UNIVERSIDADE FEDERAL DE ALFENAS

GUSTAVO APARECIDO DA CUNHA

PRÓPOLIS: UM AGENTE ANTIOXIDANTE NEUTRALIZADOR DE RADICAIS LIVRES E REDUTOR DO ESTRESSE OXIDATIVO

Alfenas/MG

2022

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PRÓPOLIS: UM AGENTE ANTIOXIDANTE NEUTRALIZADOR DE RADICAIS LIVRES E REDUTOR DO ESTRESSE OXIDATIVO

Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Biologia celular, molecular e estrutural das doenças agudas e crônicas.

Orientador: Prof. Dr. Pedro Luiz Rosalen
Coorientador: Prof. Dr. Masaharu Ikegaki

Alfenas/MG

2022

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Biblioteca Central

Cunha, Gustavo Aparecido da.
Própolis: um agente antioxidante neutralizador de radicais livres e redutor do estresse oxidativo / Gustavo Aparecido da Cunha. - Alfenas, MG, 2022.
74 f. : il. -

Orientador(a): Pedro Luiz Rosalen.
Dissertação (Mestrado em Ciências Biológicas) - Universidade Federal de Alfenas, Alfenas, MG, 2022.
Bibliografia.

1. Produtos naturais. 2. Flavonoides. 3. Apis mellifera. 4. Revisão sistemática. I. Rosalen, Pedro Luiz, orient. II. Título.

Ficha gerada automaticamente com dados fornecidos pelo autor.

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Aprovada em: 24 de fevereiro de 2022.

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Documento assinado eletronicamente por **Livia Maris Ribeiro Paranaiba Dias**, **Professor do Magistério Superior**, em 03/03/2022, às 14:28, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **PEDRO LUIZ ROSALEN**, **Usuário Externo**, em 03/03/2022, às 19:24, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Josy Goldoni Lazarini**, **Usuário Externo**, em 03/03/2022, às 21:16, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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À minha família.

AGRADECIMENTO

Em primeiro lugar agradeço à minha família pelo apoio, carinho, paciência e incentivo em todos os momentos.

À minha mãe, Maria do Carmo Cunha, pelo amor, incentivo, por acreditar no meu potencial e ser a pessoa mais importante em minha vida. Esse trabalho é nossa vitória, conquistamos juntos, mãe. Muito obrigado.

Ao meu orientador, professor Dr. Pedro Luiz Rosalen, por sua acolhida, críticas, sugestões, por me guiar pelos bons princípios de pesquisa e boas conversas durante nossas reuniões.

Ao meu coorientador, Dr. Masaharu Ikegaki, por sua generosidade, auxílios durante o uso do laboratório, críticas e valiosas sugestões.

Ao professor, Dr. Marcelo Franchin, por sua dedicação, generosidade, por ser o intermediário entre ESALQ e UNIFAL e por sempre estar disposto a nos ajudar.

Ao professor, Dr. Severino Alencar, por sua generosidade em nos ceder todas as estruturas de seu laboratório.

À senhora, Carlota Rios, proprietária do Apiário Taquaruçu, pela gentileza em nos ceder a amostra de própolis verde utilizada neste estudo.

À aluna de doutorado, Ana Sofia Martelli Chaib Saliba, pela paciência, por ser minha mentora na execução dos ensaios, pelas boas risadas e disposição em todos os momentos.

Aos amigos do Laboratório de Bioprocessos, Namuhell Oliveira, Rafaella Bruzadelli e Leonardo Araújo, pelas boas risadas, pelas conversas divertidas, valorosa contribuição na execução dos ensaios e pelo “cafezin” nos fins de tarde.

Ao técnico de laboratório, Gustavo Silveira, por sua generosidade, prontidão em nos ajudar, dedicação durante os ensaios e por ser o nosso professor de Química durante os terríveis cálculos de concentração das amostras.

À Mikaela, por sua amizade, carinho, boas risadas, por ter entrado em minha vida em um momento tão difícil e deixar tudo mais leve e colorido.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Por fim, a aqueles que de alguma forma possam ter contribuído na execução deste trabalho.

Agradeço imensamente a todos.
Muito obrigado!!!

RESUMO

O objetivo do presente estudo foi avaliar os efeitos da propriedade antioxidante presente nas própolis sobre o estresse oxidativo e espécies reativas de oxigênio. Esta dissertação foi elaborada na forma de dois artigos (1º- revisão sistemática e 2º- estudo *in vitro*). A revisão sistemática avaliou a relevância da administração de própolis sobre os parâmetros oxidativos do diabetes mellitus (DM) induzido em modelos animais. Ocorreu grande heterogeneidade quanto a origem dos estudos e própolis utilizadas, indicando o aumento significativo no número de casos de DM ao redor do mundo e a busca por novas moléculas com potencial antidiabético. A administração de própolis induziu ao restauro das defesas antioxidantes endógenas dos animais tratados, processo acompanhado pelo aumento na secreção de insulina, redução dos níveis glicêmicos e da peroxidação lipídica. Todos os resultados bioquímicos/fisiológicos obtidos foram decorrentes da redução de espécies reativas de oxigênio e aumento nos níveis plasmáticos de enzimas antioxidantes, demonstrando que as própolis desempenham um efeito benéfico sobre o diabetes induzido em animais, sem apresentar toxicidade e fornecendo parâmetros para seu uso em seres humanos de forma isolada ou em associação a drogas de referência. No estudo *in vitro* avaliamos a atividade antioxidante em duas formulações à base de própolis verde do sul de Minas Gerais. A partir de uma amostra de própolis verde sul mineira foram desenvolvidas duas formulações a 11% (p/v) de: 1) extrato hidroetanólico (EEP) e 2) gel mucoadesivo aquoso (MuAd-P). Primeiramente, o perfil químico da própolis verde foi determinado por RP-HPLC e o derivado do ácido cinámico - artepelin C - foi o composto majoritário identificado. Em seguida, determinados o conteúdo fenólico, teor de flavonoides e atividade antioxidante de ambas as formulações. EEP e MuAd-P diferiram estatisticamente ($p<0.05$) em seus conteúdos fenólico e de flavonoides, resultado possivelmente influenciado pelos polifenóis do óleo de linhaça presente exclusivamente no gel mucoadesivo. Nos ensaios DPPH, $ABTS^{\bullet+}$ e FRAP não existiram diferenças estatísticas significativas entre as formulações ($p>0.05$) e ambas apresentaram forte atividade redutora. Com os resultados obtidos foi possível determinar que EEP e MuAd-P apresentam forte atividade antioxidante e que os processos farmacotécnicos utilizados na produção da mucoadesiva não alteraram sua capacidade de neutralização de radicais livres. ***Conclui-se que a propriedade antioxidante das própolis reduz o estresse oxidativo, reestabelecendo os parâmetros enzimáticos aliado a um forte efeito neutralizante sobre espécies reativas de oxigênio. Destacamos a forte atividade antioxidante observada na própolis verde do sul de Minas Gerais tanto na forma de extrato etanólico, quanto na forma de gel mucoadesivo.***

Palavras-chave: Produtos naturais; Flavonoides; *Apis mellifera;* Revisão sistemática.

ABSTRACT

The aim of the present study was to evaluate the effects of the antioxidant property present in propolis on oxidative stress and reactive oxygen species. We prepared this dissertation as two articles (1st - systematic review and 2nd - *in vitro* study). The systematic review evaluated the relevance of propolis administration on the oxidative parameters of diabetes mellitus (DM) induced in animal models. There was great heterogeneity regarding the origin of the studies and propolis used, showing a significant increase in the number of DM cases around the world and the search for new molecules with antidiabetic potential. The administration of propolis induced the restoration of the endogenous antioxidant defenses of the treated animals, a process accompanied by an increase in insulin secretion, a reduction in glycemic levels and lipid peroxidation. All the biochemical/physiological results got were because of the reduction of reactive oxygen species and increase in plasma levels of antioxidant enzymes, demonstrating that propolis has a beneficial effect on induced diabetes in animals, without presenting toxicity and providing parameters for its use in humans, alone or in association with reference drugs. In the *in vitro* study, we evaluated the antioxidant activity in two formulations based on green propolis from the south of Minas Gerais. From a sample of this propolis, we developed two formulations at 11% (w/v), such as 1) hydroethanolic extract (EEP) and 2) aqueous mucoadhesive gel (MuAd-P) to be compared. First, the chemical profile of green propolis was determined by RP-HPLC, and the cinnamic acid derivative - artepelin C - was the major compound identified. Then, the phenolic and flavonoid total contents and antioxidant activity of both formulations were determined. EEP and MuAd-P differed statistically ($p<0.05$) in their phenolic and flavonoid total contents, a result possibly influenced by the linseed oil polyphenols present only in the MuAd-P gel. In the DPPH, $ABTS^{\bullet+}$ and FRAP assays, there were no statistically significant differences between the formulations ($p>0.05$) and both showed strong reducing activity. With the results got, it was possible to determine that EEP and MuAd-P have strong antioxidant activity and that the pharmacotechnical processes used in the production of mucoadhesive did not change their ability to neutralize free radicals. ***We concluded that the antioxidant property of propolis reduces oxidative stress, restoring enzymatic parameters combined with a strong neutralizing effect on reactive oxygen species. We also highlight the strong antioxidant activity observed in green propolis from the south of Minas Gerais, both as ethanolic extract and as mucoadhesive gel.***

Keywords: Natural products; Flavonoids; *Apis mellifera*; Systematic review.

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LISTA DE ABREVIATURAS E SIGLAS

ROS/EROs	Reactive oxigen species/Espécies reativas de oxigênio
OS/EO	Oxidative Stress/Estresse oxidativo
EEP	Extrato etanólico de própolis verde
MuAd-P	Formulação mucoadesiva
HPLC	High Performance Liquid Cromatography
DPPH	2,2-difenil-1-picrilhidrazil
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric Reducing Antioxidant Power
Nrf2	Nuclear factor erythroid 2-related factor 2
SOD	Superóxido dismutase
CAT	Catalase
GPx	Glutationa peroxidase
GSH	Glutationa

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1 INTRODUÇÃO GERAL

Espécies reativas de oxigênio (EROs) são moléculas, átomos ou íons derivados de oxigênio, caracterizadas por sua alta eletronegatividade e instabilidade. O termo EROs engloba radicais livres de oxigênio, como ânions superóxido ($O_2^{\cdot-}$) e radical hidroxila (·OH), e oxidantes não radicalares, como peróxido de hidrogênio (H_2O_2) e oxigênio singlete ($^1O_2$). Produzidas naturalmente nas reações da cadeia transportadora de elétrons, são importantes reguladoras do ciclo de vida celular, atuando sobre os processos de proliferação, diferenciação e apoptose (HYBERTSON *et al*., 2011; VONA *et al*., 2019; ZOROV; JUHASZOVA; SOLLOTT, 2014).

Apesar de sua importância bioquímica, em excesso no organismo as EROs promovem alterações no gradiente eletroquímico, morte celular e necrose tecidual (ZOROV; JUHASZOVA; SOLLOTT, 2014). Como forma de neutralizar os efeitos deletérios mediado pelas espécies reativas, o organismo conta com um sistema antioxidante enzimático [ex. superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx)] capaz de converter esses compostos a moléculas não prejudiciais. Entretanto, fatores externos como tabagismo, radiação UV, alcoolismo e poluição podem induzir a superprodução de EROs, promovendo um desequilíbrio entre os níveis teciduais destes compostos e o de enzimas antioxidantes (KIM; BYZOVA, 2014; MOLDOGAZIEVA *et al*., 2019; TAN; NORHAIZAN; LIEW, 2018;).

O desbalanço gerado pelo acúmulo de EROs promove alterações severas no ambiente celular comprometendo o funcionamento de órgãos afetados. Retinopatias, nefropatias, aterosclerose, diabetes e Alzheimer são doenças diretamente associadas a esse quadro, muitas delas de caráter crônico (CHEN; ZHONG, 2014; MOLDOGAZIEVA *et al*., 2019). A ingestão de antioxidantes exógenos pode contribuir na redução do dano oxidativo e na melhora clínica de muitos destes casos. Vitamina C, terpenos, quinonas e compostos fenólicos são importantes agentes redutores encontrados nos alimentos e em produtos naturais, como a própolis (BANKOVA, 2005; MARCUCCI *et al*., 2001; TIVERON *et al*., 2016).

Na literatura a propriedade antioxidante presente nas própolis é amplamente discutida, esses produtos resinosos produzidos por abelhas *Apis mellifera* ou abelhas sem ferrão apresenta uma rica composição química decorrente de seus inúmeros compostos fenólicos (BUENO-SILVA *et al*., 2017; COELHO *et al*., 2017; PARK; ALENCAR; AGUIAR, 2002). Portanto, o uso de própolis pode ser eficaz no controle das EROs, reduzindo o dano oxidativo e contribuindo com a melhora clínica em muitas doenças. *O objetivo do presente*

estudo foi avaliar os efeitos da propriedade antioxidante presente nas própolis sobre o estresse oxidativo e espécies reativas de oxigênio.

Esta dissertação foi elaborada na forma de dois artigos, conforme as determinações da UNIFAL-MG[1], sendo o primeiro uma revisão sistemática sobre própolis e seu efeito sobre o estresse oxidativo e parâmetros bioquímicos do diabetes e o segundo artigo, um estudo *in vitro* avaliando a capacidade de neutralização de radicais livres pela própolis verde do Sul de Minas Gerais.

[1] https://www.unifal-mg.edu.br/ppgcb/elaboracao-de-teses-e-dissertacoes/
https://www.unifal-mg.edu.br/bibliotecas/templates

2 ARTIGO 1 - EFFECTS OF PROPOLIS ON OXIDATIVE STRESS AND LIPID PROFILE IN EXPERIMENTALLY INDUCED DIABETES: A SYSTEMATIC REVIEW OF PRECLINICAL EVIDENCE[2]

[2] Artigo a ser submetido a revista "*Diabetes and Metabolic Syndrome: Clinical Research and Reviews*". Fator de impacto 2021 (2.462). Versão redigida conforme diretrizes do periódico

The effects of propolis on oxidative stress and lipid profile in experimentally-induced diabetes: a systematic review of preclinical evidence

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Abstract

In this systematic review, we evaluated the impact of the administration of propolis on oxidative stress and lipid profile in experimentally-induced diabetes mellitus (DM). Three databases (PubMed / Medline, Scopus, and Web of Science) were searched for eligible articles by applying search filters and specific keywords. A methodological bias assessment was performed using the SYRCLE tool. Our primary search returned 198 studies, of which 14 were considered eligible after removing duplicates and applying the exclusion criteria. Among the selected studies, 42% were from countries that are known to have a high prevalence of diabetics (China, Malaysia, and Nigeria), while the remainder were more heterogeneously distributed. Intraperitoneal injections of streptozotocin in male rats were the main method of DM induction. Propolis administration started after the onset of DM and caused a significant decrease in glycemic levels in diabetic animals, which likely results from a reduction of pancreatic oxidative stress associated with the restoration of endogenous antioxidant defenses. There was an improvement in the lipid profile of treated animals, although the corresponding metabolic pathways were not discussed in the studies. Our risk of bias assessment showed a methodological quality score of less than 40% due to lack of randomization, blinding, and appropriate allocation of animals. To conclude, treatment with propolis induced a significant hypoglycemic effect in diabetic animals compared to untreated controls, which is likely to be associated with a reduction of pancreatic oxidative stress.

Keywords: Flavonoids; Natural products; Blood glucose levels; Oxidative stress.

1 Introduction

Diabetes mellitus (DM) is a chronic endocrine disease characterized by constant hyperglycemia that results from a deficiency in insulin production (type 1 DM) or sensitivity (type 2 DM). Currently, over 420 million individuals worldwide have diabetes and approximately 1.6 million deaths are directly related to this disease each year [1,2].

Constant hyperglycemia in diabetic individuals stimulates the generation of reactive oxygen species (ROS). Oxygen-derived molecules are highly reactive and electronegative and act as oxidizing agents across different tissues. ROS can cause alterations in DNA, proteins, and lipids; induce cell apoptosis; destroy pancreatic β cells, and lead to exacerbated inflammatory processes. Despite the direct influence of ROS on the pathogenesis of DM, none of the current drugs have reducing mechanisms that can directly inhibit them. Therefore, the exploration of antioxidant sources could, at least in theory, provide new drug and development opportunities to manage DM [3,4].

In folk medicine, natural products such as plant extracts, teas, and, more recently, bee products, have been historically used to treat and/or prevent several diseases. Propolis is a chemically diverse resinous product produced by *Apis mellifera* or stingless bees that is made of plant exudates as well as wax, pollen, and honey. Over the years, several studies have highlighted the biological properties of bee resins and their applicability in the management of different diseases. Among the biological properties described for propolis, there are reports of antioxidant, anti-inflammatory, anticancer, antimicrobial, and immunomodulatory activity. More recently, propolis samples were also found to have antiviral activity against SARs-CoV-2, which is the virus that causes COVID-19 [5–10].

Therefore, the administration of propolis is a potential alternative for the treatment of DM, considering the antioxidant, anti-inflammatory, and immunomodulatory properties of the resins [3]. While the effects of propolis on DM have been previously reported, all available evidence is fragmented. Hence, a systematic review is needed to provide a comprehensive view of the data as well as possible limiting factors and methodological biases [11].

Thus, we carried out a systematic review to determine the relevance and impact of propolis administration on experimentally induced diabetes in animal models, mainly on outcomes related to redox balance and glycemic profile. Our study provides relevant insights into natural molecules with hypoglycemic mechanisms and their potential targets.

2 Methods

2.1 Guiding question and search strategy

This systematic review was designed to answer the following guiding question: Is the administration of propolis effective in controlling hyperglycemia and oxidative stress in animals with experimentally induced diabetes when compared to untreated diabetic animals?

To answer the guiding question, primary studies were selected based on the PRISMA strategy - Preferred Reporting Items for Systematic Reviews and Meta-analysis [12]. Relevant studies were selected from three databases, namely: PubMed / Medline, Scopus, and Web of Science. First, a search filter based on the PubMed platform was set, containing three levels of information: (i) biological condition (diabetes), (ii) intervention (propolis), (iii) study groups (animal models). The search filters were structured using standardized descriptors and keywords based on the Medical Subject Headings (MeSH) database. Searches were performed by associating Boolean operators (AND / OR / NOT) and search algorithms [MeSH Terms] and [TIAB]. Search filters were applied in PubMed and later in the Scopus and Web of Science platforms with the association of search algorithms and terms suitable for each database. This systematic review was registered in the International Prospective Registry of Systematic Reviews - PROSPERO (registration number: CRD42021290848).

2.2 Study selection

Literature searches were structured into two levels of information (primary and secondary) to ensure access to the greatest number of relevant studies. Initially, the studies were identified in the three electronic databases. In the Scopus platform, the descriptor NOT INDEX MEDLINE was associated with the search terms to ensure the removal of duplicate studies from PubMed/Medline. Identified primary studies were managed in the Mendeley Reference Management Program (Mendeley, London, Westminster, UK) and duplicates were removed using the "Check for Duplicates" tool. Retrieved studies were then screened for eligibility. Studies falling out of the scope of this review were excluded.

In the secondary search, the reference lists of relevant articles that were selected in the primary search were checked manually to identify possible additional studies. These search strategies are described in the PRISMA flowchart [12].

The other studies were accessed in full and included in the eligibility analysis, in

which well-defined inclusion and exclusion criteria were applied. Studies that addressed the effects of propolis administration on diabetic animals were considered relevant. The following studies were excluded: (i) not available in full; (ii) studies that were not written in English; (iii) gray literature (not indexed and not published in formal scientific peer-reviewed journals); (iv) studies that did not disclose the outcomes of interest; (v) secondary studies (*e.g.*, letters to the editor, conference abstracts, commentaries, notes, and books); (vi) studies that did not have at least one control group; (vii) Studies of diabetic disorders (retinopathies, nephropathies, or diabetic wounds).

Two researchers (Cunha, GA, and Carlstrom, PF) independently completed the screening for eligibility and study selection. Any disagreements between the examiners were resolved by consulting with a third examiner (Rosalen, PL).

2.2 Data extraction

After study selection, the data were structured in graphs and tables to facilitate the visualization and identification of outcomes. Two examiners (Cunha, GA, and Carlstrom, PF) evaluated the survey data, and differences were resolved by consensus in consultation with the third examiner (Rosalen, PL). The following descriptive levels were adopted:

I) Study characteristics: year, author, country of origin, DM induction method, study groups;

II) Characteristics of the animal model(s): age, species, lineage, body weight, sex;

III) Characteristics of propolis: origin, chemical profile, the form of administration, dosage, period of intervention, and;

IV) Measured outcomes: oxidative parameters, glycemic levels, and lipid profile.

2.3 Risk of Bias Assessment

The risk of bias was determined using the SYRCLE risk of bias tool for animal studies [13]. This tool was developed following the Cochrane Risk of Bias (ROB) tool, with adjustments for specific aspects of bias with a relevant impact on intervention animal studies. The SYRCLE tool is stratified into ten topics related to potential sources of bias, such as (i) selection, (ii) performance, (iii) detection, (iv) friction, (v) reporting, and (vi) additional sources of bias not covered by other domains. Based on the SYRCLE criteria, the risk of bias was categorized as: (i) High, (ii) Low, or (iii) Unclear. The overall and individual result obtained with the SYRCLE strategy was graphically expressed using the Review Manager

software (RevMan), version 5.3 (Cochrane Collaboration, 2014).

3 Results

3.1 Prisma-guided study selection

Searches in the three databases returned a total of 198 studies (PubMed/Medline n = 36; Scopus n = 112; Web of Science n = 50), from which 9 literature reviews were directly excluded. The other studies were imported into the Mendeley reference manager and 76 duplicates were removed by the "Check for duplicates" tool. The titles and abstracts of 109 articles were read, of which 62 were excluded for not falling into the scope of the systematic review. Forty-seven studies were considered eligible for full-text analysis, but 9 of them could not be retrieved. Hence, 38 articles were read in full and screened for the study criteria, of which 14 articles were selected for this systematic review. A detailed flowchart of the search strategy is shown in Figure 1. The search filters used in the databases are available in Supplementary Table S1.

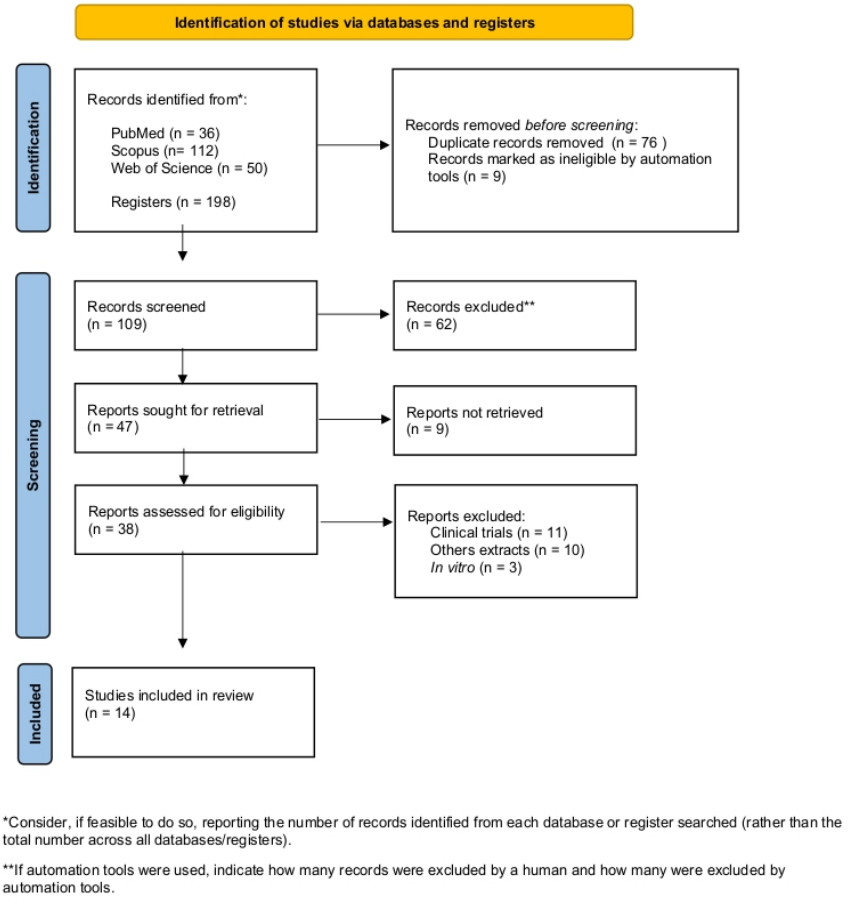

Figure 1 - PRISMA flowchart describing the stages of selection of eligible studies

3.2 Characteristics of selected studies

Studies from China, Malaysia, and Nigeria accounted for 42% of the publications in this review. The other studies (58%) were produced by authors from different countries, including Egypt, Indonesia, Japan, Morocco, Mexico, Saudi Arabia, Taiwan, and Turkey.

The administration of streptozotocin was the DM induction method in 78.57% of the selected studies, followed by the injection of alloxan (21.43%). The main route of drug administration was intraperitoneal (71.42%), followed by the intravenous route (28.58%). The data described above can be viewed in detail in Supplementary Table S2.

3.3 Characteristics of the animal models

Overall, rats and mice were used in 85.8% and 14.2% of the selected studies, respectively. Sprague-Dawley rats were most often used (42.7%), followed by Wistar rats (35.9%). The rat strain was not described in 7.2% of the articles. CD1 mice were used in 14.2% of the studies. Male animals were mostly used (85.8%) as compared to females (7.1%). In 7.1% of the studies, the authors did not disclose the sex of animals.

The average weight of animals was 260 g for rats and 37.5 g for mice. The age of the animals was omitted in 57.1% of the studies; 28.4% described the age in days (mean of 60 days), and 14.5% of them considered the animals to be adults (criterion established by the authors). Detailed characteristics of the animal models are presented in Supplementary Table S3.

3.4 Characteristics of propolis

The most frequent origin of propolis was Chinese, Nigerian, and Malay, which accounted for 42.9% of the samples in the selected studies. The other propolis types (49.9%) were Brazilian, Indonesian, Taiwanese, Mexican, Moroccan, Egyptian, and Turkish. In 7.2% of the studies, two different samples were used (Chinese and Brazilian).

Propolis extracts were administered mostly orally (92.8%) and less frequently via the intragastrical route (7.2%) at doses ranging from 10 to 919.5 mg/kg. The most common doses were 300 mg/kg (50%) and 200 mg/kg (35.7%). The effectiveness of propolis administration was dose-dependent, and there were no reports of toxicity.

The shortest exposure time of the animals to propolis extracts was 7 days, and the maximum was 70 days. In 57.2% of the studies, there was no chemical characterization of the

extract, indicating that animals were exposed to crude extracts whose chemical composition was unknown. Alarmingly, only 42.8% of the authors performed the identification of compounds by chromatographic methods. All results described above can be viewed in detail in Supplementary Table S4.

3.4.1 Identified compounds

The propolis samples that were chemically identified showed great heterogeneity in their composition. Table 1 shows the major compounds tentatively identified in the samples of the selected studies.

Table 1 - Major compounds tentatively identified in the propolis samples of the selected studies

Authors	Propolis origin	Chemical profile*
Matsushige et al. 1996	Brazil	Clerodane diterpenoid Quercetin
Usman et al. 2017	Malaysia	Glucuronic acid derivatives Ellagic acid Gallic acid derivatives
Chen et al. 2018	Taiwan	Propolin (D, F, C, H, and G)
Yañes et al. 2018	Mexico	Naringin Quercetin Luteolin Kaempferol
Hegazy et al. 2020	Egypt	2-[3,4-(Methylenedioxy) Phenyl]-1- Cyclopentanone 3-(2h)-Pyridazinone,4,5-Dihydro-4-(4-Methoxyphenyl) 7-Methoxy-3,6-Dimethyl-2-Tetralone 2'-Hydroxy-2,3,4',6'-Tetramethoxychalcone
Taleb et al. 2020	Turkey	Chrysin Caffeic acid phenyl ester

* As described by the authors

3.5 Measured outcomes

In this review, we chose to assess outcomes related to oxidative parameters, glycemic index, and lipid profile. The main molecules measured to describe changes in the redox balance in the animals' bodies were MDA (malondialdehyde) and TBARS (thiobarbituric acid

reactive substances). The antioxidant potential of propolis is related to the occurrence of phenolic compounds, whose chemical structure allows for the donation of electrons to unstable molecules (ROS or RNS), thereby reducing oxidative damage. However, measuring their levels and qualifying which antioxidant agents are acting in the organism is unfeasible, hence quantifying the activity or levels of endogenous antioxidant enzymes is preferred. Antioxidant enzymes such as SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), and the tripeptide GSH (glutathione) were the main reducing molecules evaluated in the selected studies.

The administration of propolis decreased MDA levels in all studies and increased the levels or activity of endogenous antioxidant enzymes on a dose-dependent basis.

The glycemic index was measured in 92.8% of the studies. The animals were characterized as diabetic when they had blood glucose levels above 11.1 mmol/L or greater than 200 mg/dL.

Dyslipidemias are a set of metabolic changes that lead to a progressive increase in blood lipid levels, a process that affects around 85% of diabetic individuals [14] Although lipid alterations are recurrent in DM, only 42.8% of the studies measured blood levels of low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Propolis administration reduced LDL levels and increased HDL levels in a dose-dependent fashion. All results described above can be seen in Supplementary Table S5.

3.5 Risk of methodological bias analyzed by the Syrcle's tool

In general, all studies analyzed presented a quality score of less than 30% based on the SYRCLE tool due to lack of relevant information for methodological development (inadequate georeferencing of propolis and lack of chemical characterization) and neglect of criteria such as randomization, allocation of animals and blinding of examiners. However, variables such as animal weight, propolis administration routes, DM induction method, and exposure time were described in 100% of the studies (Figure 2).

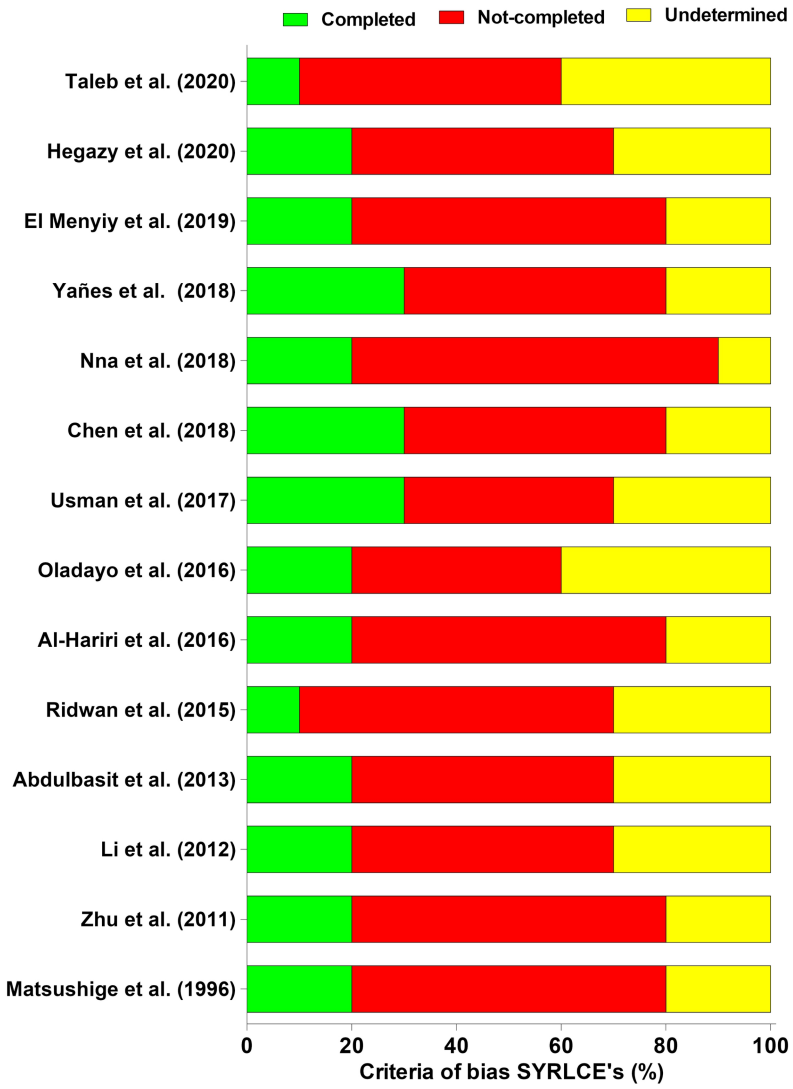

Figure 2 - Methodological bias analysis by the SYRCLE'S tool

4 Discussion

In this review, most studies that investigated the effects of propolis on DM were Chinese, Malaysian, and Nigerian. According to the International Diabetes Federation [2], these countries have a high prevalence of diabetes. China and Malaysia are in the western Pacific, a region whose growth in the number of DM cases will exceed 30% by the year 2045. Moreover, a 143% increase in the number of cases is expected for the African continent until that same year. Asian and African countries have been exploring the use of natural products for several centuries, particularly herbal infusions and medicinal extracts [15, 16]. We also note that all these studies explored local bee resins in their raw form and that isolated compounds from propolis were not evaluated.

Despite the alarming situation observed with the global increase in DM cases, intervention in humans requires robust evidence from preclinical *in vivo* trials to ensure the safety and efficacy of the active ingredients. The literature indicates rodent models as a species of choice to mimic the diabetic effects that would be observed in humans, as they present high similarity with our DNA (85% in coding regions) and are easy to handle [17,18]. For DM induction, intraperitoneal injection with 60 mg/kg streptozotocin (STZ) was the main method used by the authors, STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose). The toxicity of STZ is dependent on the DNA alkylating activity of its methylnitrosourea moiety [19,20]. Diabetic effects were also induced by intraperitoneal injections of alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxiacyl), whose dosages were greater than 100 mg/kg. Alloxan acts by selectively inhibiting glucose-induced insulin secretion through specific inhibition of glucokinase. According to Lenzen (2008) [20], streptozotocin is the agent of choice for DM induction in animals due to its chemical characteristics and high stability. Alloxan, on the other hand, is an excellent compound for ROS-mediated beta-cell toxicity models, although its effects are reported to be more severe in rat beta cells than in humans [19].

The oral administration of propolis effectively controlled the glycemic levels of the animals. These findings are intriguing since the flavonoids present in propolis (and in any other food of natural origin) have low bioavailability (10% or less), suggesting they have strong antidiabetic activity [21,22]. Those effects were dose-dependent and observed at all tested doses, with optimal effectiveness between 200 and 300 mg/kg. Higher dosages did not show significantly greater benefits.

The chemical composition of propolis is variable depending on environmental characteristics and extraction methods, which may have affected the final biological response. Therefore, the chemical characterization of the selected samples is necessary to elucidate which molecules are responsible (alone or synergistically) for the antidiabetic effects and/or to purify through chromatographic methods by the most active ones, if applicable [23–25].

Even though propolis samples were not chemically characterized in most studies, the main antidiabetic agents in propolis are flavonoids. Based on the literature, quercetin, naringin, luteolin, kaempferol, and chrysin have hypoglycemic effects by inducing insulin secretion, increasing the sensitivity of skeletal muscles to glucose, and selectively inhibiting α-amylase and α-glucosidase, although other metabolic pathways are also involved [26-29]. The effective propolis doses (200 to 300 mg/kg) are not toxic, according to the Food and Drug Administration, because propolis is generally recognized as safe (GRAS) [26]. These doses

are lower than those used in the standard treatment of type 2 DM with the drug metformin, whose doses vary from 500 mg/kg twice daily to 800 mg/kg daily [30].

In our review, the hypoglycemic effect observed in propolis was due to the neutralization of ROS in the pancreatic tissue and the increase of endogenous antioxidant defenses. Hyperglycemia activates numerous metabolic pathways that culminate in the generation of ROS, which can induce DNA changes, promote peroxidation of the phospholipid bilayer and lead to ATP deficit. Altogether, these mechanisms promote the necrosis of pancreatic β cells, resulting in insulin deficiency [31,32]. According to Newsholme et al. 2016 [31], it is extremely difficult to measure changes in ROS levels in the body, as reactive species have an extremely short half-life in biological fluids, cells, and tissues. Consequently, researchers have developed other techniques to determine the redox state, and these usually involve the assessment of stable by products of oxidative stress in the blood. In this review, MDA was the main marker for oxidative stress. In biological systems, this molecule is a byproduct of the lipid peroxidation of cell membranes as a consequence of the reaction of polyunsaturated fatty acids (PUFAs) and radical species. Compared to ROS, MDA has a relatively long half-life (minutes-hours) and an uncharged structure, making it a potentially more destructive compound [33,34]. Induced DM promoted alterations in the lipid peroxidation rate, as demonstrated by the plasma levels of MDA, which were reduced following the administration of propolis. Although the main antioxidant mechanism of propolis is the donation of electrons to ROS with their consequent stabilization, the studies also reported a reduction of lipid peroxidation, with the restoration of the body's endogenous enzyme antioxidant system.

The enzymes SOD, CAT, GPx, and the tripeptide GSH had their plasma levels increased in all studies. There were no reports of the possible causes that led to this result; however, the activation of the nuclear factor erythroid 2-related factor 2 (*Nrf2*) was pointed out in previous studies. *Nrf2* is a transcription factor that acts as the main regulator of the antioxidant response. In situations of oxidative stress, it migrates into the cell nucleus and activates genes involved in the expression of endogenous antioxidant enzymes and other ROS scavenging mechanisms [32,35]. Hotta et al. (2020) [35] demonstrated that treatment with Brazilian red propolis increased the mRNA levels of *Nrf2*, *Nqo1*, *Hmox1* genes responsible for the activation of endogenous antioxidant defenses. While it may not be pertinent to extrapolate these findings to the propolis types described in this review, the data suggest the *Nrf2* activation pathway is likely to be involved.

Lastly, the administration of propolis induced an increase in HDL and a decrease in

LDL levels in all the studies that examined these variables. Yet, further research is needed to elucidate the metabolic pathways and possible molecular targets involved in both processes. A possible route of action would be to control the expression of apolipoproteins, protein subunits responsible for the stabilization and transport of cholesterol molecules. In *in vitro* assays, quercetin and isoquercitrin positively modulated the expression of apolipoprotein A-I (apoA-I), a subunit present in HDL [37]. On the other hand, animals treated with naringenin showed a 36% reduction in the secretion of apolipoprotein B (apoB), a protein is related to LDL synthesis [38]. The compounds occurring in the propolis samples described in our review are likely to have similar effects in controlling the plasma levels of high and low-density lipoproteins. The changes observed in the lipid profile of animals provide parameters to explore the effects of propolis administration on other diseases, especially cardiovascular disorders that are directly linked to fat deposition in the blood vessel wall [39].

The methodological consistency of preclinical studies must be considered when examining the quality of evidence to support future clinical trials [40]. Surprisingly, none of the analyzed studies met all the methodological criteria proposed by the SYRCLE (Figure 2), presenting variable scores without chronological influence (year of publication). This result indicates that the reporting bias was systematically reproduced through the mechanistic research process, without interpretations of possible sources of bias. The main neglected aspects were randomization, precise georeferencing of the origin of propolis, animal allocation, randomization, the chemical composition of propolis, comments on study limitations, and generalizability to human biology [41,42]. Finally, we make it clear that our objective was not to confront the current results, nor to devalue them, but to verify the possible sources of current methodological bias and, from such notes, provide support for data consistency and reproducibility.

Conclusions

We conclude that propolis induced a significant hypoglycemic effect in diabetic animals when compared to untreated controls. This effect was associated with a reduction in pancreatic oxidative stress, a process mediated by ROS neutralization and restoration of endogenous antioxidant defenses. Propolis reestablished plasma levels of HDL and reduced those of LDL, possibly by modulating the transcription of apolipoproteins.

We also emphasize the need to review some methodological aspects to mitigate the sources of bias in preclinical approaches and ensure reproducibility in future studies, especially of criteria such as randomization, blinding, and characterization of propolis samples.

Ethics approval and consent to participate

Not Applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

Funding

None

Acknowledgments

None

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ARTIGO 1 - APÊNDICES

Table S1. Complete search strategy with search filters and number of studies recovered in databases PubMed-Medline†, Scopus, and Web of Sciences.

†PubMed-MEDLINE- Search filters	*Records*
#1 Propolis: (“Propolis”[MeSH Terms] OR “Bee Glue”[TIAB])	**2430**
#2 Biological condition (Diabetes): (“Diabetes mellitus”[MeSH Terms] OR “Diabetes”[TIAB] OR “Diabetes mellitus, type 1”[TIAB] OR “Diabetes mellitus, type 2”[TIAB] OR “Diabetes mellitus, experimental”[MeSH Terms])	**716669**
#3 Study groups (animal models): ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSHTerms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSHTerms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSHTerms:noexp] OR "primates"[MeSHTerms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSHTerms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSHTerms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSHTerms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongopygmaeus"[MeSH Terms])	**7074117**
#3 Combined search: #1 AND #2 AND #3 AND #4	**36**

Database search was concluded in November 20, 2021 at 17:20:56 p.m.

Table S1 (*continuation*). Complete search strategy with search filters and number of studies recovered in databases PubMed-Medline, Scopus† and Web of Sciences.

†SCOPUS – Search filters	*Records*
#1 Propolis: (TITLE-ABS-KEY("Propolis") OR TITLE-ABS-KEY("Bee Glue"))	**7826**
#2 Biological condition (Diabetes): (TITLE-ABS-KEY("Diabetes mellitus") OR TITLE-ABS-KEY("Diabetes") OR TITLE-ABS-KEY("Diabetes mellitus, type 1") OR TITLE-ABS-KEY("Diabetes mellitus, type 2") OR TITLE-ABS-KEY("Diabetes mellitus, experimental"))	**40437**
#3 Combined search: (#1 AND #2) AND NOT INDEX (medline)	**760**
#4 Search limits (Keyword): Animal	**112**

Database search was concluded in November 20, 2021 at 17:30:53 p.m

†WEB OF SCIENCE – Search filters	*Records*
#1 Propolis: TS=Propolis OR TS=Bee Glue	**8643**
#2 Biological condition (Diabetes): TS=Diabetes mellitus OR TS=Diabetes OR TS=Diabetes mellitus, type 1 OR TS=Diabetes mellitus, type 2 OR TS=Diabetes mellitus, experimental	**121345**
#3 Combined search: (#1 AND #2) AND NOT INDEX (medline)	**50**

Database search was concluded in November 20, 2021 at 17:45:33 p.m

Table S2 - Study characteristics

Authors	Country of origin	DM induction method	Study groups
Matsushige et al. 1996	Japan	Single intravenous injection of various doses (30-70 mg/kg) of streptozotocin (STZ)	I) Control (normal group) II) Diabetic (STZ 30 - 70 mg/kg) III) Nicotinamide (200 mg/kg) IV) Propolis water extract (200 mg/kg) V) Propolis methanolic extract (200 mg/kg)
Zhu et al. 2011	China	Injected intravenously through the tail vena with a single dose of 2% STZ (50 mg kg^{-1})	I) Normal group (saline) II) Diabetic (STZ 50 mg/kg) III) Positive group (saline) IV) Chinese propolis (10 mg/100g bw) V) Brazilian propolis (10 mg/100g bw) VI) Glucobay (10 mg/kg bw)
Li et al. 2012	China	Rats were fed with higth-fat diet and injected intravenously with low-dose STZ (10, 5, 20, and 10 mg/kg)	I) Diabetic (STZ) II) Normal group (normal diet) III) Propolis (50 mg/kg) IV) Propolis (100 mg/kg) V) Propolis (200 mg/kg)
Abdulbasit et al. 2013	Nigeria	Single intraperitoneal injection of alloxan monohydrate (100 mg/kg)	I) Normal group (saline) II) Propolis non-diabetic (200 mg/kg) III) Diabetic control (saline) IV) Diabetic treated (150 mg/kg metformin) V) Propolis (200 mg/kg) VI) Propolis (300 mg/kg)

Table S2 (*Continuation*) - Study characteristics

Authors	Country of origin	DM induction method	Study groups
Ridwan et al. 2015	Indonesia	Single intraperitoneal injection of alloxan monohydrate (200 mg/kg)	I) Normal group II) Diabetic control (alloxan 200 mg/kg) III) Propolis(50 mg/kg) IV) Propolis (100 mg/kg) V) Propolis (200 mg/kg) VI) Propolis (175 mg/kg)
Al-Hariri et al. 2016	Saudi Arabia	Single intraperitoneal injection of STZ (60 mg/kg)	I) Normal group II) Diabetic control (STZ 60 mg/kg) III) Propolis(0.3 g/kg)
Oladayo et al . 2016	Nigeria	Single intraperitoneal injection of alloxan (110 mg/kg)	I) Normal group (saline) II) Diabetic control (alloxan 200 mg/kg) III) Propolis(200 mg/kg) IV) Propolis (300 mg/kg) V) Metformin (150 mg/kg)
Usman et al. 2017	Malaysia	Single intraperitoneal injection of STZ (60 mg/kg)	I) Normal group (water) II) Diabetic control (STZ 60 mg/kg) III) Propolis(300 mg/kg) IV) Propolis (600 mg/kg) V) Metformin (100 mg/kg)
Chen et al. 2018	Taiwan	Intraperitoneally injected with streptozotocin (STZ) at 15 mg/kg every two days fromweek 1 to week 4	I) Diabetic untreated (water) II) Propolis (183.9 mg/kg) III) Propolis(919.5 mg/kg)

Table S2 (*Continuation*) - Study characteristics

End

Authors	Country of origin	DM induction method	Study groups
Nna et al. 2018	Malaysia	Single intraperitoneal injection of STZ (60 mg/kg)	I) Diabetic control (water) II) Propolis (300 mg/kg) III) Meformin (300 mg/kg) IV) Propolis + metformin(300 mg/kg)
Yañes et al. 2018	Mexico	Single intraperitoneal injection of STZ (130 mg/kg)	I) Normal control (water) II) Diabetic group (130 mg/kg) III) Meformin (0.3 g/kg)
El Menyiy et al. 2019	Marocco	Single intravenous injection of STZ (60 mg/kg)	I) Normal group (water) II) Glibenclamide non-diabetic (2.5 mg/kg) III) Propolis non-diabetic (50 mg/kg) IV) Propolis non-diabetic (100 mg/kg) V) Diabetic untreated (water) VI) Glibenclamide diabetic (2.5 mg/kg) VII) Propolis diabetic (50 mg/kg) VIII) Propolis diabetic (100 mg/kg)
Hegazy et al. 2020	Egypt	Single dose intraperitoneal injection (35 mg/ kg STZ) for 3 successive days	I) Normal group (saline) II) Diabetic untreated (STZ 35 mg/kg) III) Propolis (300 mg/kg) IV) CSA-PAA (300 mg/kg) V) Propolis + CSA-PAA(300 mg/kg) VII) Metformin (100 mg/kg)
Taleb et al. 2020	Turkey	Intraperitoneal injection of STZ (65 mg/kg)	I) Normal group (water) II) Diabetic untreated (STZ 65 mg/kg) III) Propolis (30% mg/kg) IV) CSA-PAA (15% mg/kg)

Table S3 - Characterisitics os animals models

Authors	Species	Linage	Sex	Body weigth	Age
Matsushige et al. 1996	Rats	Sprague-Dawley	Male	220-240 g	56 days
Zhu et al. 2011	Rats	Sprague-Dawley	Male	230-310 g	(-)
Li et al. 2012	Rats	Sprague-Dawley	Male	270-370 g	(-)
Abdulbasit et al. 2013	Rats	Wister	Male	200-250 g	Adult (?)
Ridwan et al. 2015	Mice	Wistar	Male	30-45 g	56 days
Al-Hariri et al. 2016	Rats	Wistar	Male	150-250 g	(-)
Oladayo et al . 2016	Rats	(-)	(-)	160-200 g	(-)
Usman et al. 2017	Rats	Sprague-Dawley	Female	190-220 g	56-70 days
Chen et al. 2018	Rats	Sprague-Dawley	Male	270 g	(-)
Nna et al. 2018	Rats	Sprague-Dawley	Male	250-300 g	(-)
Yañes et al. 2018	Mice	CD1	Male	(-)	49 days
El Menyiy et al. 2019	Rats	Wistar	Male	150-220 g	Adult (?)
Hegazy et al. 2020	Rats	Wistar	Male	(-)	(-)
Taleb et al. 2020	Rats	Wistar	Male	250-300 g	(-)

Table S4 - Characteristics of propolis

Authors	Origin	Route of administration	Dosage	Intervention time	Majority molecules*
Matsushige et al. 1996	Brazil	Oral	200 mg/kg	7 days	Clerodane diterpenoid Quercetin
Zhu et al. 2011	China/Brazil	Intragastrically	10mg/100g bw	56 days	(-)
Li et al. 2012	China	Oral	50, 100 and 200 mg/kg	70 days	(-)
Abdulbasit et al. 2013	Nigeria	Oral	200 and 300 mg/kg	28 days	(-)
Ridwan et al. 2015	Indonesia	Oral	50, 100 and 175 mg/kg	21 days	(-)
Al-Hariri et al. 2016	China	Oral	300 mg/kg	14 days	(-)
Oladayo et al . 2016	Nigeria	Oral	200 and 300 mg/kg	42 days	(-)
Usman et al. 2017	Malaysia		200 and 300 mg/kg	28 days	Glucoronic acid derivatives Ellagic acid Gallic acid derivatives
Chen et al. 2018	Taiwan	Oral	189.3 and 919.5 mg/kg	56 days	Propolin (D, F, C, H and G)
Nna et al. 2018	Malaysia	Oral	300 mg/kg	28 days	(-)
Yañes et al. 2018	Mexico	Oral	300 mg/kg	15 days	Naringin Quercetin Luteolin Kaempeferol
El Menyiy et al. 2019	Marocco	Oral	50 and 100 mg/kg	15 days	(-)
Hegazy et al. 2020	Egypt	Oral	300 mg/kg	30 days	2-[3,4-(Methylenedioxy) Phenyl]-1-Cyclopentanone 3-(2h)-Pyridazinone, 4,5-Dihydro-4- (4-Methoxyphenyl) 7-Methoxy-3,6-Dimethyl-2-Tetralone 2'-Hydroxy-2,3,4',6'-Tetramethoxychalcone
Taleb et al. 2020	Turkey	Oral	30% from EEP 15% from 30% EEP	14 days	Crysin Caffeic acid phenyl ester

(-) Data not reported
* Established by authors

Table S5 - Measured outcomes*

Authors	Oxidative parameters	Glycemic levels	Lipid profile
Matsushige et al. 1996	(-)	Propolis decreased blood glucose levels (mg/dL) compared to diabetic group: Diabetic: (352.7 ± 36.6) Ethanol extract (200mg/kg): (261.2 ± 45.3) Water extract (200 mg/kg):(169.6 ± 17.3)	(-)
Zhu et al. 2011	Propolis decreased MDA (nmol L^{-1}) compared to diabetic group: Diabetic: (5.15 ± 0.55) Chinese propolis: (3.61 ± 0.80) Brazilian propolis: (4.80 ± 2.11) Propolis increased SOD (U mL^{-1}), CAT U mL^{-1}), GSH-Px (μmol L^{-1}) compared to diabetic group: Diabetic: SOD (39.42 ± 14.30); CAT (9.65 ± 0.83); GSH-Px (687.88 ± 48.29) Chinese propolis: SOD (44.46 ± 11.66); CAT (9.97 ± 1.04); GSH-Px (682.35 ± 48.89) Brazilian propolis: SOD (54.53 ± 3.41); CAT (11.04 ± 1.07); GSH-Px (663.38 ± 80.87)	Propolis decrease fasting blood glucose (mmol/L) compared to diabetic group: Diabetic: (29.3) Chinese propolis (10 mg/100 g): (19.8) Brazilian propolis (10 mg/ 100 g): (18.63)	(-)
Li et al. 2012	(-)	Propolis decrease fasting blood glucose (mmol/L) compared to diabetic group: Diabetic:(9.05 ± 1.06) Propolis (50 mg/kg):(7.88 ± 0.46) Propolis (100 mg/kg):(7.51 ± 0.50) Propolis (200 mg/kg):(7.37 ± 0.68)	Propolis decreased LDL cholesterol (mmol/L) and increased HDL cholesterol (μmmol/L): Diabetic: LDL (0.32 ± 0.07); HDL (1.09 ± 0.26) Propolis (50 mg/kg): LDL (0.27 ± 0.05); HDL (1.14 ± 0.21) Propolis (100 mg/kg): LDL (0.29 ± 0.06); HDL (1.12 ± 0.19) Propolis (200 mg/kg): LDL (0.26 ± 0.05); HDL (1.11 ± 0.17)

Table S5 (*Continuation*) - Measured outcomes

Authors	Oxidative parameters	Glycemic levels	Lipid profile
Abdulbasit et al. 2013	Propolis decreased MDA (nmol/ mg protein) compared to diabetic group: Diabetic: (1.1 ± 0.16) Propolis (200 mg/kg): (0.58 ± 0.03) Propolis (300 mg/kg): (0.53 ± 0.02) Propolis increased SOD (U/g protein), GSH (U/mL) compared to diabetic group: Diabetic: SOD (26.2 ± 3.0); GSH (5.1 ± 1.2); Propolis (200 mg/kg): SOD (60.5 ± 3.3); GSH (12.9 ± 2.1) Propolis (300 mg/kg): SOD (83.1 ± 4.8); GSH (15.3 ± 1.8)	Propolis decreased blood glucose levels (mg/dL) compared to diabetic group: Diabetic: (397.8) Propolis (200 mg/kg): (159.4) Propolis (300 mg/kg): (141.3)	Propolis decreased LDL cholesterol (mg/dL) and increased HDL cholesterol (mg/dL): Diabetic: LDL (46.6 ± 5.8); HDL (22.7 ± 2.6) Propolis (200 mg/kg): LDL (23.3 ± 3.2); HDL (33.6 ± 0.21) Propolis (300 mg/kg): LDL (23.3 ± 2.5); HDL (31.1 ± 3.8)
Ridwan et al. 2015	Propolis decreased ROS density (mm^2/mg tissue) in the pancreas compared to diabetic group: Diabetic: (129.1) Propolis (50 mg/kg): (61.8) Propolis (100 mg/kg): (32.5) Propolis (100 mg/kg): (30.4)	(-)	(-)
Al-Hariri et al. 2016	Propolis decreased TBARS (µmol/L) compared to diabetic group: Diabetic: (71.9 ± 11.5) Propolis (300 mg/kg): (58.3 ± 9.7)	Propolis decrease fasting blood glucose (mgl/dL) compared to diabetic group: Diabetic: (221.1 ± 15.6) Propolis (300 mg/kg): (197.7 ± 50.6)	Propolis decreased LDL cholesterol (mmol/L) and increased HDL cholesterol (mmol/L): Diabetic: LDL (0.32 ± 0.07); HDL (1.09 ± 0.26) Propolis (50 mg/kg): LDL (0.27 ± 0.05); HDL (1.14 ± 0.21) Propolis (100 mg/kg): LDL (0.29 ± 0.06); HDL (1.12 ± 0.19) Propolis (200 mg/kg): LDL (0.26 ± 0.05); HDL (1.11 ± 0.17)

Table S5 (*Continuation*)- Measured outcomes

Authors	Oxidative parameters	Glycemic levels	Lipid profile
Oladayo et al. 2016	(-)	Propolis decreased blood glucose levels (mg/dL) compared to diabetic group: Diabetic: (455.1 ± 2.03) Propolis (200 mg/kg): (140.1 ± 22.20) Propolis (300 mg/kg): (126.08 ± 4.44)	Propolis decreased LDL cholesterol (mg/dL) and increased HDL cholesterol (mg/dL): Diabetic: LDL (50.85 ± 1.57); HDL (19.21 ± 1.07) Propolis (200 mg/kg): LDL (45.90 ± 1.64); HDL (41.00 ± 1.51) Propolis (300 mg/kg): LDL (37.36 ± 1.25); HDL (33.00 ± 0.73)
Usman et al. 2017	Propolis decreased MDA (nmol/ mg protein) and PCO (nmol/ mg protein) compared to diabetic group: Diabetic: MDA (1.88 ± 0.42); PCO (3.26 ± 0.57) Propolis (300 mg/kg): MDA (0.85 ± 0.35); PCO (1.58 ± 0.11) Propolis (600 mg/kg):MDA (0.76 ± 0.18); PCO (1.40 ± 0.08)	Propolis decreased blood glucose levels (mg/dL) compared to diabetic group: Diabetic: (541.88 ± 62.45) Propolis (300 mg/kg): (307.50 ± 33.63) Propolis (600 mg/kg): (270.88 ± 86.25)	(-)
Chen et al. 2018	Propolis decreased TBARS (?) compared to diabetic group: Diabetic: (2.52 ± 0.08) Propolis (183.9 mg/kg): (2.12 ± 0.08) Propolis (919.5 mg/kg): (1.3 ± 0.11) Propolis increased SOD (?), GPx (?) levels compared to diabetic group: Diabetic: SOD (1.88 ± 0.42); GPx (3.26 ± 0.57) Propolis (300 mg/kg): SOD (0.85 ± 0.35); GPx (1.58 ± 0.11) Propolis (600 mg/kg):SOD (0.76 ± 0.18); GPx (1.40 ± 0.08)	Propolis decreased blood glucose levels (mg/dL) compared to diabetic group: Diabetic: (415.30 ± 50.30) Propolis (183.9 mg/kg): (214.20 ± 19.00) Propolis (919.5 mg/kg): (144.20 ± 8.10)	Propolis decreased LDL cholesterol (mg/dL) and increased HDL cholesterol (mg/dL): Diabetic: LDL (8.4 ± 0.16); HDL (15.3 ± 0.14) Propolis (200 mg/kg): LDL (7.8 ± 0.30); HDL (17.2 ± 0.20) Propolis (300 mg/kg): LDL (5.3 ± 0.19); HDL (22.2 ± 0.39)

Table S5 (*Continuation*)- Measured outcomes

Authors	Oxidative parameters	Glycemic levels	Lipid profile
Nna et al. 2018	Propolis decreased MDA (nmol/mg protein) levels compared to diabetic: group: Diabetic:(8.66 ± 0.94) Propolis (300 mg/kg): (3.68 ± 0.64) Propolis increased SOD, CAT, GPx, and GSH activity (unit/mg protein): Diabetic: SOD (1.26 ± 0.23); CAT (11.40 1.58); GPx (10.41 ± 1.85); GSH (0.86 ± 0.17) Propolis (300 mg/kg): SOD (3.03 ± 0.40); CAT (33.20 ± 3.58); GPx (27.34 ± 2.92); GSH (1.83 ± 0.39)	Propolis decreased fasting blood glucose (mmol/L) compared to diabetic group: Diabetic:(28.02 ± 1.73) Propolis (300 mg/kg): (12.02 ± 0.82)	(-)
Yañes et al. 2018	Propolis increased SOD, CAT, and GPx activity (nmol/min/mL): Diabetic: SOD (0.009 ± 0.001); CAT (1.65 0.78); GPx (32.81 ± 6.93) Propolis (300 mg/kg): SOD (0.013 ± 0.004); CAT (2.74 ± 0.40); GPx (53.56 ± 8.75)	Propolis decreased fasting blood glucose (mg/dL) compared to diabetic group: Diabetic:(586.43 ± 15.80) Propolis (300 mg/kg): (364.70 ± 19.70)	(-)
El Menyiy et al. 2019	(-)	Propolis decreased fasting blood glucose (mg/dL) compared to diabetic group: Diabetic: (441 ± 12) Propolis (50 mg/kg): (315 ± 11) Propolis (100 mg/kg): (198 ± 15)	Propolis decreased LDL cholesterol (mg/dL) and increased HDL cholesterol (mg/dL): Diabetic: LDL (77 ± 3.2); HDL (20.1 ± 2.0) Propolis (50 mg/kg): LDL (48 ± 3.7); HDL (36.6 ± 1.5) Propolis (100 mg/kg): LDL (31.5 ± 3.2); HDL (42.0 ± 2.1)

Table S5 (*Continuation*) - Measured outcomes

Authors	Oxidative parameters	Glycemic levels	Lipid profile
Hegazy et al. 2020	(-)	Propolis decreased fasting blood glucose (mg/dL) compared to diabetic group: Diabetic: (295.61 ± 33.69) Propolis (300 mg/kg): (156.58 ± 16.09) Propolis + CS-PAA (300 mg/kg): (111.21 ± 13.49)	(-)
Taleb et al. 2020	(-)	Propolis decreased fasting blood glucose (g/L) compared to diabetic group: Diabetic: (5.04) Propolis (15%): (3.3) Propolis (30%): (1.5)	(-)

(-) Data not reported *End*

*Data taken directly from the publications, when presented in graphs, the extraction took place automatically with the help of ImageJ software (Schneider, C.A., Rasband, W.S., Eliceiri, K.W. "NIH Image to ImageJ: 25 years of image analysis". Nature Methods 9, 671-675, 2012.)

Table S6- SYRCLE's risk of bias tool for animal studies

Authors	Was the allocation sequence adequately generated and applied?	Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Was the allocation to the different groups adequately concealed during?	Were the animals randomly housed during the experiment?	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	Were animals selected at random for outcome assessment?	Was the outcome assessor blinded?	Were incomplete outcome data adequately addressed?	Are reports of the study free of selective outcome reporting?	Were the molecules responsible for the observed biological effect identified by the authors?	**Quality score (%)**
Matsushige et al. 1996	No	No	Unclear	No	No	No	No	Unclear	Yes	Yes	20
Zhu et al. 2011	No	Yes	Unclear	No	No	No	No	Unclear	Yes	No	20
Li et al. 2012	No	Yes	Unclear	Unclear	No	No	No	Unclear	Yes	No	20
Abdulbasit et al. 2013	Unclear	Yes	Unclear	No	No	No	No	Unclear	Yes	No	20
Ridwan et al. 2015	No	Unclear	Unclear	No	No	No	No	Unclear	Yes	No	10
Al-Hariri et al. 2016	No	Yes	Unclear	No	No	No	No	Unclear	Yes	No	20
Oladayo et al . 2016	Unclear	Yes	Unclear	Unclear	No	No	No	Unclear	Yes	No	20
Usman et al. 2017	No	Yes	Unclear	Unclear	No	No	No	Unclear	Yes	Yes	30

Table S6 (*Continuation*) - SYRCLE's risk of bias tool for animal studies

Authors	Was the allocation sequence adequately generated and applied?	Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Was the allocation to the different groups adequately concealed during?	Were the animals randomly housed during the experiment?	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	Were animals selected at random for outcome assessment?	Was the outcome assessor blinded?	Were incomplete outcome data adequately addressed?	Are reports of the study free of selective outcome reporting?	Were the molecules responsible for the observed biological effect identified by the authors?	**Quality score (%)**
Chen et al. 2018	No	Yes	Unclear	No	No	No	No	Unclear	Yes	Yes	30
Nna et al. 2018	No	Yes	Unclear	No	No	No	No	Unclear	Yes	No	20
Yañes et al. 2018	No	Yes	Unclear	No	No	No	No	Unclear	Yes	Yes	30
El Menyiy et al. 2019	No	Yes	Unclear	No	No	No	No	Unclear	Yes	No	20
Hegazy et al. 2020	No	Yes	Unclear	No	No	No	No	Unclear	Unclear	Yes	20
Taleb et al. 2020	No	Unclear	Unclear	No	No	No	No	Unclear	Unclear	Yes	10
Quality score / itens-Yes (n)	0	11	0	0	0	0	0	0	12	6	
Quality score / itens (%)	0	78.5	0	0	0	0	0	0	85.7	42.8	

End

3 ARTIGO 2 - PHENOLIC COMPOSITION AND ANTIOXIDANT ACTIVITY OF MUCOADHESIVE FORMULATION (MuAd-P) AND ETHANOL EXTRACT (EEP) BASED ON GREEN PROPOLIS FROM SOUTHERN MINAS GERAIS[3]

[3] Artigo a ser submetido a revista "Journal of Ethnopharmacology". Fator de impacto 2021 (4.360). Versão redigida conforme diretrizes do periódico

PHENOLIC COMPOSITION AND ANTIOXIDANT ACTIVITY OF MUCOADHESIVE FORMULATION (MuAd-P) AND ETHANOL EXTRACT (EEP) BASED ON GREEN PROPOLIS FROM SOUTHERN MINAS GERAIS

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Abstract

In the present study, we aimed to determine the total phenolic composition, total flavonoid content, and antioxidant activity of an aqueous mucoadhesive gel (MuAd-P) and an ethanolic extract (EEP) of green propolis from southern Minas Gerais. Through RP-HPLC we identified the compounds present in propolis, and artepelin C was the major molecule found. The content of phenolic compounds was determined by the Folin-Ciocalteu method. In this trial, MuAd-P and EEP showed a total phenolic content of 268.0 and 234.8 mg GAE/g, respectively ($p<0.05$). Total flavonoid content was also statistically different between products, MuAd-P was 159.3 mg QE/g and EEP was 105.5 mg QE/g ($p<0.05$). The linseed oil polyphenols have influenced the higher MuAd-P activity in both assays present only in this formulation. Finally, the antioxidant activity of MuAd-P and EEP was determined by the DPPH, $ABTS^{\bullet+}$, and FRAP methods. MuAd-P showed slightly higher activity than EEP in these assays, with 1165.1 µmol TE/g in DPPH, 2911.6 µmol TE/g in $ABTS^{\bullet+}$, and 2474.6 µmol FS /g in FRAP. For the three methodologies, EEP reached values of 1144.1 µmol TE/g, 2708.8 µmol TE/g, and 2413.1 µmol FS/g, respectively. Despite this small difference, MuAd-P and EEP did not differ statistically in their reducing activity in any of the three trials ($p>0.05$). In conclusion, both formulations (MuAd-P and EEP) showed a rich phenolic and flavonoid composition and strong antioxidant activity. Future trials will elucidate whether other biological properties of this formulation remain unchanged. Providing reliable parameters for its use in *in vivo* studies, especially those related to the treatment of mucosal tissue lesions whose pathological origin is multivariate.

Keywords: Flavonoids; Chemical profile; *Baccharis dracunculifolia*; Biological properties.

1 Introduction

Over the years, green propolis from Minas Gerais has stood out in the national and international market as a non-toxic food with many health benefits. Its consumption became popular as an agent to treat colds and throat infections, however, other biological properties are described, among them antioxidant, anti-inflammatory, anticancer, and immunomodulatory, all resulting from the rich chemical composition of this variety of bee resin (Park, Alencar & Aguiar, 2002; Cabral et al., 2012; Cavalaro et al., 2020) All these characteristics make green propolis from Minas Gerais a potential target for the development of pharmaceutical products or the isolation of molecules (Burdock, 1998; Sforcin; Bankova, 2011; Tiveron et al., 2016; Bueno-Silva et al., 2017; Nani et al., 2020).

The main form of commercialization of propolis-based products is through extracts, alcoholic formulations intended for oral use, or as a spray. Despite being effective in the treatment of several diseases, they contraindicate their use on mucosal lesions because of their alcohol content and the low oral bioavailability of the flavonoids found in propolis (Thilakarathna, Vasantha Rupasinghe, 2013). In this sense, we developed a non-alcoholic mucoadhesive formulation based on green propolis from southern Minas Gerais. In this way, phenolic compounds are concentrated on the region and released gradually, exercising their biological function for a longer time (Abbasi et al., 2018; Alqahtani et al., 2021).

Many compounds can be lost in the production processes of propolis extracts, altering their chemical composition and the effectiveness of the biological properties present. These changes are major limiting factors for manufacturing propolis-based pharmaceuticals, as they make standardization unfeasible (Marcucci et al., 2001; Bankova, 2005). In these cases, it is advisable to check whether variations in the chemical composition of the formulations occur and are significant to the point of inducing the loss of properties. Therefore, *in vitro* assays provide parameters to verify if such alterations can compromise the effectiveness of the product as a therapeutic agent (Sforcin; Bankova, 2011; Vieira de Morais et al., 2021).

Among the biological properties present in green propolis, the antioxidant is one of the best described, as it has therapeutic, industrial, and cosmetic potential. An antioxidant is a compound capable of inhibiting the oxidation of an oxidizable substrate through the donation of electrons. In organic systems, redox reactions occur naturally, however, some pathologies result in the redox's destabilization environment and compromise the functioning of cellular functions, requiring the ingestion of exogenous antioxidants to reestablish homeostatic parameters (Cabral et al., 2012; Cavalaro et al., 2019; Moldogazieva et al., 2019).

The present study aimed to determine the total phenolic composition, total flavonoid content, and antioxidant activity of a aqueous mucoadhesive gel (MuAd-P) and an ethanolic extract (EEP) of green propolis from southern Minas Gerais.

2 Methodology

2.1 Collection and extraction of propolis

The green propolis sample (300 mg) was purchased in the city of Gaxupé, state of Minas Gerais (-21°16'20.1"S 46°42'52.4"W) in March 2020. Then taken to the laboratory of Bioprocesses from the Department of Food and Medicines at the Federal University of Alfenas, where it was kept in a freezer at -20ºC until the moment of extraction.

The extraction process took place according to Tiveron et al. (2016), green propolis was extracted in 80% ethanol (v/v) for 30 min at 70°C. After extraction, the mixture was centrifuged for the separation of waxes and then the excess solvent was removed in rota evaporator. The extraction process resulted in a 30% yield. After extraction, the ethanolic extract of green propolis (EEP) and aqueous mucoadhesive gel (MuAd-P) were prepared.

EEP at 11% (v/v) was prepared by diluting 5.5 g of lyophilized propolis in 50 mL of 80% ethanol. It was stored in a refrigerator and used as a stock solution for serial dilutions in each assay.

MuAd-P at 11% (v/v) was prepared by diluting 2.97 g of lyophilized propolis in 27 g of vehicle (surfactants, stabilizers, and linseed oil), stored under ambient conditions, and used as a stock solution for serial dilutions in each assay. This formulation is under patent confidentiality, therefore its description was limited to protect the invention.

The mucoadhesive gel vehicle was isolated and submitted to all tests to verify its influence on this formulation.

2.2 Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

HPLC separation method was performed as described by Tiveron et al. (2016), with slight modifications, using reversed phase RP-HPLC in a chromatograph equipped with Agilent Eclipse column (XDB-C18, column size 4.6 × 250 mm; particle size 5 μm), DAD detector (SPD-M10AVp, Shimadzu Co., Kyoto, Japan), and ultraviolet-visible (UV-Vis) detector (SPD-20AV, Shimadzu).The lyophilized extract of propolis (4% (m/v) in methanol 80%) was filtered through a 0.22 μm filter (Millex PTFE) prior to injecting 5-μL aliquots into the HPLC system. The mobile phase consisted of water/acetic acid (99.5/0.5 v/v) (A) and methanol (100%) (B). Gradient elution was performed as follows: starting with 30% B and increasing to 40% B (15 min), 50% B (30 min), 60% B (45 min), 75% B (85 min), 95% B (95

min), and decreasing to 30% B (105 min), at a solvent flow rate of 0.8 mL/min. Data were analyzed using Shimadzu software Class-VP.

2.3 Total phenolic composition by the Folin-Ciocalteu Method

The content of total phenolic compounds was determined by Folin-Ciocalteu, as described by Al-Duais et al. (2009), adapted for micro volumes. About 20 µL of the samples (EEP, MuAd-P, Vehicle/MuAd-P or standard) were added to the microplate wells. Then, we added 100 µL of the 10% Folin-Ciocalteu reagent and, after 5 minutes, 75 µL of the 7.5% potassium carbonate solution. The reaction took place for 40 minutes at room temperature and was protected from light, the absorbance was measured in a microplate reader at 740 nm. The total content of phenolic compounds was expressed in gallic acid equivalents (GAE), calculated based on a calibration curve from 20 to 120 µg/mL. The blank was composed of 20 µL distilled water in place of the sample. The samples were analyzed in triplicate and expressed in mg of GAE/g of propolis extract.

2.4 Determination of total flavonoid content by dddition of aluminum chloride

The complex formed by the association between aluminum (III) and the flavonoids' carbonyl and hydroxyl groups promotes an increase in absorbance through the bathochromic shift of flavonoid bands I and II, a reaction that can be monitored by spectrophotometry. The analysis was conducted according to Ássimos (2014), with adaptations and adjustments for micro volumes. The 96-well microplates were divided into two quadrants, in the first, we added 50 µL of samples (EEP, MuAd-P, Vehicle/MuAd-P or standard), then 100 µL of potassium acetate (CH_3COOK), and finally 100 µL of aluminum chloride ($AlCl_3$). In the second quadrant, we followed the same procedures, however, $AlCl_3$ was replaced by 80% ethanol (v/v). The blank consisted of 50 µL of distilled water or 80% ethanol in place of the sample. The reaction took place for 40 minutes in the dark, the reading was carried out at 415 nm in a microplate reader. The content of total flavonoids was expressed in quercetin equivalent (QE), calculated based on a calibration curve between 20 and 70 µL/mL, constructed by plotting the concentration on the y-axis and the absorbance on the x-axis, the absorbance has calculated starting from the difference between the aluminum-containing quadrant and the non-aluminum quadrant.

2.5 DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is an intensely colored stable free radical. In the presence of an antioxidant, DPPH will be reduced to hydrazide, a pale yellow compound, a reaction accompanied by a decrease in absorbance. The analysis was conducted as described by Tiveron et al., (2016) with adaptations. In 96-well microplates we added 66 µL of the samples (EEP, MuAd-P, Vehicle/MuAd-P or standard), 134 µL of the ethanol solution of DPPH at 150 µM. The blank was composed of 200 µL of ethanol PA or water. The reaction medium was kept in the dark for 40 minutes, reading took place at 517 nm. A standard Trolox curve was constructed with concentrations ranging from 20 to 140 µM. The results were expressed as µmol Trolox equivalents per mg of sample (µmol TE/mg).

2.6 ABTS free radical scavenging assay

The antioxidant activity by the $ABTS^{\bullet+}$ radical scavenging method was performed according to the procedure described by Al-Duais et al. (2009) with modifications and adapted for micro volumes. The $ABTS^{\bullet+}$ radical was generated by adding 88 µL of 140 mM potassium persulfate to 5 mL of 7 mM ABTS solution, the reaction occurred in the dark for 16 hours. After this period, the $ABTS^{\bullet+}$ radical solution was corrected for absorbance of 0.7 ± 0.02 at 734 nm using potassium phosphate buffer (75 mM and pH 7.4). Subsequently, in each well of the microplate, we added 20 µL of samples (EEP, MuAd-P, Vehicle/MuAd-P or standard) and 220 µL of the solution containing $ABTS^{\bullet+}$, the reaction took place in the dark for 6 minutes. Absorbance was measured in a microplate reader at 734 nm. a standard Trolox curve was constructed with connections between 12.5 to 200 mM, all samples were analyzed in triplicate and the results were expressed as µmol Trolox equivalents per mg of sample (µmol TE/mg).

2.7 Antioxidant activity by the iron reduction method (Frap - Ferric Reducing Antioxidant Power)

The analysis consists of the ability of the antioxidants present in the sample to be tested, to reduce the Fe^{3+} /TPTZ (tripyridyltrazine) complex to Fe^{2+} /TPTZ in an acidic medium, a process that generates an intense blue color that can be detected by spectrophotometry at 595 nm (Al-Duais et al., 2009). The FRAP reagent was prepared at the time of analysis by mixing 25 mL of acetate buffer (300 mM and pH 3.6), 2.5 mL of TPTZ

solution (10 mM of TPTZ in 40 mM of HCl), and 2.5 mL of $FeCl_3$ (20 mM) in solution watery. In each microplate well, we added 20 µL of samples (EEP, MuAd-P, Vehicle/MuAd-P or standard), 30 µL of distilled water, and 200 µL of FRAP reagent. The plate was incubated at 37°C for 30 minutes, the analysis was carried out at 595 nm in a microplate reader. We constructed a standard analytical curve for ferrous sulfate (SF) with concentrations between 100 to 700 µM, all samples were analyzed in triplicate and expressed in mg of SF/g of propolis extract.

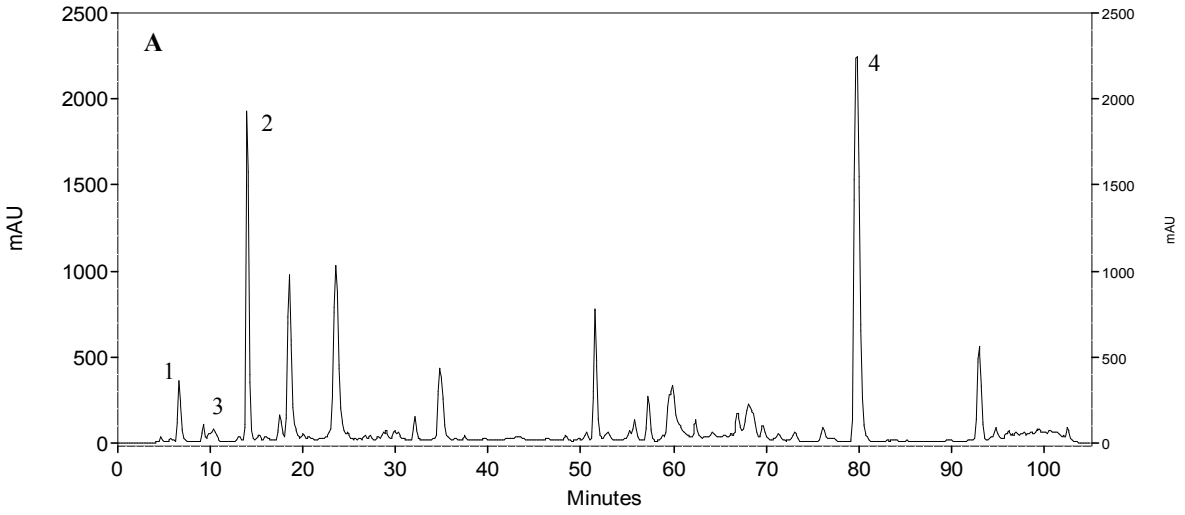
4 Statistical Analysis

The data obtained were submitted to analysis of variance (ANOVA) and Tukey's test for the comparison of means at a 5% significance level. Results were expressed as mean and standard deviation (Mean ± SD).

3 Results

We conducted a high-performance reversed-phase liquid chromatography (RP-HPLC) to identify the compounds present in the green propolis used as the basis for both formulations (EEP and MuAd-P). Among the compounds, we found three phenolic acids (caffeic acid, ρ-coumaric acid, and ferulic acid) and a prenylated derived from cinnamic acid (artepelin C) (Figure 1A). Artepelin C was the major compound identified, with a concentration of 89.24 ± 0.45 mg/g of extract, followed by p-coumaric, caffeic, and ferulic acids, whose concentrations were 15.82 ± 0.07, 1.04 ± 0.01, and 0.35 ± 0.00, respectively (Figure 1B).

Figure 1- (A) HPLC of green propolis and compounds detected at 308 nm; (B) Characterization and quantification of the compounds found



B

Compound #	Compound name	Retention time (min)	UV máx	mg/g extract
1	Caffeic acid	9.50	309	1.04 ± 0.01
2	ρ-Coumaric acid	14.30	325	15.82 ± 0.07
3	Ferulic Acid	15.45	325	0.35 ± 0.00
4	Artepelin C	80.25	312	89.24 ± 0.45

After identifying the molecules present in green propolis, we started the tests to determine the antioxidant activity, first quantifying the content of phenolic compounds and total flavonoids present in the samples. The content of phenolic compounds was determined by the Folin-Ciocalteu spectrophotometric method. In this test, the EEP presented a phenolic composition of 234.8 ± 10.1 mg GAE/g, while the MuAd-GP 268.0 ± 8.8 mg GAE/g, the analysis of variance and the Tukey test showed a statistically significant difference between both formulations ($p<0.05$). In this same test, the Vehicle/MuAd-P did not show activity. Then, we quantified the content of flavonoids present in the sample through the reaction with aluminum chloride, whose concentration of EEP was 105.5 ± 1.2 mg QE/g and MuAd-GP was 159.3 ± 20.2 mg QE/g, differing statistically ($p<0.05$). Vehicle/MuAd-P did not show activity (Figure 2).

Figure 2- Total phenolic content, flavonoid content and free radical scavenging activity given formulations based on southern green propolis from Minas Gerais

Compounds	TP (mg GAE/g)	TF (mg QE/g)	DPPH (µmol TE/g)	$ABTS^{\bullet+}$ (µmol TE/g)	FRAP (µmol FS/g)
EEP	234.8 ± 10.1[a]	105.5 ± 1.2[a]	1144.1 ± 253.9[a]	2708.8 ± 360.3[a]	2413.1 ±175.4[a]
MuAd-P	268.0 ± 8.8[b]	159.3 ± 20.2[b]	1165.1 ± 1.8[a]	2911.6 ± 56.9[a]	2474.6 ±153.1[a]
Vehicle (MuAd-P)	ND	ND	ND	ND	ND

Mean ± SD

TP - Total phenolic compounds (expressed in Gallic acid equivalents)

TF - Total flavonoid content (expressed in quercetin equivalents)

TE - Trolox equivalent

FS - Ferrous sulfate equivalent

EEP - Ethanolic Extract of Green Propolis.

MuAd-P - Mucoadhesive formulation of Green Propolis

ND - activity not detected

Means followed by the same letter in the column do not show statistical differences by Tukey's Test ($p > 0.05$)

After determining the content of phenolic compounds and flavonoid content, we started to evaluate the antioxidant potential of the samples. First, we verified the reducing capacity of the compounds against the stable radical DPPH, in this assay the EEP presented the activity of 1144.1 ± 253.9 µmol TE/g, while the MuAd-P reached 1165.1 ± 1.8 µmol TE/g, the statistical analysis did not present significance at a level of 5% of probability ($p>0.05$), in this trial the Vehicle/MuAd-P did not perform reducing activity. Afterward, the samples had their antioxidant potential measured with the $ABTS^{\bullet+}$ radical, when submitted to this test, the EEP reached a value of 2708.8 ± 360.3 µmol TE/g, while the MuAd-GP performed activity of 2911.6 ± 56.9 µmol TE/g, results not statistically significant ($p>0.05$), in this trial the Vehicle/MuAd-P had no activity. Finally, the reducing power of the samples against ferric ion was evaluated, in this essay, the EEP and MuAd-GP performed activities equivalent to 2413.1 ± 175.4 and 2474.6 ± 153.1 µmol FS/g, respectively, non-significant results ($p>0.05$). Vehicle/MuAd-P was inactive in this trial (Figure 2).

4 Discussion

The green propolis used as a base for both formulations showed a chemical profile rich in phenolic acids, with artepelin C as the main compound, a derivative of cinnamic acid with strong antioxidant and anticancer activity. Artepelin C is considered a chemical marker for green propolis from Minas Gerais, as this compound appears in high concentration in this variety of resin and its botanical origin (Moise and Bobiş, 2020). The chemical profile found in the propolis used in this study is in accordance to Park et al. (2002), which classified green propolis from southeastern Brazil, as group 12, based in physicochemical properties, whose botanical origin is the plant *Baccharis dracunculifolia* DC. Also, the chemical profile of our green propolis sample was qualitatively similar to that got by Szliszka et al. (2013), but was quantitatively different. According to Bueno-Silva et al. (2017), seasonality influences the chemical composition of the same propolis, so the compounds present in extracts can be similar and have the same biological property, but their concentration will always be different.

Cabral et al. (2012), analyzing the total phenolic content (TP) of a sample of green propolis from the south of Minas Gerais, found results of 169.6 mg GAE/g, a lower value than our formulations, whose TP was 268.0 and 234.8 mg GAE/ g, for MuAd-P and EEP, respectively. These divergent results between studies reinforce the influence of seasonality on the chemical composition of propolis. Analyzing the molecules identified in the studies, Artepelin C was the main compound in both cases. However, its concentration was higher in our propolis and possibly potentiated the phenolic content of our formulations.

We also observed a significant variation in the phenolic content between the formulations based on the same green propolis, the MuAd-P presented 268.0 ± 8.8 mg GAE/g, while the EEP presented a TP of 234.8 ± 10.1 mg GAE/g, this statistically significant difference was influenced by the components of the mucoadhesive formulation. We plan MuAd-P in a lipid base with linseed oil to prevent its immediate dissociation in saliva and promote fixation in the oral epithelium. Flaxseed has low phenolic acid content, around 9 mg/g, however, the association with other antioxidant agents such as vitamins of complex C and E, commonly found in this seed, may have been able to act on the reagent Folin-Ciocalteu reduces it and interferes with the measurement of the phenolic content of the mucoadhesive formulation (Everette et al., 2010; Herchi et al., 2014, 2011; Matić et al., 2017). When we analyzed the Vehicle (MuAd-P), it had no activity despite containing linseed oil in its composition, thus showing an inherent error of the method, the detection limit. The National Health Surveillance Agency – ANVISA (Agência Nacional de Vigilância Sanitária,

2003) establishes that the detection limit is the smallest amount of analyte present in a sample that can be detected, but not quantified under the established experimental conditions, therefore, linseed oil interfered in the quantification of the phenolic content of the sample, but did not present a quantifiable polyphenol content by the method in the Vehicle (MuAd-P).

The total flavonoid content was different between the formulations, MuAd-P and EEP showed results of 159.3 ± 20.2 and 105.5 ± 1.2 mg QE/g, respectively. Three hypotheses could explain this difference. First, many flavonoids react with $AlCl_3$ and absorb at different wavelengths, a characteristic that can lead to an underestimation of the real concentration of these compounds (Mammen and Daniel, 2012), possibly the reading at 415 nm, could not map all the flavonoids in the EEP. Second hypotheses, linseed oil in MuAd-P, which, as we showed above, has a small content of polyphenols capable of influencing the concentration of phenolic compounds, but not detectable, possibly the same applies to flavonoids in the mucoadhesive. Reinforcing this hypothesis, we observed the inactivity of the Vehicle (MuAd-P) in this assay. Third hypothesis, is the influence of quercetin on the analytical curve, in the literature variations in the compounds used as standard lead to different results in the flavonoid content in the same sample of propolis (Marcucci, Woisky and Salatino, 2008) Probably our test has a greater sensitivity to flavonols (group of quercetin), molecules found in lower concentration in green propolis, reducing TF in EEP, but in good quantity in flaxseeds, potentiating TF in MuAd-P.

Although MuAd-P and EEP have different phenolic and flavonoid content, even though they are formulations based on the same green propolis, both products meet the requirements proposed by Brazilian legislation for propolis, such as: > 5.0% phenolic content, and > 2.5% of flavonoids (Ministério da Agricultura e do Abastecimento, 2001). These characteristics are responsible for the various biological properties of green propolis from Minas Gerais and highlight it in the national and international market. In addition, the state is the largest producer of propolis in Brazil, with an estimated production of 80 tons per year, corresponding to 90% of the total produced in the country (Berretta, 2021). In this study, we evaluate the presence and permanence of the antioxidant property in MuAd-P and EEP, for that we conducted three tests based on oxidation-reduction reactions mediated by compounds present in the formulations.

MuAd-P and EEP had a strong DPPH radical-scavenging capacity reaching values of 1165.1 ± 1.8 and 1144.1 ± 253.9 µmol TE/g, respectively. Although our formulations have a strong antioxidant activity, this was lower than that obtained by Andrade et al. (2017), where green propolis from northeastern Brazil reached 4554 ± 80.20 µmol TE/g in this same trial.

According to Sforcin and Bankova (2011), the chemical composition of propolis is influenced by local phytogeography. The divergent results found between our study and Andrade et al. (2017) may be due to the behavior of bees and the source plant used to collect plant resins. These characteristics demonstrate the great Brazilian biodiversity and the need for adequate georeferencing when describing the origin of propolis. We also emphasize that the pharmacotechnical processes used in the production of MuAd-P did not change its effectiveness with an antioxidant agent. Skaba et al. (2013), demonstrated that a green propolis-based toothpaste from Minas Gerais performed activity of 1230.07 ± 135.55 μmol TE/g in the DPPH test, a result similar to that obtained in our study and which reaffirms the potential of propolis green with raw material for the production of pharmaceutical products and personal hygiene.

In the $ABTS^{\bullet+}$ radical scavenging assay, MuAd-P and EEP showed activities of 2911.6 ± 56.9 and 2708.8 ± 360 μmol TE/g, respectively ($p>0.05$). Our results are superior to those got by Cavalaro et al. (2019), in this same assay, with the ethanolic extract of green propolis from Minas Gerais, whose result was 2417.4 ± 7.3 μmol TE/g. According to Vieira de Morais et al. (2021), temperature, extraction time and extractor liquid can affect the phenolic content of bee resins and alter the final biological response. Possibly, the extraction conditions adopted potentiated the antioxidant activity of our propolis. Andrade et al. (2018), evaluating the microencapsulated ethanolic extract of green propolis, found values higher than ours, reaching 13044.48 ± 70.34 μmol TE/g in this same assay. A standardized and reproducible method is necessary both in the extraction processes and in the $ABTS^{\bullet+}$ assay, so that the results obtained result from the biological activity of the propolis and not from external influences.

Finally, MuAd-P and EEP had their antioxidant property evaluated in the FRAP assay, this method is based on the reduction of Fe^{3+} into Fe^{2+} by antioxidant compounds in the presence of 2,4,6-tris- (2-pyridyl)-s-triazine (Vieira de Morais et al. 2021). In this assay, MuAd-P and EEP showed a reducing power of 2474.6 ± 153.1 and 2413.1 ± 175.4 μmol FS/g, respectively, while Vehicle (MuAd-P) was inactive. Our results are superior to those got by Ding et al. (2021), where Chinese propolis reached 290.34 ± 10.80 μmol FS/g of extract, the authors credit this value to the extraction conditions adopted. In the study by Cavalaro et al. (2020), the green propolis extract from Minas Gerais, after ultrasonic extraction, the performed activity of 36231.0 μM FS/g, the authors' credit this result with the optimization of the extraction process and the influence on seasonality in the chemical profile of propolis.

5 Conclusion

In conclusion, both formulations (MuAd-P and EEP) showed a rich phenolic and flavonoid composition and strong antioxidant activity. The pharmacotechnical processes used in the production of the mucoadhesive gel did not affect the effectiveness of the biological property. However, the components of the formula potentiated its polyphenolic content. Future trials will explain whether the other properties of this formulation remain unchanged. Future trials will elucidate whether other biological properties of this formulation remain unchanged. Providing reliable parameters for its use in *in vivo* studies, especially those related to the treatment of mucosal tissue lesions whose pathological origin is multivariate.

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4 CONSIDERAÇÕES FINAIS

Conclui-se que a propriedade antioxidante das própolis reduz o estresse oxidativo, reestabelecendo as defesas antioxidantes endógenas, bem como os níveis das enzimas antioxidantes e exercendo um forte efeito neutralizante sobre espécies reativas de oxigênio. Dessa maneira a administração de própolis pode ser eficaz no manejo de doenças cuja patologia esteja relacionada o estresse oxidativo, atuando sobre a expressão gênica ou como um agente redutor. Destacamos a forte atividade antioxidante observada na própolis verde do sul de Minas Gerais tanto na forma de extrato etanólico, quanto na forma de gel mucoadesivo. Portanto, o uso de própolis verde como matéria-prima para o desenvolvimento de fármacos ou isolamento de moléculas deve ser estimulado em função de sua potente atividade antioxidante, que pode ser empregada nas formas *in natura,* em solução (etanólica ou aquosa), ou na adição a medicamentos, cosméticos e alimentos.

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