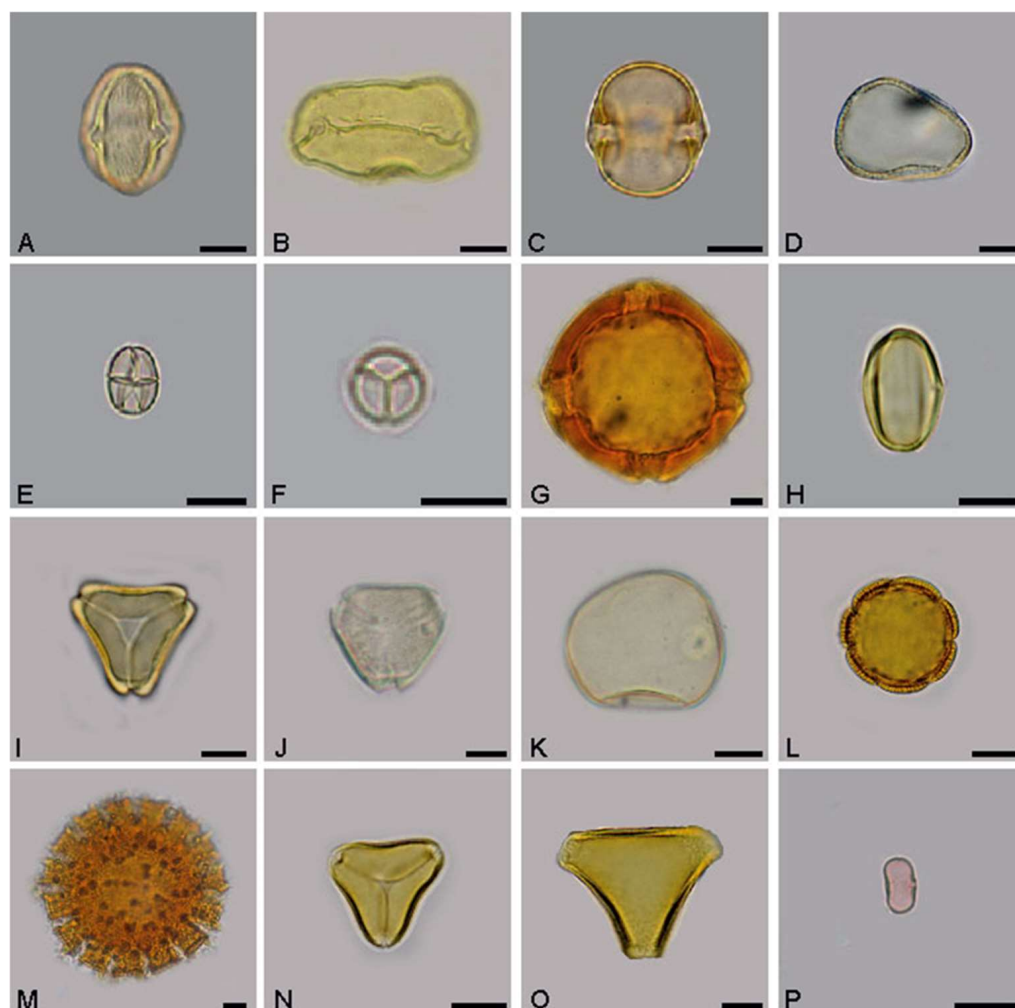
The quantification and separation of phenolic compounds by HPLC-UV was performed based on the procedure described by Rubilar et al. [26], with some modifications. The chromatograph used was a Shimadzu (LabSolutions Software, version 5.3) with column oven (CTO-10AS vp), injection system (LC-20AD), auto sampler (SIL-20A ht), and photodiode array detector (SPD-M20A), equipped with a Phenomenex Gemini C18 reverse-phase column (250 × 4.6 mm; 5 µm). The extracts were filtered through 0.22 µm nylon filters (Millipore) and a volume of 20 µL was injected. The mobile phase consisted of water (solvent A) and methanol (solvent B), both acidified with 0.1% (v/v) formic acid, at a flow rate of 1.0 mL/min in gradient mode as follows: 0 min, 15% of B in A; 20 min, 30% of B in A; 40 min, 45% of B in A; 45 min, 50% of B in A; 50 min, 55% of B in A; 65 min, 70% of B in A; 75 min, 100% of B; and 100% of B. This was maintained (75–80 min); the mobile phase composition returned to the initial conditions within the next ten minutes and was kept for another 10 min for stabilization before the next injection. The eluents were filtered through a nylon filter (0.20 µm pore size, Supelco, Bellefonte, PA, USA) and degassed. During the analyses, the column temperature was maintained at 25 °C. Detection of phenolic compounds was performed via scanning between 190 and 600 nm, and quantification was performed at 280 nm for monomeric flavan-3-ols (catechin and epicatechin), hydroxybenzoic acids (gallic, vanillic, protocatechuic, syringic, and β-resorbic acids), naringin, naringenin, and cinnamic acid; at 320 nm for hydroxycinnamic acids (caffeic, chlorogenic, p-coumaric, ferulic, and sinapic); and at 360 nm for rutin, quercetin, and kaempferol. The analytes in each extract were identified through comparison of their retention times and UV-Vis spectra with those of the standards. The purity of each peak was checked to exclude any contribution from interfering peaks. The concentration of each phenolic compound identified in the extracts was obtained from the calibration curve, which was constructed using a mixture of the abovementioned standards in a concentration range of between 1 and 50 mg/L in methanol:water (50:50). The results were expressed in milligrams of compound per liter (mg/L).

### 3. Results

#### 3.1. Palynological Analysis

In the samples analyzed, 75 pollen types were found and 59, belonging to 28 botanical families, were properly identified. The *Fabaceae* family was the most prevalent, followed by the *Myrtaceae* family with seven types. The *Rubiaceae* family also had a great representation, with the presence of five types in total (Figure 1). The types *Cecropia* (*Urticaceae*, Figure 1P), *Eucalyptus* (*Myrtaceae*, Figure 1I), *Mimosa pudica* (*Fabaceae*, Figure 1F), and *Myrcia I* (*Myrtaceae*, Figure 1J) were present in all samples analyzed. The types *Protium heptaphyllum* (*Buseraceae*, Figure 1C) and *Schinus terebinthifolius* (*Anacardiaceae*, Figure 1A), both considered sources of resin for bees, were obtained—56.25% and 81.25% of distribution frequency, respectively. In addition, nine other types, *Borreria verticillata* (Figure 1L), *Miconia* (Figure 1H), *Mimosa tenuiflora* (Figure 1E), *Myrcia II*, *Poaceae* (Figure 1K), *Rhynchospora cephalotes* (Figure 1D), *Senna*, *Serjania* (Figure 1O), and *Solanum paniculatum*, were also classified as very frequent as they had distribution frequency values greater than 50%.

Overall, the geopropolis from the rainy season presented 53 pollen types, and that from the dry season, 45 types. There were 38 common pollen types: *Schinus terebinthifolius*, *Mimosa pudica*, *Mimosa tenuiflora*, *Miconia-Melastomataceae*, *Eucalyptus*, *Myrcia I*, *Poaceae*, and *Cecropia*.



**Figure 1.** Pollen types found in geopropolis samples collected in an Atlantic Forest area in the state of Bahia (Northeast Brazil). (A) Anacardiaceae: *Schinus terebinthifolius*. (B) Arecaceae: *Syagrus coronata*. (C) Burseraceae: *Protium heptaphyllum*. (D) Cyperaceae: *Rhynchospora cephalotes*. (E,F) Fabaceae: (E) *Mimosa pudica*; (F) *Mimosa tenuiflora*. (G) Malpighiaceae: *Stigmaphyllon blanchetii*. (H) Melastomataceae: *Miconia*. (I,J) Myrtaceae: (I) *Eucalyptus*; (J) *Myrcia* I. (K) Poaceae. (L,M) Rubiaceae: (L) *Borreria verticillata*; (M) *Richardia grandiflora*. (N,O) Sapindaceae: (N) *Cupania*; (O) *Serjania* I. (P) Urticaceae: *Cecropia*. (Bar = 10  $\mu$ m).

### 3.2. Physicochemical Analysis

Table 1 summarizes the results obtained for the physicochemical analyses from the geopropolis samples collected in the rainy and dry seasons. Regarding the values obtained for pH (acidic samples) ash, moisture, and waxes, these were identical in the samplings carried out in both seasons. The electrical conductivity and total phenols and flavonoids of the geopropolis samples collected in the rainy season had the highest values and were statistically different from those quantified in the dry season (Tukey's test). This result can be explained by its chemical variability as a result of edaphoclimatic differences that affect the resin foraging and pollen profile [27,28].

**Table 1.** Physicochemical characteristics of geopropolis samples produced in an Atlantic Forest area, Bahia State, Brazil (mean and standard deviation).

ASamples	pH	Conductivity (mS/cm)	Moisture (%)	Ash (%)	Waxes (%)	Phenolic Compounds (%)	Flavonoid Compounds (%)
Rainy geopropolis (summer) *	4.110 ± 0.060 a	36.000 ± 0.000 a	2.630 ± 0.070 a	76.130 ± 0.860 a	3.370 ± 0.290 a	25.130 ± 0.140 a	3.920 ± 0.090 a
Dry geopropolis (winter) *	4.200 ± 0.040 a	34.000 ± 0.820 b	2.660 ± 0.020 a	76.340 ± 0.070 a	3.170 ± 0.720 a	19.30 ± 0.150 b	2.090 ± 0.370 b

\* Different letters (a, b) indicate significant differences (Tukey's test).

### 3.3. Determination of Phenols

Table 2 shows the HPLC quantified values for the phenols of the geopropolis samples collected in the wet and dry seasons. The composition of geopropolis in phenolic compounds assessed using HPLC is abundant and diverse (Table 2). In our samples, hydroxybenzoic acids, flavonoids, citrus flavonoids, and carboxylic acids were quantified. The HPLC chromatogram displaying the mix of standard phenolic compounds, along with the chromatograms obtained for the rainy geopropolis propolis sample and the dry geopropolis sample, are available in the supplementary material, specifically in Figures S1 and S2, respectively. It was found that for most compounds, the amount observed in the sample collected in the rainy season was almost double that quantified in the dry season.

**Table 2.** Phenolic compounds identified and quantified in propolis and geopropolis extracts using HPLC-UV; results are expressed in mg/L (ND: not detected; LOQ: limit of quantification; LOD: limit of detection).

Compound	Retention Time (min)	Rainy Geopropolis	Dry Geopropolis
gallic acid	7.14	5.820	5.840
protocatechuic acid	12.74	2.530	2.460
Catechin	18.30	8.390	2.640
vanillic acid	22.79	2.430	ND
caffeic acid	24.40	ND	ND
Epicatechin	25.38	1.720	1.320
p-coumaric acid	31.48	<LOQ	<LOD
ferulic acid	34.38	ND	ND
Naringin	42.17	45.390	29.870
Rutin	45.56	ND	ND
cinnamic acid	49.71	2.540	<LOD
Naringenin	55.25	2.110	<LOQ
Quercetin	54.07	ND	ND

It was also found that gallic acid, protocatechuic acid (hydroxy benzoic acids), catechin (flavonoids), and naringin (citrus flavonoid) were present in both samples analyzed. Quercetin, rutin, and caffeic acid were not observed in any of the samples analyzed. Of the compounds mentioned, naringin stands out, whose content in the geopropolis samples obtained in the rainy season (45.39 mg/L) was 1.5 times higher than the values observed in the dry season samples (29.87 mg/L) (Table 2).

### 3.4. Biological Activities

The results obtained for the antioxidant capacity, hyaluronidase enzyme inhibition, and acetylcholinesterase inhibition induced by geopropolis extracts are summarized in Table 3.

**Table 3.** Values obtained for antioxidant capacity, hyaluronidase enzyme inhibition, and acetylcholinesterase inhibition in geopropolis samples, collected in the rainy and dry seasons.

	Scavenging of DPPH Radicals—IC <sub>50</sub> (mg/mL)	Reducing Power—IC <sub>50</sub> (mg/mL)	β-Carotene/Linoleic Acid (%)	Hyaluronidase (30 mg/mL) (%)	Acetylcholinesterase—IC <sub>50</sub> (μg/mL)
Rainy Geopropolis *	0.058 ± 0.005 a	0.310 ± 0.008 a	89.52 ± 0.57 a	30.95 ± 1.28 a	0.28 ± 0.01 b
Dry Geopropolis *	0.064 ± 0.004 a	0.220 ± 0.001 b	75.54 ± 1.10 b	26.62 ± 0.90 b	0.354 ± 0.006 a
Ascorbic acid	0.015 ± 0.001	0.074 ± 0.013	-	-	-
BHA	-	-	92.450 ± 0.001	-	-
Eserine					0.005 ± 0.001

\* Different letters (a, b) indicate significant differences (Tukey's test).

### 3.4.1. Antioxidant Capacity

Regarding antioxidant capacity, the observed values ranged from IC<sub>50</sub> = 0.058 mg/mL (DPPH) to 89.52% (β-carotene). It should be noted that the higher the CI values<sub>50</sub> were, the lower the antioxidant capacity was. The geopropolis collected in the rainy season showed a higher antioxidant capacity than the samples obtained in the dry season. The antioxidant capacity assessed using both the DPPH and reducing power methods was approximately three and a half times lower than that obtained for the standard (ascorbic acid). There were significant differences in antioxidant capacity between the samples when analyzed using the different methods, apart from DPPH. For both samples, the method used to evaluate the antioxidant capacity overall significantly influenced the results obtained.

### 3.4.2. Anti-Inflammatory Activity

The inhibition of hyaluronidase enzyme activity allows the indirect assessment of the effect of geopropolis extracts on anti-inflammatory activity.

It was found that the anti-inflammatory activity induced by geopropolis collected in the dry season was lower (26.62 ± 0.90%), with significant differences observed compared to that of the rainy season (30.95 ± 1.28%).

### 3.4.3. Inhibition of Acetylcholinesterase

The link between acetylcholinesterase and Alzheimer's disease results from decreased levels of acetylcholine in the brain. In this study, we tested the effect of geopropolis extracts on this enzyme. The concentrations of extract required to cause a 50% inhibition of the activity of this enzyme were found to be 0.281 ± 0.01 and 0.3542 ± 0.0006 μg/mL for wet and dry season geopropolis, respectively, with statistically significant differences between the effects induced by the two samples. Compared to the geopropolis extract, eserine (control) had a more pronounced effect on the inhibition of this enzyme.

### 3.4.4. Antimicrobial Activity

From the analysis in Table 4, it was found that all the extracts of the samples analyzed showed antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts (Table 4). Overall, *Staphylococcus aureus* ESA 321, a clinical isolate from pus (MIC = 0.05 ± 0.02 and MIC = 0.12 ± 0.01 mg/mL for the rainy and dry seasons, respectively), was the most sensitive microorganism to the action of both samples under study, and *Candida albicans* ESA115, isolated from a sputum (MIC = 2.5 ± 0.11 and MIC = 5.00 ± 0.01 mg/mL for the rainy and dry seasons, respectively), was the most resistant. For *Proteus mirabilis*, MIC values were between 0.7 and 1.0 mg/mL [29].

**Table 4.** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of geopropolis extracts, gentamicin, and amphoterecin against isolated (ESA) and reference (ATCC) microorganisms (\*—not applicable to the microorganism).

Microorganisms	Rainy Geopropolis (mg/mL)		Dry Geopropolis (mg/mL)		Gentamicin (mg/mL)	Amphoterecin (mg/mL)
	MIC	MBC	MIC	MBC		
Gram-positive bacteria						
<i>Staphylococcus aureus</i> ATCC 4330	0.05 ± 0.12	2.01 ± 0.07	0.10 ± 0.02	1.25 ± 0.06	0.03 ± 0.01	*
<i>Staphylococcus aureus</i> ESA 321	0.05 ± 0.02	2.25 ± 0.01	0.12 ± 0.01	1.75 ± 0.01	0.13 ± 0.02	*
Gram-negative bacteria						
<i>Proteus mirabilis</i> ATCC 25933	1.00 ± 0.03	1.50 ± 0.03	0.50 ± 0.15	1.34 ± 0.04	0.06 ± 0.01	*
<i>Proteus mirabilis</i> ESA 229	0.07 ± 0.01	1.76 ± 0.07	0.10 ± 0.05	1.12 ± 0.01	0.005 ± 0.001	*
Fungi						
<i>Candida albicans</i> ATCC 10231	1.35 ± 0.01	5.01 ± 0.17	2.5 ± 0.04	5.00 ± 0.06	*	0.002 ± 0.001
<i>Candida albicans</i> ESA 115	2.50 ± 0.11	5.22 ± 0.02	5.00 ± 0.01	5.00 ± 0.04	*	0.021 ± 0.008

Regarding the minimum bactericidal concentration (MBC), the following values were obtained for *S. aureus*: rainy season— $2.25 \pm 0.01$  mg/mL and  $1.75 \pm 0.01$  mg/mL for the dry season. The values observed for this parameter in *C. albicans* were  $5.20 \pm 0.02$  mg/mL and  $5.00 \pm 0.04$  mg/mL for the rainy and dry seasons, respectively. Overall, the inhibition induced on the various microorganisms under study by the samples collected in the rainy season was higher than that induced by the samples obtained in the dry season.

The difference in the efficiency of propolis on the control of Gram-positive and Gram-negative bacteria may be due to the difference in the structure of the cell wall, wherein the Gram-negative ones are more complex, preventing the action of compounds with an antimicrobial effect. Note that this behavior was observed in both samples tested [11].

### 3.4.5. Antimutagenic Activity

Table 5 shows the results obtained for the antimutagenic activity induced by geopropolis extracts. All samples were found to have anti-genotoxic action, with values ranging from  $23.01 \pm 2.05\%$  to  $88.59 \pm 1.71\%$ ; in fact, both extracts decreased gene conversion frequencies (Table 5). The percentage-of-survival results showed a significant interaction term between the dry/rainy geopropolis samples (factor 1) and geopropolis extract amount (factor 2) ( $p$ -value = 0.031) in the two-way ANOVA model ( $R^2 = 0.914$ ;  $p$ -value < 0.001; residual standard error = 4.70). Geopropolis extract, regardless of harvest time, was found to reduce the survival rate of *S. cerevisiae* D7 (ATCC 201137), an effect that became more pronounced as the concentration of extract added increased.

When *S. cerevisiae* cells were incubated in the presence of geopropolis and EMS (ethyl methanesulfonate; mutagen), both samples were found to have anti-genotoxic action, causing a reduction in both mutant colonies and gene conversion.

The dry geopropolis had higher gene-conversion and colony values compared to rainy geopropolis. Furthermore, the increase in geopropolis extract concentration from 2.5 to 5.0 mg/mL significantly decreased the colony values (two-way ANOVA;  $R^2 = 0.893$ ;  $p$ -value < 0.001; residual standard error = 3.14; interaction term  $p$ -value = 0.148). As for the mutant colony results, the interaction term was significant ( $p$ -value = 0.018) in the two-way ANOVA model ( $R^2 = 0.960$ ;  $p$ -value < 0.001; residual standard error = 9.45). The results

showed that as the geopropolis-extract concentration increased, the effects on mutant colonies appeared to converge for the two samples.

**Table 5.** Effects of the hydroethanolic extract of geopropolis of Me on the survival percentage of yeast cells (*Saccharomyces cerevisiae*) (diploid line D7 ATCC 201137), conversion of genes, and mutant colonies.

Sample	Treatment		Survivals (%)	Gene Conversion Colonies/10 <sup>5</sup>	Mutant Colonies/10 <sup>6</sup>
	[Extract] (mg/mL)	EMS (mg/mL)			
Dry geopropolis	0	5.0	88.18 ± 1.60	51.55 ± 0.60 a	380.56 ± 7.67
	2.5	5.0	36.24 ± 2.72	37.33 ± 1.27 b	323.07 ± 1.36
	5.0	5.0	28.84 ± 2.19	31.07 ± 0.98 c	302.58 ± 3.43
Rainy geopropolis	0	5.0	88.59 ± 1.71	51.34 ± 0.63 d	404.04 ± 9.46
	2.5	5.0	30.64 ± 2.38	43.82 ± 3.95 e	354.95 ± 17.78
	5.0	5.0	23.01 ± 2.05	37.21 ± 1.87 f	311.88 ± 1.52

Different letters (a, b, c, d, e, f) indicate significant differences (multiple comparisons using Tukey's HSD).

## 4. Discussion

### 4.1. Palynological Analysis

In this study, where two samples of geopropolis from the Atlantic biome were evaluated, 75 different pollen types were detected. This was much more than the number detected by other studies, for example, the study performed by Barros et al. [30], who analyzed 16 samples of geopropolis produced in a resting area in the state of Maranhão and only identified 38 pollen types. These results reveal the diversity of plant taxa available in this region as sources for *Mellipona scutellaris* throughout the year. Among these various types, *Eucalyptus* (*Myrtaceae*) emerges as particularly significant due to its widespread presence and distribution. A study conducted by Barth et al. [31] in Southeastern Brazil, focusing on geopropolis, classified the *Eucalyptus* type as the dominant pollen source, accounting for more than 45% of the analyzed samples. Notably, plant species associated with this type frequently appear in research related to propolis and other beekeeping/melipon products in Brazil.

Despite not being a native species to the Brazilian flora, *Eucalyptus* has demonstrated remarkable adaptability to the country's climate and soil conditions. Since the 1990s, it has been actively utilized in an extractive manner across various regions of Brazil, as documented by Araújo et al. [32]. Resin constitutes a noteworthy component of geopropolis, yet the factors influencing bees' preferences for specific resin sources remain a mystery, even though they exhibit discerning choices in this regard [30]. Stingless bee species exhibit regional variations in collecting resins from different plants, making the presence of their pollen in geopropolis a valuable clue for potential resin sources [33]. Notably, *Schinus terebinthifolius* (*Anacardiaceae*) and *Cecropia* (*Urticaceae*) species, as identified by [13], are potential resin providers for *Apis mellifera* propolis in the North Coast region of Bahia, coinciding with the study area. Pollen referring to these species was very frequent in the analyzed samples and may also be indicative of sources of resin used by *M. scutellaris* to produce geopropolis in this region.

### 4.2. Physicochemical Analysis

The composition of geopropolis is complex as it contains mineral particles or ash from the soil. These particles are incorporated into this product when bees encounter the soil during the collection of resins and other materials used in its production. Geopropolis is mainly known for its pharmacological and therapeutic properties. Despite the great multiplicity of bee species capable of producing this product, studies on its physical-chemical characteristics and biological properties are recent, and some of them scarce or non-existent. In this context, delving into research concerning the chemical composition and biological activity of stingless bee geopropolis holds pivotal significance. Such investigations not only

aim to establish a quality benchmark but also seek to unravel the mechanisms of action inherent to this remarkable natural product.

According to the literature, geopropolis is generally slightly acidic, with pH values ranging between 3.5 and 5.5. Phenolic acids and flavonoids, which are relevant in the chemical composition of geopropolis, are the main factors responsible for this characteristic [1]. However, it is important to note that pH can vary with the origin of geopropolis and even within the same bee colony. Although this parameter has not been contemplated in the legislation, the values obtained in our study corroborate those described by da Silva Cruz et al. [2]. According to this researcher, pH is one of the main factors responsible for the stability of this product, as it influences the multiplication of its microbiota.

Specific studies on the electrical conductivity of geopropolis are insufficient or non-existent. However, it is possible to infer that the conductivity is probably low, particularly due to the high content of waxes and resins that are part of its composition.

Regarding ashes, Oliveira et al. [34] analyzed geopropolis from different stingless bee species, reporting values ranging from  $75.40 \pm 0.23\%$  to  $85.43 \pm 0.53\%$  (m/m), which corroborate the results obtained in this study. According to Pereira et al. [35], the high values of this parameter compared to those observed in other bee products are justified by the fact that geopropolis is a mixture of clay/earth and resin, and consequently, its content of minerals and trace elements is higher. However, it is important to note that the concentration and specific composition of minerals in ash can vary depending on the region and the plants used by bees [36]. Sawaya et al. [33] reported that high ash contents indicate that the product has not been adulterated.

Concerning moisture, the values obtained in our study were identical to those reported by Araújo et al. [37] for *Melipona scutellaris* and *M. fasciculata* species (1.40% to 1.76%), suggesting that the moisture content of stingless bees is low. Indeed, da Silva Cruz et al. [2] found that the moisture content of geopropolis was six and eleven times lower than those of honey and pollen produced by *M. scutellaris*, respectively.

The waxes in geopropolis are important components of the structures of hives, also possessing therapeutic properties. The results obtained in this study are identical to those observed by Araújo et al. [37] in geopropolis of *M. scutellaris* (4.33%). However, they are higher than those described by the same author in geopropolis of *M. fasciculata* (0.95%) and by Cardozo et al. [38] in geopropolis of *M. Jataí*, *M. Mandaçaia*, and *M. Mandurí*. Araújo et al. [39] evaluated propolis collected in the same apiary and obtained values for this parameter that ranged between 25.45 and 24.09%, demonstrating that the product produced by *Apis mellifera* had higher values than the geopropolis of stingless bees. These differences can be justified by the fact that the chemical composition of geopropolis varies with the flora and the geographical region where it is collected [39].

Phenolic compounds are present in several natural products, namely geopropolis, and are responsible for many of the biological properties associated with these products. However, it is important to note that the quantities, qualities, and beneficial effects of these substances depend on the specific chemical composition of each sample and the concentration of compounds present [40,41]. In samples of geopropolis from *Melipona fasciculata* collected by Dutra et al. [42] in the savannah of Maranhão state, the values of this parameter ranged from 14.14 to 67.46%. These values were higher than those observed by these authors in the geopropolis of *M. scutellaris* obtained in the state of Bahia (Brazil). According to Araújo et al. [39], the flora of the region and the species of stingless bee can quantitatively influence the content of total phenols in this bee product.

Regarding flavonoids, Cardozo et al. [38] obtained lower contents (0.66 to 0.02%) than those quantified in our study. This variation in flavonoid content between different geopropolis samples has already been reported by other researchers, for example, ref. [42], who evaluated the flavonoid content in geopropolis produced in Maranhão and obtained values between 0.17 and 6.0%. Sawaya et al. [33] observed flavonoid contents in extracts of geopropolis of *Melipona fasciculata* from the same state ranging from 0.85% to 1.85%. According to these researchers, the variations in the content of flavonoid compounds in

the different studies may result from differences in the time and time of collection of the geopropolis, vegetation, or the type of soil collected by the bees, since the extraction methods and the extracting solvents used were the same.

#### 4.3. Quantification of Phenolic Compounds

According to Righi et al. [43], when dealing with complex mixtures, chromatographic methods are essential for assessing active compounds. In our study, we noted variations in phenolic compound levels between samples collected in different seasons. Naringin, the most abundant compound in both samples, was about 1.5 times lower in concentration during the dry season compared to the rainy season. Kasote et al. [44] found a similar phenolic compound profile in geopropolis from various Indian states, with naringin being the predominant compound. In contrast, gallic acid was a primary component in propolis samples from stingless bees collected in Brazilian states like Pernambuco, Parana, and São Paulo [45]. Santos et al. [6] also reported that the hydroalcoholic extracts of geopropolis from *Melipona orbignyi* stood out for presenting flavonoids, aromadendrin, and naringenin. Naringin is a glycosylated flavanone commonly found in citrus fruits and grapes. This compound possesses antioxidant, anti-inflammatory, antimutagenic, and antimicrobial properties [46,47].

#### 4.4. Antioxidant Activity

According to the literature, antioxidant activity depends on the chemical composition of geopropolis, particularly the quantity and quality of phenols and flavonoids [48]. Free radical scavenging is one of the methodologies frequently used to assess antioxidant capacity [20]. DPPH free radical scavenging measures the ability of the analyzed substances to donate hydrogen ions [49]. The reducing power of a given product indicates its antioxidant potential [20]. The  $\beta$ -carotene/linoleic acid system measures the antioxidant potential of a substance as the ability to sequester the free radical generated by the peroxidation of linoleic acid [50].

Santos et al. [6], when analyzing the antioxidant activity of geopropolis from *Melipona orbignyi* using the DPPH method, obtained IC<sub>50</sub> values of 18.3  $\mu$ g/mL. These values were lower than those determined in our study, suggesting that this biological property is influenced by the local flora and bee species. However, Araújo et al. [39], in their investigation of geopropolis from *Melipona scutellaris* collected in an area with vegetation identical to that in our study, reported IC<sub>50</sub> values determined by the DPPH method that were remarkably similar to those observed in our work. The values obtained by Batista et al. [51] in hydro-ethanolic extracts of geopropolis produced by *M. fasciculata* in Maranhão also corroborate our determinations. Even though our samples had the same geographical origin, differences were also observed regarding the botanical origin and the amount and type of phenolic compounds.

#### 4.5. Anti-Inflammatory Activity

The enzyme hyaluronidase breaks down hyaluronic acid, a key component found in the extracellular matrix of connective tissue. The assessment of anti-inflammatory activity based on this enzyme is used to determine its ability to reduce inflammation and modulate the inflammatory response. These assays are important to understand the mechanisms of action of hyaluronidase and may pave the way for the development of new therapeutic approaches in the treatment of inflammatory diseases [23]. Its activity is relevant in physiological and pathological processes, and it is also used as a therapeutic adjuvant in several clinical contexts. The effect of hyaluronidase enzyme inhibition induced by geopropolis used in our study was lower than that reported by Santos et al. [6] in geopropolis collected in Mato Grosso do Sul, Brazil.

#### 4.6. Inhibition of Acetylcholinesterase

Acetylcholinesterase (AChE) is vital for breaking down acetylcholine, a neurotransmitter involved in nerve signaling. Compounds that inhibit or reactivate AChE have potential uses in Alzheimer's disease and organophosphate poisoning treatment [37]. In our study, there was a greater inhibition of this enzyme by the sample collected in the rainy season. This fact may be associated with the higher content of phenols present in this sample; however, further studies involving larger numbers of samples are needed to clarify the effect of this bee product, produced almost exclusively by stingless bees, on acetylcholinesterase, as we are not aware of other studies carried out in this area.

#### 4.7. Antimicrobial Activity

In this study, all samples showed antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts. Overall, *Staphylococcus aureus* ESA 321, a clinical isolate from pus, was the most sensitive microorganism to the action of both matrices under study, and *Candida albicans* ESA115, isolated from a sputum, was the most resistant. The geopropolis extract showed greater efficacy against *Proteus mirabilis* than previously reported by Campos et al. [29] in a study involving a geopropolis extract produced by stingless bees (*Tetragonisca fiebrigi*) in Grandes Dourados. These authors observed MIC values for this bacterium of between 2.25 and 3.08 mg/mL, i.e., higher than those obtained in our study. The fact that yeasts are eukaryotic organisms may justify their behavior towards bacteria. The higher resistance of Gram-negative bacteria can be explained by their more complex cell wall composition, which makes it more difficult for antimicrobials to enter. In all cases, the resistance of the reference microorganisms was higher than that of the clinical isolates, probably because the clinical isolates had already acquired antimicrobial resistance. Our results are identical to those reported by Santos et al. [6] in geopropolis produced in Grandes Dourados by *Melipona orbignyi*.

Liberio et al. [52] found that geopropolis samples produced by *M. fasciculata* inhibited the growth of *C. albicans*. Da Cunha et al. [53] studied the antimicrobial effect of geopropolis samples of *M. scutellaris*, collected in the study area where the samples used in our study were from, against Gram-positive and Gram-negative bacteria and found that the growth of *S. aureus* was inhibited for concentrations higher than 50 µg/mL while the MIC for *Pseudomonas aeruginosa* was higher than 1600 µg/mL. These observations corroborate our results, indicating that Gram-positive bacteria are more sensitive than Gram-negative bacteria. Valcanaia et al. [54] reported that natural products showing antimicrobial activity with MIC (minimum inhibitory concentration) values below 100 µg/mL can be considered good antimicrobial agents. In this context, the results obtained in this work suggest that geopropolis can be used as an antibacterial agent, with its antifungal effect being more limited.

According to what has been reported in the literature, phenolic compounds are said to be mainly responsible for the biological properties of hive products, particularly geopropolis. Polyphenol derivatives common in propolis have been shown to exert a wealth of therapeutic benefits [55], having antioxidant capacity (can interrupt the chain reactions caused by free radicals but can also inhibit their formation), antimicrobial (can induce the permeabilization of the microbial cytoplasmic membrane and inhibition of the synthesis of nucleic acids in Gram-negative/Gram-positive bacteria and synthesis of ATP and interruption of electron transport) and anti-inflammatory activity and pro-inflammatory enzymes [6] such as nitric oxide synthase and cyclooxygenase-2 [56].

#### 4.8. Antimutagenic Activity

The use of the yeast *Saccharomyces cerevisiae* ATCC 20113–D7 in the evaluation of antimutagenic activity involves the analysis of the effect of geopropolis in preventing or reducing genetic mutations induced by mutagens. In the present study, three concentrations of geopropolis were used: 0.0, 0.25, and 0.5, with methyl methanesulfonate (EMS) at a concentration of 0.5%. EMS is an alkylation agent that produces random mutations in

genetic material via nucleotide substitution. Geopropolis, depending on the concentration, caused a decrease in the frequency of gene conversion and the number of mutant colonies induced by the EMS mutagen.

## 5. Conclusions

The analysis of the geopropolis samples gathered within the Atlantic Forest of Bahia revealed that *Schinus terebinthifolius* and *Cecropia* species served as the resin providers utilized by *Melipona scutellaris* bees to produce this product. In this study, for the first time, we demonstrated that the timing of harvest influences the chemical profile and bioactivity of *M. scutellaris* geopropolis. Antioxidant, antibacterial, antimutagenic, anti-cholinesterase, and inflammatory capabilities, particularly evident in the samples obtained during the rainy season, suggest the potential of this product for preventing inflammatory, oxidative, and infectious diseases. Although the meliponary under study is in an area where extractive agriculture is practiced, the maintenance of the vegetation ensures resource availability throughout the year. However, it is necessary to protect these species to ensure that the production of this natural product becomes a sustainable source of income. Additionally, these findings show the importance of studies that search for effective alternative therapies in facing the global increase in resistance to antibiotics currently used and point out the extracts as possible candidates for the development of new treatments against bacterial infections. On the other hand, the high variability of the chemical profile of geopropolis of *Melipona* species is a factor requiring strategies aiming at the standardization of the product.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms11112779/s1>, Figure S1: HPLC chromatogram obtained for the rainy geopropolis propolis sample at 280 nm; Figure S2: HPLC chromatogram obtained for the dry geopropolis sample at 280 nm; Table S1: Phenolic compounds analysed by HPLC.

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# **Capítulo 4**

## **Considerações Finais**

#### 4.1. Considerações Finais

Este estudo fornece contributos para a compreensão da diversa composição botânica e química das amostras de geoprópolis da Mata Atlântica da Bahia, salientando o seu potencial enquanto fonte de compostos bioativos. A deteção de 75 tipos de pólen diferentes, superando largamente estudos anteriores, realça a rica biodiversidade de plantas disponíveis para a *Melipona scutellaris* ao longo do ano, com o *Eucalyptus* a destacar-se como uma fonte de pólen dominante. A adaptabilidade de espécies não nativas, como o *Eucalyptus*, aliada a variações regionais nas fontes de resina, revela as complexas interações entre a flora e as abelhas sem ferrão na produção de geoprópolis.

A presença de vários compostos bioativos, nomeadamente ácidos fenólicos e flavonoides, indica o potencial farmacológico da geoprópolis, conferindo-lhe propriedades antioxidantes, antimicrobianas e anti-inflamatórias. As diferenças nas concentrações de compostos fenólicos entre as estações seca e chuvosa evidenciam igualmente a influência dos fatores ambientais na composição química e na atividade biológica deste produto apícola.

Apesar dos resultados promissores, são necessárias investigações adicionais sobre certos aspetos, nomeadamente os fatores que influenciam a recolha de resina pelas abelhas e os efeitos específicos da geoprópolis sobre enzimas como a acetilcolinesterase. A atividade antimicrobiana demonstrada pelas amostras, em particular contra *Staphylococcus aureus* e *Proteus mirabilis*, sugere que a geoprópolis poderia ser desenvolvida como um agente antibacteriano natural, embora o seu potencial antifúngico permaneça limitado.

Em suma, esta investigação contribui para uma compreensão mais aprofundada da geoprópolis, reforçando o seu papel enquanto produto natural valioso com potencial terapêutico. Estudos adicionais sobre as suas propriedades químicas e biológicas são essenciais para compreender os seus mecanismos de ação e otimizar a sua utilização em aplicações farmacêuticas.

## 4.2. Perspetivas Futuras

As perspetivas futuras deste trabalho apontam para várias áreas de investigação e desenvolvimento que podem aprofundar o conhecimento sobre a geoprópolis e o seu potencial uso:

- Explorar mais detalhadamente as preferências das abelhas *Melipona scutellaris* por diferentes fontes de resina, tendo em conta a flora regional e as variações sazonais;
- Ampliar a amostragem, e estudar a geoprópolis de outras regiões e espécies, a fim de fornecer dados mais robustos com possíveis aplicações terapêuticas;
- Investigar mais aprofundadamente as propriedades antimicrobianas da geoprópolis, especialmente no que se refere à resistência a antibióticos;
- Desenvolver estratégias de padronização do produto para otimizar a sua utilização em tratamentos para doenças inflamatórias e oxidativas;
- Abordar a viabilidade da produção sustentável de geoprópolis, assegurando a preservação das abelhas sem ferrão e das plantas que fornecem os recursos essenciais;
- Criar normas de qualidade para a padronização da geoprópolis, dada a sua variabilidade química, que permitam a sua utilização mais ampla na indústria farmacêutica.

Com isto, pretende-se abrir caminhos para uma investigação mais aprofundada da geoprópolis enquanto produto natural com um grande potencial terapêutico e de aplicação em várias áreas da saúde.