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Transepithelial transport of isoflavonoids from Brazilian red propolis in Caco-2 cell monolayers

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To my black family who has passed but whose spirit still lives.

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“You only are free when you realize you belong no place - you belong every place - no place at all. The price is high. The reward is great.”

Maya Angelou

ABSTRACT

MORAIS, D. M. **Transepithelial transport of isoflavonoids from Brazilian red propolis in Caco-2 cell monolayers**. 2020. 71 p. Tese (Doutorado) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

The study of natural products in Brazil provides relevant information on plant species and raw materials, which can be used sustainably to benefit society while supporting several development programs for biodiversity conservation. Over the past years, several studies have elucidated the mechanisms underlying the overproduction of free radicals and oxidative stress in systemic diseases. To overcome this issue, naturally-occurring bioactive compounds (*e.g*., polyphenols) have been shown to be effective as ROS/RNS scavengers. Among them, Brazilian Red Propolis (BRP) extract and its isolated isoflavones were shown to have a remarkable antioxidant capacity, even though their potential bioactivity after human consumption remains largely unknown. In the first study, we investigated the best extraction conditions for recovery of phenolic compounds with antioxidant activity from BRP extracts. LC-QTOF-ESI-MS/MS analysis was used in negative mode to identify the extract composition. In the second study, the optimized BRP extract was examined for its ROS/RNS scavenging capacity throughout gastrointestinal digestion and absorption across Caco-2 cell monolayers. Our findings revealed that the optimal extraction conditions were 90 % ethanol at 80 °C for 30 min. Interestingly, thirteen substances were tentatively identified for the first time in BRP, including four flavones (tricin; genkwanin; hispidulin and 8-Hydroxy-5-methoxyflavanone), one flavanol (7-Hydroxy-6-methoxydihydroflavonol), two flavanones (5,6-Dihydroxy-3',4'-dimethoxyflavanone and 6-Hydroxyflavanone), two chalcones (2',4'-Dihydroxychalcone and 2',6'-dihydroxy-4'-methoxydihydrochalcone) and four isoflavonoids (dihydrobiochanin A, dimethyl medicarpin, 5,4′-Dihydroxy-7-methoxyisoflavone and 3,9-Dimethoxypterocarpan). In the gastrointestinal model, a significant decrease in the amount of phenolic compounds in the BRP extract was observed throughout different digestion phases as well as in the antioxidant capacity. Eleven compounds could be absorbed through Caco-2 monolayers, which showed significant peroxyl radical (ROO•) scavenging activity. Due to the complexity of the human gastrointestinal system, further studies will be necessary to understand the contribution of other variables, such as the presence of colonic bacteria, to the absorption of the flavonoids present in the BRP extract. This is a pioneer study using an *in vitro* digestion/Caco-2 cell model to evaluate the antioxidant activity of an optimized BRP extract. Collectively, our findings provide important insights to understand the bioavailability of bioactive compounds from propolis and other natural products.

Keywords: Antioxidant compounds. *in vitro* digestion. Propolis.

RESUMO

MORAIS, D. M. **Transporte transepitelial de isoflavonoides de própolis vermelha brasileira através de monocamadas de células Caco-2**. 2020. 71 p. Tese (Doutorado) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

O estudo dos produtos naturais presentes no Brasil não só fornece informações relevantes sobre espécies e /ou materiais que apresentam potencial para serem sustentavelmente usados pela sociedade, mas também contribuem para os programas de políticas de conservação da biodiversidade. Nos últimos anos, os avanços científicos puderam descrever e elucidar os mecanismos envolvidos no processo de produção de radicais livres e estresse oxidativo em doenças sistemáticas, ocasionando, consequentemente, um aumento nas pesquisas direcionadas ao estudo de substâncias bioativas, principalmente polifenóis, presentes em produtos com capacidade sequestrante de espécies reativas de oxigênio e nitrogênio ROS/RNS. Extratos de própolis vermelha brasileira (EEP) e suas isoflavonas isoladas têm se destacado por suas intrínsecas capacidades antioxidantes. Ainda assim, desconhece-se o potencial da sua bioatividade após o seu consumo. No primeiro estudo, foi investigada a melhor condição de extração para obtenção dos compostos fenólicos com atividade antioxidante em EEP. Além disso, LC-QTOF-ESI-MS/MS em modo negativo foi utilizado para identificar sua composição química. No segundo estudo, os EEP otimizados foram avaliados em relação à sua capacidade de sequestro de ROS / RNS ao longo da digestão gastrointestinal e absorção através de monocamadas de células Caco-2. Os resultados revelaram como a condição ideal para extração: etanol 90% a 80 ° C durante 30 min. Curiosamente, treze substâncias foram identificadas pela primeira vez em EEP, incluindo quatro flavonas (Tricina; genkwanin; hispidulina e 8-hidroxi-5-metoxiflavona), um flavanol (7-hidroxi-6-metoxididroflavonol), duas flavanonas (5,6- Diidroxi-3', 4'-dimetoxiflavanona e 6-hidroxiflavanona), duas chalconas (2', 4'-Diidroxichalcona e 2 ', 6'-dihidroxi-4'-metoxididrocalcona) e quatro isoflavonoides (Diidrobiochanina A, dimetil medicarpina, 5, 4′ - Diidroxi - 7 - metoxiisoflavona e 3,9-Dimetoxipterocarpan). No ensaio gastrointestinal, foi observado que a composição fenólica do EEP diminuiu significativamente ao longo das diferentes fases da digestão. Contudo, a capacidade antioxidante para todas as frações foi mantida. Onze compostos foram absorvidos através da monocamada de células Caco-2. Além disso, a bioatividade foi detectada pelo sequestro do radical peroxil (ROO•). Considerando a complexidade do sistema gastrointestinal humano, novos estudos precisam ser realizados com a finalidade de compreender os efeitos de outras variáveis, como bactérias presentes cólon, por exemplo, durante a absorção dos flavonoides identificados neste trabalho. Uma vez que esse é o primeiro estudo a avaliar a capacidade antioxidante de EEP utilizando um sistema de digestão *in vitro*/Caco-2 células, os resultados aqui apresentados fornecem um *insight* às pesquisas sobre biodisponibilidade de compostos bioativos presentes em própolis e produtos naturais.

Palavras-chave: Compostos antioxidantes. Digestão *in vitro*. Própolis

SUMMARY

1. INTRODUCTION

In recent years, biodiversity protection and preservation has been a relevant topic of concern worldwide. Several public policies have been established to mitigate the impact of natural resource exploitation on ecosystems. Concomitantly, the biological properties of natural products and their potential use as therapeutic agents have aroused significant interest[1], particularly as an alternative to the side effects of synthetic drugs and substances added to food products as well as to minimize health and environmental burdens.

Brazil stands out for its extraordinary biodiversity, which accounts for nearly 15% of all living species and 18% of the world's total flora[2]. This translates into a large number of plant species which can be potentially used as a bee pasture, contributing to a great diversity of bee products, such as honey and propolis, whose medicinal properties are directly related to their originating botanical species. Consequently, the biodiversity resulting from different environmental conditions has dramatically contributed to the production of different propolis types in Brazil[3,4].

Propolis or bee glue is a resinous balsamic substance which is collected by bees from plant exudates and processed thereafter. Propolis is used by bees as a cement to seal cracks and maintain the internal temperature of the hive. As a natural antiseptic, it is deposited inside the alveolus to protect the eggs and prevent contamination of the hive. Furthermore, propolis is commonly used by the bees to “mummify” the bodies of killed invaders[5,6].

The discovery and use of propolis for various purposes dates to ancient Egypt (1700 BC; termed then as "black wax"), when it was used to mummify human bodies. Greeks and Romans regarded propolis as an effective natural antiseptic and healing medicine. Moreover, the records indicate that Persians used propolis to treat rheumatism, eczema and myalgia[7]. Its medicinal benefits have been validated scientifically over the past decades, which substantially increased its value in the pharmaceutical, cosmetic and food industries.

The major biological properties of propolis are attributed to its chemical composition, which may include over 300 constituents, such as chalcones, fatty acids, phenolic compounds (caffeic acid, ferulic acid, esters of caffeic acid etc.), flavonoids (apigenin, chrysin, rutin, quercetin, myricetin, naringin, galangin, daidzein etc.), terpenes, β-steroids and alcohols[8–10]. Nevertheless, identifying and quantifying the

phytochemical composition of propolis samples is a challenging task, which varies substantially according to the bees' harvesting season[11].

Although propolis was discovered millennia ago, its bioactive composition remains attractive until the present day. Therefore, several approaches have been used to validate the health-promoting properties of different propolis types, with a special focus on their reactive oxygen (ROS) and nitrogen species (RNS) scavenging capacity.

Both ROS and RNS are essential for human homeostasis and wellness. Nonetheless, their overproduction has been implicated in the pathogenesis of cardiovascular, neurodegenerative and digestive conditions, and cancer [12]. When critical molecules that modulate gene expression and the inflammatory response (*e.g.*, proteins, lipids, carbohydrates, and nucleic acids) are affected by oxidative stress, they can activate mechanisms that trigger the onset and/or progression of chronic inflammatory conditions[13]. Even though ROS/RNS concentration can be regulated by antioxidants generated under physiological conditions, exogenous antioxidants obtained from natural products can also have a beneficial effect[14].

Brazilian red propolis (BRP) is a remarkable polyphenol-rich natural product. Our research group has published several reports on the biological properties of BRP, such as anti-inflammatory, antimicrobial, antioxidant and anticancer[16,17]. Isoflavonoids are the major bioactive compounds present in BRP samples. However, other isolated compounds, such as vestitol and neovestitol, have shown potential for cancer treatment by downregulating the expression of cancer-related genes, *i.e*., alpha-tubulin, tubulin in microtubule, histone H3, and prostaglandin E synthase. Hence, future research in oncological therapy[15] should focus on understanding the effects of propolis constituents on cancer-related molecular targets.

The immune-modulatory effects of neovestitol isolated from BRP were previously examined in LPS activated macrophages. The results showed that neovestitol inhibits NF-κB activation, thereby inhibiting several downstream pathways. Neovestitol was found to modulate multiple signalling pathways, leading to reduced levels of oxygen reactive products and pro-inflammatory cytokines[16]. Moreover, other bioassay-guided studies revealed that xanthochymol, formononetin, biochanin A and liquiritigenin, showed antimicrobial, antitumoral and antioxidant properties[11,17,18].

The use of natural products is strongly associated with dietary habits worldwide. Most of them are consumed fresh, such as fruits, grains and vegetables, or processed, such as teas, juices, and flours. According to their nutritional and therapeutic properties, some natural products may be considered both functional food and medicine, with important benefits to human[19].

As previously discussed, screening natural products for their chemical composition and biological properties (*e.g.*, antioxidant, anti-inflammatory and antimicrobial) is utterly important. However, their impact on human health should actually be established based on the amount of substances that are effectively absorbed and assimilated by the organism[20].

Although certain endogenous antioxidants are able to regulate biochemical pathways that lead to the proper functioning of the organs, some results show that antioxidants exert no favorable effects on disease control. Therefore, the interactions between ROS and different antioxidants should be investigated to restore the redox balance under pathological conditions[12].

The analysis of digestion of health-associated compounds is a relevant approach to understand the relationship between nutrients and their sources[21]. *In vitro* digestion methods have the advantage of reproducing the host's physicochemical properties (*e.g.*, pH, enzymes, temperature and texture of the matrix), as well as of identifying the effects of food preparation and processing on micronutrient absorbability[22,23]. For instance, the *in vitro* digestion/Caco-2 cell culture model has been successfully applied in the study of intestinal absorption of bioactive compounds such as flavonoids. Caco-2 cells are derived from the human colon and share morphological and functional properties of mature enterocytes when fully differentiated into a monolayer. This model is relevant to study the mechanisms underlying the absorption, transport, and metabolism of any substance[24–25]. Furthermore, this has been a good alternative to animal studies which is accepted by both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) to investigate the intestinal permeability of drugs and foods.

A diverse group of natural products are commonly used in folk medicine, but scientific evidence about the biological properties of chemical constituents following human consumption is still lacking. Hence, the study of bioprospection and bioaccessibility/bioavailability of naturally-occurring products may provide evidence of their health benefits and help understand their impact on the local's bioeconomy while

serving as a basis for preservation policies. This Ph.D. thesis focuses on the importance of the BRP extract regarding all these aspects. The main botanical source of the red propolis used herein is located in the mangroves and along coastal areas in northeastern Brazil. Propolis production is an activity that impacts the local economy and also contributes to biodiversity and ecosystem preservation. Collectively, our results are expected to add scientific and commercial value to Brazilian natural products.

1.1 Aims

1.2 General aims

The general aims of this study were (i) to establish the optimal conditions for extraction of phenolic compounds from BRP; (ii) to recover the highest percentage of bioactive compounds from BRP extract after gastrointestinal digestion; and (iii) to evaluate the transepithelial transport of isoflavonoids from BRP extract during digestion *in vitro*.

1.3.1 Specific aims

1.To Establish the optimal extraction conditions to recovery active phenolic compounds from BRP;

2. To determine the reaction oxygen (ROS) and species nitrogen (RNS) scavenging capacity of the optimized BRP;

3. To assess the gastrointestinal digestion of BRP ethanolic extracts;

4. To assess the transport of digested BRP extract across Caco-2 cells monolayers;

5. To identify digested/transported polyphenols from BRP extract across Caco-2 cell monolayers (LC-ESI-QTOF-MS).

1.4 Hypothesis

Our study hypothesis was that the phenolic composition of BRP extract enhances after the bioaccessibility by using *in vitro* digestion/Caco-2 cells model.

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2. ACTIVE PHENOLICS FROM BRAZILIAN RED PROPOLIS: AN OPTIMIZATION STUDY FOR RECOVERY AND IDENTIFICATION BY LC-ESI-QTOF-MS/MS

Abstract

Brazilian red propolis (BRP) is a natural product widely known for its phenolic composition and strong antioxidant properties. In this study, we used the Box–Behnken Design (BBD) with Surface Response Methodology to optimize the extraction conditions for total phenolic content (TPC) and antioxidant activity (TEAC) of bioactive phenolics from BRP. The variables extraction time, ethanol concentration and temperature, were tested. All variables had significant effects ($P \leq 0.05$), with a desirability coefficient of 0.88. Under optimized conditions (90 % ethanol at 80 °C for 30 min), the BRP extract showed a TPC of 129.00 ± 2.16 mg GAE/g and a TEAC of 3471.76 ± 53.86 µmol TE/ g. Moreover, FRAP and ORAC assays revealed that the optimized BRP extract had 861.62 ± 42.34 µmol Fe^{2+}/ g and 2538.67 ± 67.07 µmol TE/ g, respectively. Thirty-two phenolic compounds were tentatively identified by LC-QTOF-ESI-MS/MS, of which thirteen were found for the first time in BRP, including four flavones, one flavanol, two flavanones, two chalcones and four isoflavonoids. Thus, our results highlight the importance of BRP as a source of a wide variety of phenolic compounds with significant antioxidant properties.

Keywords: Isoflavonoids. Antioxidant compounds. Propolis. *Apis mellifera.*

2.1 Introduction

Propolis or bee glue is a resinous and balsamic substance collected by bees from plants exudates. It is naturally used in to protect the hive and as an efficient antiseptic[1,2]. Historically, propolis has been long used as a therapeutic substance in folk medicine, but recent advances in science and technology have been increasing its commercial value in the food and pharmaceutical industries[3].

Among the different types of propolis occurring worldwide, Brazilian red propolis (BRP) stands out for its health benefits, which are attributed to a phenolic-rich composition, mainly isoflavonoids. The mechanisms of action of some BRP compounds, such as formononetin, vestitol and neovestivol, were recently examined.

These constituents were found to have strong antioxidant, anti-inflammatory and antimicrobial properties[4,5].

To date, more than 200 compounds have been identified in BRP[4,6–9], most of them belonging to polyphenols group. Propolis composition is causally related to both its botanical source and environmental conditions. The main botanical sources of BRP are *Dalbergia ecastaphyllum*, a rich source of isoflavonoids, and *Symphonia globulifera*, a rich source of polyprenylated benzophenones (guttiferone E and oblongifolin B) and triterpenoids (*β*-amyrin and glutinol).

Although several studies have correlated the presence of phenolics with the biological activity of BRP, mainly antimicrobial, anti-inflammatory, and anti-cancer[10–12], an optimization of extraction conditions has not been carried out thus far. Chemical extraction is the initial procedure for recovery of polyphenols from a natural product. Thus, choosing appropriate extraction conditions (*e.g.*, sample-to-solvent ratio, solvent concentration, temperature, and extraction time) is utterly important, as these may affect the final extract composition and bioactivity[13].

As stated by Riswanto et al.[14], the Response Surface Methodology (RSM) is a technique widely applied in the optimization of natural products due to its advantages compared to the traditional one-variable-at-a-time design. When combined with an experimental design like the Box–Behnken Design (BBD), it can be employed as a mathematical and statistical tool in natural product research. Due to its capacity of reducing the number of experiments required to find optimal conditions, BBD has been effectively used to optimize polyphenol extraction[13,15].

In this study, BBD was used to establish the optimal extraction conditions for recovery of antioxidant compounds from BRP. The antioxidant activity and phenolic composition of the optimized BRP extracts were further investigated by biological assays and LC-ESI-QTOF-MS/MS, respectively.

2.2 Materials and methods

2.2.1 Chemicals

The following chemicals were used in this study: Folin–Ciocalteau reagent (Dinamica Quimica Contemporanea, Diadema, SP, Brazil); sodium carbonate, potassium chloride, ethanol; monobasic and dibasic potassium phosphate, the standards ({±})-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox),

gallic acid, diammonium salt (ABTS) and potassium peroxydisulfate, fluorescein sodium salt and 2,20-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of analytical grade.

2.2.2 Propolis collection and extraction

BRP samples were collected from the internal parts of *Apis mellifera L*. (Apidae) beehives located in the city of Maceió (9°40′S, 35°41′W), Alagoas State, Northeastern Brazil. Access to the Brazilian genetic heritage was previously obtained in accordance with the Brazilian legislation SECEX/CGEN Ordinance No. 1. Approval for sample collection was obtained via the SISGEN platform under accession number A5A0509.

Propolis samples were crushed with liquid nitrogen, weighted (0.5 g) and mixed with 50 mL of solvent and remained in sealed tubes in a bath shaker (Gyromax 929, Amerex) for the time and temperature established in the experimental design (Table 1). After that, BRP ethanolic extracts were kept overnight at - 20 °C until complete wax decantation. The supernatant solution was filtered, concentrated on a rotary evaporator at 110 mbar and 50 °C, and analyzed for its antioxidant activity and total phenolic content. All extraction procedures were carried out in triplicate.

2.2.3 Experimental design and optimization

The following independent variables were considered: time (X_1) (30-60 min), temperature (X_2) (30-80 °C), and percentage of ethanol/water (X_3) (30-90 %, v/v). Following the Box-Behnken design, 15 experiments with 3 central points were performed to determine the effects of these independent parameters on two dependent responses (TEAC and TPC) (Table 1). RSM was performed to investigate the relationship between the independent and dependent variables. The quadratic polynomial model is represented by the following equation:

$$\boldsymbol{Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_{ij} + \sum_{i<1}^{3} b_{ij} X_i X_j} \qquad (1)$$

where Y is the dependent variable (TEAC or TPC) for the independent responses (X_1-X_3); The constant coefficients for intercept, linear, quadratic, are β_0, β_i, β_{ii}, β_{ij}.

Table 1 - Box-Behnken Design for the extraction of antioxidants compounds from Brazilian red propolis

Run	Independent variables						Dependent variables	
	Coded values			Real values			TEAC (µmol TE/ g)	TPC (mg GAE/ g)
	Time (min)	Temp. °C	EtOH (%)	Time (min)	Temp. °C	EtOH (%)		
1	-1	-1	0	30	30	75	2560.36	125.76
2	1	-1	0	90	30	75	2386.42	132.80
3	-1	1	0	30	80	75	2804.74	136.17
4	1	1	0	90	80	75	2918.30	118.82
5	-1	0	-1	30	55	60	2370.61	120.30
6	1	0	-1	90	55	60	2719.93	116.09
7	-1	0	1	30	55	90	3106.62	127.64
8	1	0	1	90	55	90	3156.93	123.38
9	0	-1	-1	60	30	60	3169.87	118.82
10	0	1	-1	60	80	60	2827.74	116.53
11	0	-1	1	60	30	90	3200.06	109.49
12	0	1	1	60	80	90	3471.75	126.25
13	0	0	0	60	55	75	2544.55	133.00
14	0	0	0	60	55	75	2635.12	137.06
15	0	0	0	60	55	75	2622.18	134.98

TEAC= Trolox equivalent antioxidant activity; TPC= Total phenolic compounds.

2.2.4 Total phenolic compounds

The analysis of total phenolic content (TPC) was performed according to the Folin–Ciocalteau spectrophotometric method, with some modifications. Aliquots of 20 µL of the standard solution (gallic acid) or BRP extract and 100 µL of the Folin–Ciocalteau solution (10% in water) were pipetted into the wells of a microplate. After 5 min, 75 µL of a 7.5% sodium carbonate aqueous solution were added to each well. A control was prepared by replacing the sample with distilled water. The absorbance was measured at 740 nm in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) after 40 min. The TPC was calculated by linear regression using gallic acid as a standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract[16]. All samples were analysed in triplicate.

2.2.5 Antioxidant activity

2.2.5.1 Ferric reducing antioxidant power (FRAP) assay

Briefly, 20 µL of BRP extract were mixed with 30 mL of water and 200 µL of FRAP reagent (prepared fresh daily) in a 96-well microplate. The FRAP reagent consisted of 10 volumes of 300 mmol/L acetate buffer (pH 3.6), one volume of 20 mmol/L $FeCl_3$ and one volume of 10 mmol/L TPTZ in 40 mmol/L HCl[16]. The absorbance was measured at 595 nm in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) after 8 min. Water was used as a blank; ferrous sulphate solutions (100 to 700 µM) were used for calibration; and the FRAP value was calculated by linear regression. The assay was performed in triplicate, and the results were estimated as µmol Fe^{2+}/ g of lyophilized sample.

2.2.5.2 Peroxyl radical (ROO•)

Briefly, 30 µL of BRP extract plus 60 µL of fluorescein and 110 µL of an AAPH solution were transferred to a microplate. The reaction was performed at 37 °C and the absorbance was measured every minute for 2 h at 485 nm (excitation) and 528 nm (emission) in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Trolox standard was used at concentrations ranging from 12.5 to 400 µM. The results were expressed as µmol/Trolox equivalents (TE) per g of lyophilized sample[17]. The assay was carried out in triplicate.

2.2.5.3 Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant capacity of the BRP extract was determined based on free radical ABTS, with modifications[16]. The ABTS radical was diluted in 75 mM potassium phosphate buffer (pH 7.4) to an absorbance of 0.700 ± 0.01 at 734 nm. Aliquots of 20 µL of Trolox or BRP extract and 220 µL of ABTS radical solution were transferred to wells and kept at room temperature protected from light. After 6 min of reaction, the absorbance was read at 734 nm using the potassium phosphate buffer as a blank. Trolox was used as a standard at concentrations ranging from 12.5 to 200 µM, and the results were expressed as µmol Trolox equivalents (TE) per g of dry extract.

2.2.6 High-resolution mass spectrometry analysis (LC-ESI-QTOF-MS/MS)

Liquid chromatography analysis was carried out using a chromatograph (Shimadzu Co., Tokyo) with a LC-30AD quaternary pump and SPD-20A photodiode

array detector (PDA). Reversed phase chromatography was performed using Phenomenex Luna C18 column (4.6 x 250 mm x 5 µm). A high-resolution mass spectrometer (MAXIS 3G –Bruker Daltonics - Bruker Daltonics, Bremen, Germany) was equipped with a Z-electrospray (ESI) interface operating in negative ion mode with a nominal resolution of 60.000 m/z. Twenty microliters of BRP extract were injected into the liquid chromatography system. The analytical conditions were set as follows: nebulizer at 2 Bar; dry gas at 8 L/min; temperature at 200 °C and HV at 4,500 V. The mobile phase consisted of two solvents: (A) water/acetic acid (99.5/0.5, v/v) and (B) methanol. The flow rate was 1 mL/min, and the gradient was initiated with 30% B, increasing to 40% B (15 min), 50% B (30 min), 60% B (45 min), 75% B (65 min), 75% B (85 min), 90% B (95 min), decreasing to 30% B (105 min). The run was complete after 114 min. An external calibration was carried out in MAXIS 3G –Bruker Daltonics 4.3 software to check for mass precision and data analysis. The tentative identification of the compounds was performed by comparing their exact mass (*m/z*) and MS^2 spectra in negative mode to the database available in the literature and commercial standards.

2.2.7 Data analysis

The data were submitted to multivariate factorial design (ANOVA) analysis and checked for significance of the study variables in the STATISTICA 7.0 software. The accuracy of the mathematical models was estimated by the coefficient of determination (R^2) and F test ($P < 0.05$). All assays were carried out in triplicate, and the results were expressed as mean ± standard deviation.

2.3 Results and discussion

2.3.1 Fitting the models of data

In this study, the accuracy of the RSM models was determine by analysis of variance, as shown in Table 2. The *P*-values ($P < 0.05$) observed for both responses variables were considered significant, indicating that the developed models were appropriate to represent the relationship between the independent parameters and the response variables. The F-values observed (603.14 and 34.75 for TEAC and TPC, respectively) were significant and the model fitted well, as the *P*-value was lower than 0.05. The *P*-value is used to estimate whether F is large enough to indicate any

statistical significance, and values lower than 0.05 indicate that the developed model is statistically significant[18]. Hence, the statistical analysis revealed that the independent variables significantly affected TEAC and TPC outcomes. The *P*-values for Lack of Fit in both models were greater than 0.05 (0.3673 and 0.9251 for TEAC and TPC respectively). This function is performed by comparing the variability of the residuals in the current model with the variability in the observations under repeated conditions of the factors[19]. The coefficient of determination (R^2) estimates the proportion of variation in the response that can be attributed to the model rather than to random error[20]. In our study, R^2 values of 0.9991 and 0.9843 were obtained for TEAC and TPC, respectively. The Adj-R^2 values were 0.9974 and 0.9559 for TEAC and TPC, respectively. High Adj-R^2 values indicate a strong correlation between the actual and predicted values[18]. The statistical results suggest that the developed models were fit and accurate.

The coefficients of the response variables are presented in Table 2. Our findings indicate that TEAC outcomes were significantly affected by linear (X_1, X_2 and X_3) and quadratic terms (X_2^2 and X_3^2) as well as by interactive effects (X_2 X_3). TPC was significantly affected by linear (X_1) and quadratic terms (X_1^2, X_2^2 and X_3^2) as well as by interactive effects (X_2 X_3). The other coefficients were not significant (*P* > 0.05). Equations considering only the significant terms were fitted to RSM models to predict the responses, as follows:

$$Y_{TEAC} = 13117.53 + 3.05X_1 - 60.23X_2 - 259.73X_3 + 0.31X_2^2 + 1.68X_3^2 + 0.41X_2X_3 \quad (2)$$

$$Y_{TPC} = 135.01 - 4.11X_1 - 60.23X_2 - 3.26X_1^2 - 8.24X_2^2 - 4.70X_3^2 - 0.52X_2X_3 \quad (3)$$

Where, Y represents the predicting responses and X_1, X_2 and X_3 represent time, temperature and ethanol %, respectively.

Table 2 - The effects of different parameters on TEAC and TPC outcomes during optimization of Brazilian red propolis ethanolic extracts

Term	SS	df	MS	F-value	*p*-value	
TEAC						
Model	1247423.00	9	138602.55	603.14	< 0.0001	significant
X_1	8019.54	1	8019.54	34.89	0.0183	
X_2	74849.67	1	74849.67	325.63	0.0020	
X_3	425485	1	425484.7	1851.06	0.0003	
X_1^2	83.58	1	83.58	0.3636	0.5347	
X_2^2	144174	1	144173.8	627.22	0.0010	
X_3^2	530460	1	530460.1	2307.80	0.0002	
X_1X_2	1153.35	1	1153.35	5.02	0.1092	
X_1X_3	1.87	1	1.87	0.0081	0.9254	
X_2X_3	94179.02	1	94179.02	409.73	0.0016	
Residual	1149.29	5	229.86			
Lack of Fit	846.99	3	282.33	1.87	0.3673	not significant
Pure error	302.30	2	151.15			
Total	1248572	14				
R^2	0.9991					
Adj-R^2	0.9974					
TPC						
Model	627.04	9	69.67	34.75	0.0006	significant
X_1	134.88	1	134.88	67.27	0.0004	
X_2	10.38	1	10.38	5.18	0.0719	
X_3	9.45	1	9.45	4.71	0.0820	
X_1^2	39.29	1	39.29	19.60	0.0068	
X_2^2	250.65	1	250.65	125.01	< 0.0001	
X_3^2	81.65	1	81.65	40.72	0.0014	
X_1X_2	5.97	1	5.97	2.98	0.1449	
X_1X_3	1.10	1	1.10	0.5473	0.4927	
X_2X_3	132.72	1	132.72	66.20	0.0005	
Residual	10.02	5	2.00			
Lack of Fit	1.78	3	0.5938	0.1441	0.9251	not significant
Pure error	8.24	2	4.12			
Total	637.07	14				
R^2	0.9843					
Adj-R^2	0.9559					

X_1: Time (min), X_2: Temperature (°C), X_3: EtOH (%), SS: Sum of squares, DF: Degree of freedom, MS: Mean square, R^2: Quadratic correlation coefficient; Adj-R^2: Adjusted quadratic correlation coefficient. TEAC: Trolox equivalent antioxidant capacity; TPC: Total phenolic content.

2.3.2 Response surface analysis

2.3.2.1 Effect of solvent concentration on TEAC and TPC

As shown in Figures 1 and 2, solvent concentration significantly altered the content of bioactive compounds recovered from BRP extract and its antioxidant activity. As the ethanol concentration increased, the efficiency of both phenolic extraction and antioxidant activity was greatly improved. TEAC values raised from 2700 to 3200 µmol TE/ g as the ethanol concentration was increased from 75 % to 90 %. Similarly, TPC values raised from 123 to 135 mg GAE/g as the ethanol concentration was increased from 60 % to 90%.

Our data suggest that the interaction between temperature and ethanol concentration was highly significant. Hence, the phenolic content and TEAC values can be optimized if these parameters are increased. Yet, no improvement in phenolic extraction and antioxidant activity was observed at the highest ethanol concentration (90%) and temperatures below 30 °C. A similar outcome was reported by Roselló-Soto et al.[21] during the optimization of Tiger Nuts byproducts. The authors showed that an increase in ethanol concentration significantly increased the extraction yield at temperatures above 40 °C. Oldoni et al.[22] also reported that the optimized conditions for extraction of phenolic compounds with antioxidant activity from propolis were 80 ºC and 70% ethanol. As proposed by Yang et al.[23], after modify and penetrate into the cells walls, ethanol affects cellular components and improves the chemical extraction, particularly of polyphenols.

Albeit less discussed in optimization studies, the extraction equipment plays a determining role in the efficiency of the optimization process. When experiments are carried out using non-sealed tubes, volatile solvents or nonpolar compounds may evaporate as rocking and sonification produce heat and increase the solvent temperature. Therefore, the use of an appropriate flask to perform the analysis is as important as the experimental design itself.

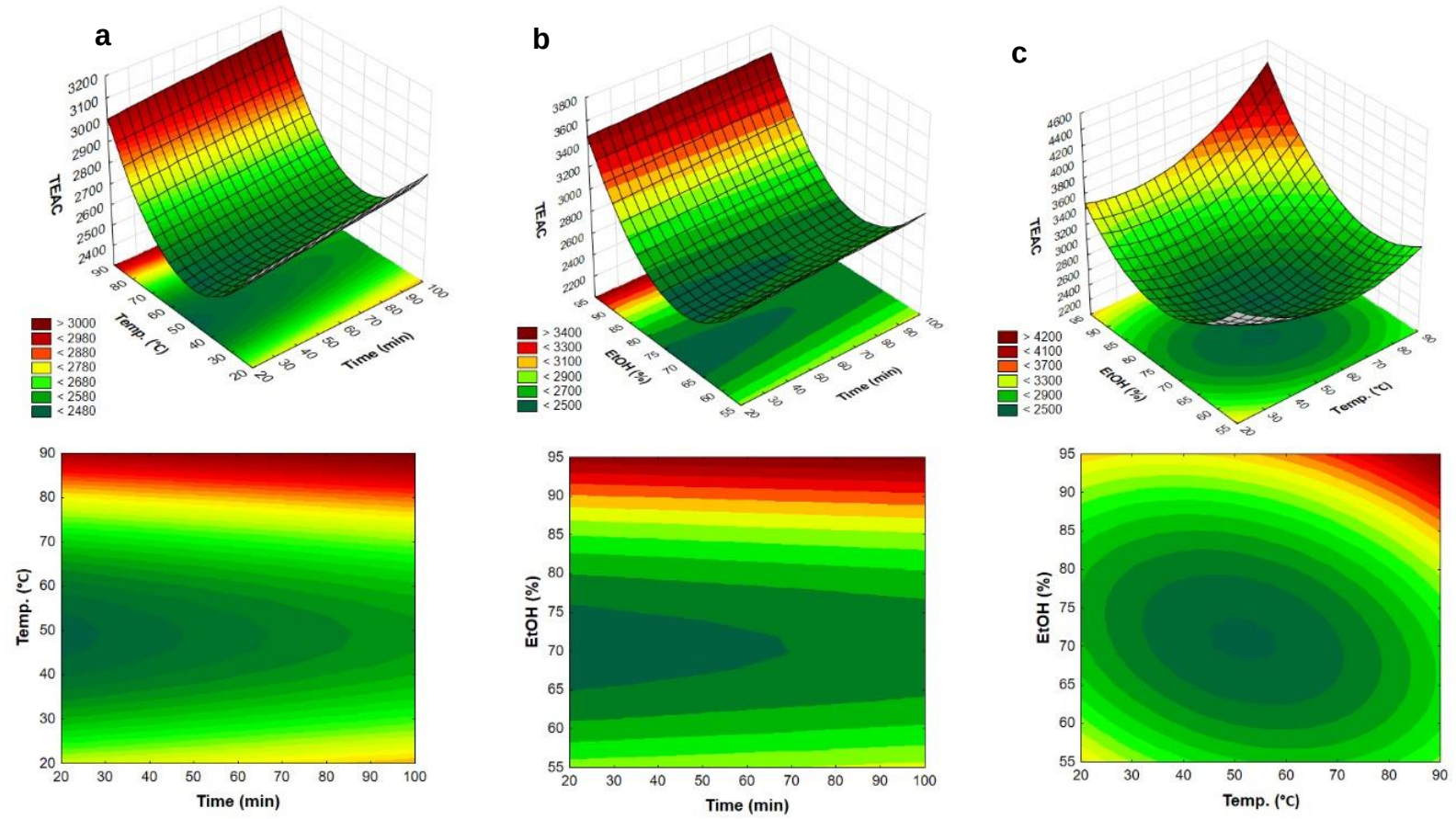

Figure 1 – Response surface plot showing the combined effect of temperature (°C) (a), time (min) (b), and EtOH (%) (c) on the TEAC of Brazilian Red Propolis extracts

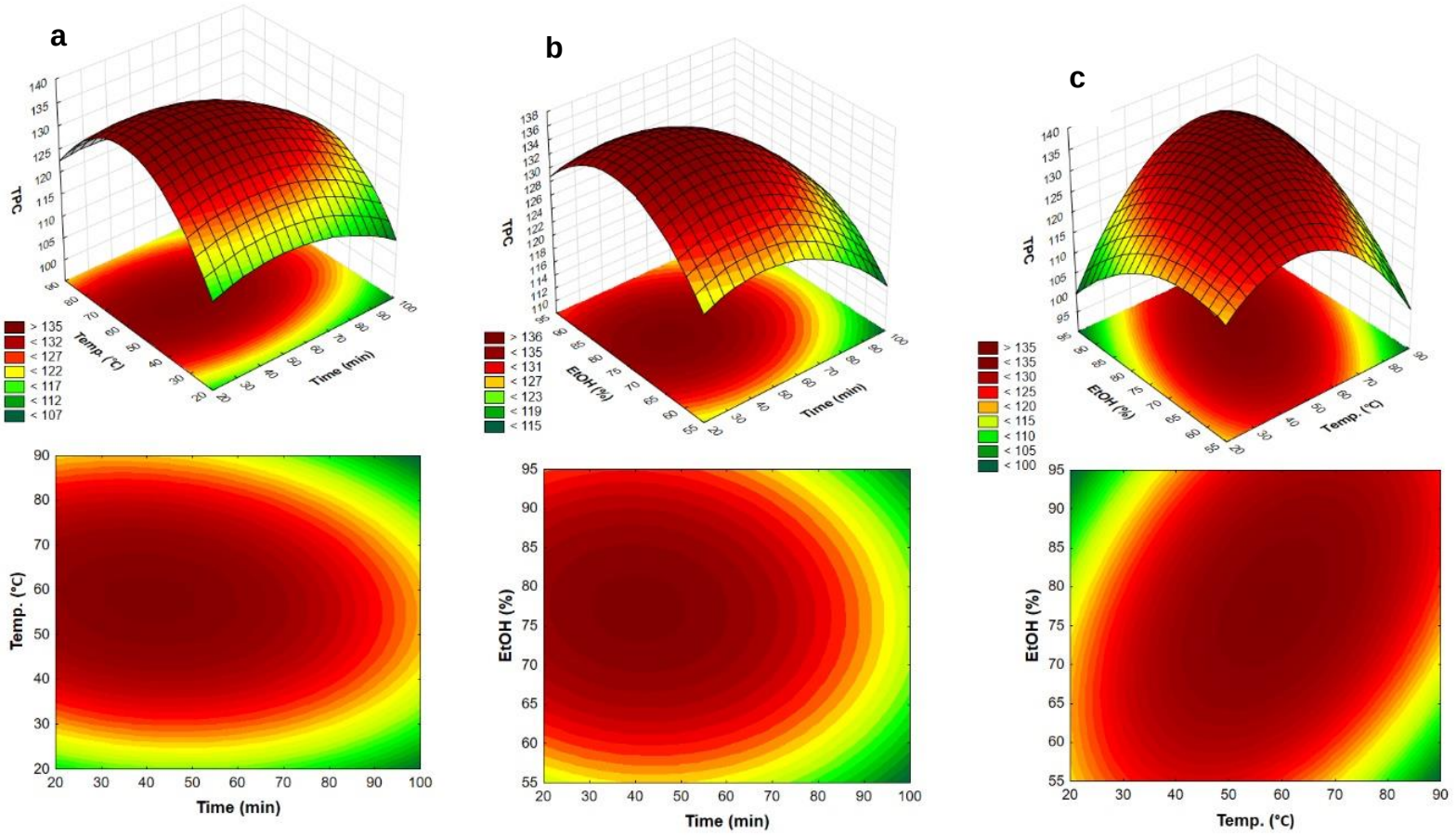

Figure 2 – Response surface plot showing the combined effect of temperature (°C) (a), time (min) (b), and EtOH (%) (c) on the TPC of Brazilian Red Propolis extracts

2.3.2.2 Effect of temperature on TEAC and TPC

Temperature is one of the variables most frequently examined in natural product optimization studies due to its effects on the content and availability of bioactive compounds, mainly polyphenols. In our study, we found that as the temperature increased so did the TEAC of the extract. The best results were obtained at the highest tested temperature (80 °C) – 3471.5 µmol TE/ g (Figure 1). This value is higher than those found by Andrade et al.[24] when testing the extraction of red, green and brown propolis at 35°C (2913.55 ± 95.26; 2214.96 ± 20.61 and 1868.45 ± 131.39 µmol TE/ g, respectively).

Moreover, the results showed that when the temperature was escalated from 30 °C to 50 °C, the TPC increased from 122 to 128 mg GAE/ g, but it decreased slightly when the temperature was further extended (Figure 2). Maran at al.[18] pointed out that higher temperatures enhance the efficiency of phenolic extraction by decreasing the viscosity and density of the extract. Thereby, higher temperatures enable the solvent to penetrate deeper into the sample matrix and have more contact with the surface area. In contrast, if the temperature is excessively elevated, it may cause bioactive compounds to decompose or vaporize.

2.3.2.3 Effect of time on TEAC and TPC

Figures 1 and 2 show the results of the variable extraction time on TEAC and TPC outcomes. Although the extraction length was not strongly associated with TEAC values, a shorter extraction time (30 min) yielded better results. As for TPC, the optimal extraction time was also 30 min. When the time was extended from 60 to 90 min, the amount of phenolic compounds recovered was drastically reduced. As stated in the literature, an extended extraction time increases the probability of oxidation, epimerization, and degradation of bioactive compounds[25]. Thus, a prolonged extraction procedure may not be appropriate for all types of natural products[21]. On the other hand, Oldoni et al.[22] reported an increase in the TPC of a propolis type produced in Southern Brazil when using 80 % ethanol at 70 °C for 45 min. Yusof et al.[26] observed that the optimal conditions for extraction of phenolics from a Malaysian propolis were 80 % ethanol at 60 °C for 25 min. Importantly, we note that the optimal extraction time reported by these authors was shorter than that found in our study, and that it was not possible to predict the extraction efficiency after 60 min.

2.3.2.4 Optimization and validation of RSM models

The optimization of the independent parameters – time (min), ethanol (%) and temperature (°C) – was carried out based on the desirability coefficient (0.8780) to obtain the highest TEAC values and TPC (Figure 3). The TEAC and TPC values predicted by the model under optimal conditions (90 % ethanol, 80°C, 30 min) were 3550.8 ± 70 µmol TE/ g and 132 ± 6.48 mg GAE/g, respectively. The RSM model was validated by comparing the experimental data (n = 3) with the predicted values. The actual TEAC and TPC values obtained under optimal conditions were 3471.76 ± 53.86 µmol TE/ g and 129.00 ± 2.16 mg GAE/g, respectively. These findings are similar to the predicted data, indicating that the method is suitable to determine the optimal conditions for extraction of phenolic compounds with antioxidant activity from BRP samples.

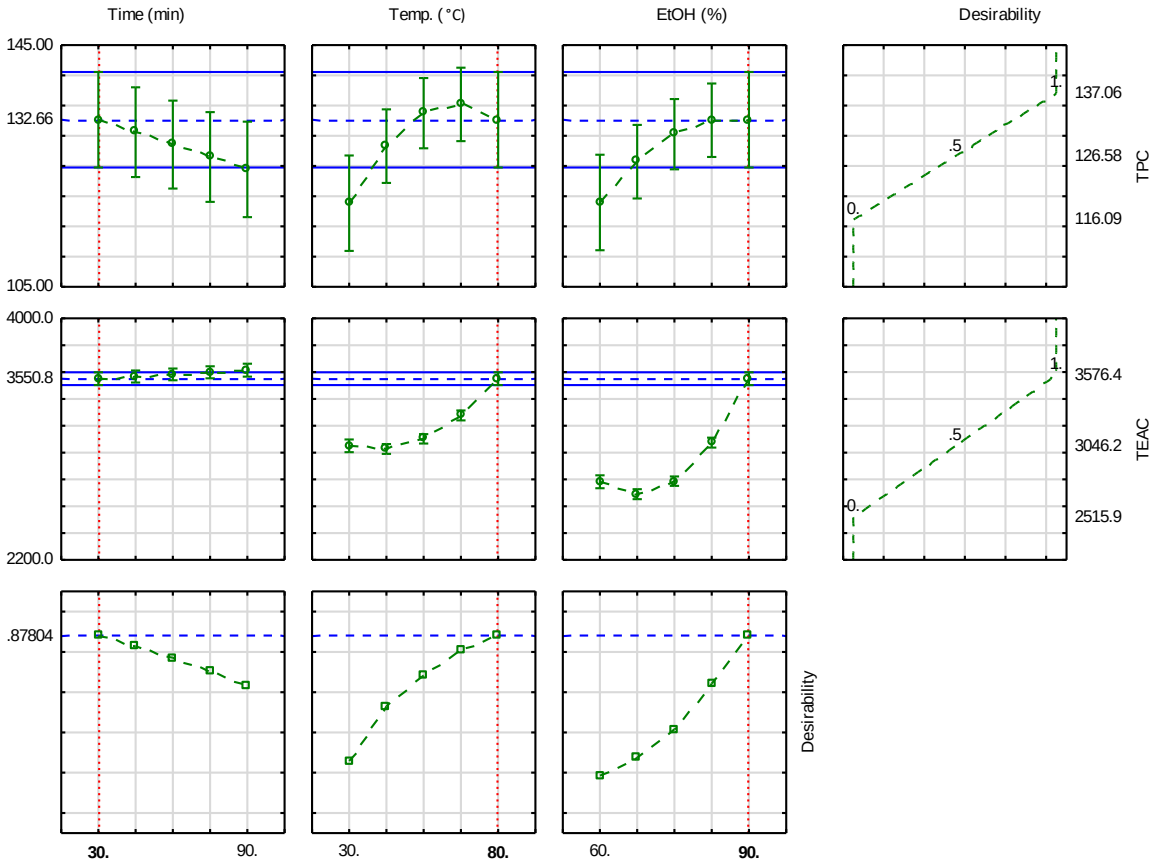

Figure 3 - Coefficient of desirability for optimization of TEAC and TPC in Brazilian red propolis extracts as a function of time (min), temperature (°C) and EtOH concentration (%)

2.3.3 Antioxidant activity

The antioxidant activity of the optimized BRP extract was evaluated *in vitro* by single electron transfer and hydrogen atom transfer assays. The FRAP 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 (TPTZ), forming a colored complex with Fe^{2+} at 593 nm[27]. The FRAP value obtained for the optimized BRP extract was 861.62 ± 42.34 µmol Fe^{2+}/ g of lyophilized sample. These values were higher than those reported by Calegari et al.[28] who determined the antioxidant activity of 30 propolis samples collected in the states of Paraná and Santa Catarina, Brazil. The authors used Fourier transform near-infrared (FTNIR) spectroscopy and obtained FRAP values ranging from 61.9 to 1770 µmol Fe^{2+}/ g of dry weight. Andrade et al.[24] reported a FRAP value of 633.18 ± 40.20 µmol TE/g of dry weight for the BRP extract, while Oldoni et al.[22] found 259.30 ± 9.50 µmol Fe^{2+}/ g of dry weight for optimized propolis samples from the state of Paraná, Brazil. When evaluating Croatian propolis from five locations in Adriatic Sea islands, Svečnjak et al.[29] observed reducing FRAP activity from 0.1 to 0.8 mmol Fe^{2+}/ dry weight.

The oxygen radical absorbance capacity (ORAC) assay measures antioxidant inhibition of the peroxyl radical via hydrogen atom transfer reactions. This method is suitable to detect both hydrophilic and hydrophobic antioxidants[30]. For that reason, it is commonly used to determine the antioxidant capacity in different types of natural products, mainly propolis. In our study, the ORAC value of the BRP extract was 2538.67 ± 67.07 µmol TE/ g. El-Guendouz et al.[31] examined 24 different samples of Moroccan propolis and found ORAC values ranging from 630.39 ± 33.79 to 1723.28 ± 33.79 µmol TE/ g of dry weight. Using 95 % ethanol for extraction, Sun et al.[32] reported that Beijing propolis extract had an ORAC value of 1433.72 ± 120 µmol TE/ g of dry weight.

2.3.4 LC-ESI-QTOF-MS/MS Characterization of Phenolic Compounds

The qualitative analysis of EEP composition was achieved by LC-ESI-QTOF-MS/MS. The compounds were identified by comparing their *m/z* values and MS^2 spectra in negative mode to the literature findings and corresponding standards.

As shown in Table 3, LC-MS/MS analysis revealed the presence of 32 phenolic compounds in the optimized BRP extract, including flavones, flavanones, flavanonols, chalcones, isoflavonoids, quinone, coumarin and their derivates.

Table 3 – Identification of phenolic compounds of Brazilian red propolis extract by LC-ESI-QTOF-MS/MS

No.	Compound	RT (min)	Molecular formula	$[M-H]^-$	MS fragments (*m/z*)
	Flavonoids				
	Flavones				
1	Chrysin	28.6	$C1_5H_{10}O_4$	253.0510	**253.0507**; 119.0483; 195.0438; 224.0481; 209.0614
2	Tricin	34.2	$C_{17}H_{14}O_7$	329.0677	**329.0667**; 299.0218; 271.0263; 243.0289
3	Genkwanin	36.2	$C_{16}H_{12}O_5$	283.0624	**268.0360**; 283.0583; 269.0397
4	Hispidulin	37.5	$C_{16}H_{12}O_6$	299.0565	**284.0331**; 227.0354;255.0301;212.0483
5	8-Hydroxy-5-methoxyflavanone	44.2	$C_{16}H_{14}O_4$	269.0831	**254.0589**; 252.0437; 195.0451; 210.0685
6	Acacetin	54.2	$C_{16}H_{12}O_5$	283.0617	**268.0382**; 211.0408; 269.042
	Flavanones				
7	Liquiritigenin	25.1	$C_{15}H_{12}O_4$	255.0667	**119.0495**; 135.0083; 255.0656; 120.0526
8	Naringenin*	32.6	$C_{15}H_{12}O_5$	271.0619	**119.0487**; 151.0029; 254.0596; 271.0609; 165.0207
9	Pinocembrin*	48.6	$C_{15}H_{12}O_4$	255.0668	**255.0678**; 240.0426; 151.0034; 133.0285; 213.0540;
10	5,6-Dihydroxy-3',4'-dimethoxyflavanone	48.7	$C_{17}H_{16}O_6$	315.0882	**315.0881**;151.0037; 235.0636; 255.1042; 121.0292;
11	6-Hydroxyflavanone	57.3	$C_{15}H_{12}O_3$	239.0722	**239.0732**; 135.0091; 197.0643
	Chalcones				
12	Isoliquiritigenin*	41.1	$C_{15}H_{12}O_4$	255.0676	**119.0496**; 135.0082; 120.0531; 151.0384; 255.0665
13	2',4'-Dihydroxychalcone	41.9	$C_{15}H_{12}O_3$	239.0723	**239.0709**; 197.0609; 135.0085; 198.0667
14	7-hydroxyflavanone	42.2	$C_{15}H_{12}O_3$	239.0719	**197.0610**; 135.0085; 239.0732; 198.0643
15	2',6'-dihydroxy-4'-methoxydihydrochalcone	45.2	$C_{16}H_{16}O_4$	271.0990	**254.0590**; 135.0444; 109.0287;
16	2'-Hydroxy-4'-methoxychalcone	49.9	$C_{16}H_{14}O_3$	253.0879	**237.0552**; 255.0665; 253.0872; 136.0169; 161.0239

(to be continue)

(continued)

Table 3 – Identification of phenolic compounds of Brazilian red propolis extract by LC-ESI-QTOF-MS/MS

	Isoflavonoids				
17	Daidzein	28.7	$C_{15}H_{10}O_4$	253.0511	**253.0513**; 208.0523; 119.0488; 135.0089
18	Calycosin	31.9	$C_{16}H_{12}O_5$	283.0617	**268.0353**; 211.0422; 224.0506; 239.0313; 267.0665
19	Dihydrobiochanin A	34.1	$C_{16}H_{14}O_5$	285.0776	**270.0541**; 109.0289; 161.0242; 285.0767
20	Vestitone	34.5	$C_{16}H_{14}O_5$	285.0776	**270.0535**; 161.0240; 109.0286; 271.0607
21	Vestitol	41.4	$C_{16}H_{16}O_4$	271.0987	**135.0450**; 109.0282; 149.0604; 147.0452; 271.0986; 256.0747
22	Neovestitol	41.8	$C_{16}H_{16}O_4$	271.0990	**135.0360**; 109.0217;256.0555; 197.0482; 212.0707
23	Formononetin	43.9	$C_{16}H_{12}O_4$	267.0666	**252.0431**; 254.0594; 223.0404; 195.0456; 253.0483
24	Demethyl medicarpin	45.2	$C_{15}H_{12}O_4$	255.0673	**255.0668**; 105.0189; 151.0032; 107.0118; 213.0532
25	Medicarpin	48.6	$C_{16}H_{14}O_4$	269.0827	**254.0594** ;225.0540; 105.0191; 121.0300; 133.0287
26	Biochanin A	52.0	$C_{16}H_{12}O_5$	283.0619	**268.0389**; 239.0354; 211.0393; 132.0202;195.4450
27	5,4′-Dihydroxy-7-methoxyisoflavone	53.1	$C_{16}H_{12}O_5$	283.0619	**268.0383**; 211.0422 ;223.0402; 224.0506;
28	3,9-Dimethoxypterocarpan	63.0	$C_{17}H_{16}O_4$	283.0988	**253.0515**;225.0564; 268.0754; 183.0456; 254.0554
	Flavonols				
29	7-Hydroxy-6-methoxydihydroflavonol	30.9	$C_{16}H_{14}O5$	285.0743	**270.0534**; 268.0383; 78.9984; 123.0078
	Other phenolic compounds				
	Neoflavonoids				
30	Dalbergin	38.3	$C_{16}H_{12}O_4$	267.0667	**252.0465**; 224.0503; 195.0451; 267.0650; 204.9615

(to be continue)

(*conclusion*)

Table 3 – Identification of phenolic compounds of Brazilian red propolis extract by LC-ESI-QTOF-MS/MS

	Polyprenylated benzophenones				
31	Guttiferone E / Xanthochymol	92.2	$C_{38}H_{50}O_6$	601.3567	**109.0291**; 108.0214; 202.9997; 177.0198; 335.1285
32	Oblongifolin B	93.8	$C_{38}H_{50}O_6$	601.3569	**109.0292**; 108.0216; 176.0146; 307.1362

Bold values indicate the main fragments; RT = retention time; $[M-H]^-$ (negative ionization mode) experimental mass of compound. As compared to an authentic standard.

2.3.4.1 Flavonoids

Flavonoids are the main class of phenolic compounds which have been identified in several natural products, including fruits, vegetables, roots, stems and flowers[33]. Multiple studies have revealed the beneficial effects of flavonoids extracted from propolis against human diseases[34]. In our study, a total of 28 flavonoids were identified in the BRP extract, which corresponded to the main chemical group present in the sample.

2.3.4.1.1 Flavones

Among the flavonoids detected in EEP, six were flavones. Chrysin (Compound **1** with [M–H]− at m/z 253.0510) yielded a fragment *m/z* 253.0507 [35]. Tricin (Compound **2**) with [M–H]− at *m/z* 329.0667, was tentatively identified based on product ions at m/z 329.0667,299.0218 [M-H-2CH_3]-, 271.0263 [M-H-$C_2H_2O_2$]−; 243.0289 [M-H-$C_4H_6O_2$]−. Genkwanin (compound **3** with [M-H]− at *m/z* 283.0624) yielded the predominant *m/z* 268 fragment due the loss of CH_2 from *m/z* 283 fragment, resulting in a stable fragment structure[36]. Hispidulin (Compound **4**) was tentatively identified based on the [M − H]− ion at *m/z* of 299.0565, with fragment ions at *m/z* 284.0331[M-CH_3], 227.0354[M-CO_2-CO]; 255.0301[M-H-CO2] and 212.0483[M-H-CO2-CO-CH3][35] Compound **5** was tentatively characterized as 8-Hydroxy-5-methoxyflavanon (m/z 269.0831) based on the *m*/*z* 254.0589 fragment. Finally, the characteristic [M–H]− ion at *m/z* 283.0617 and a major fragmentation at *m/z* 268.0382 were suggestive of acacetin (compound **6**)[37].

2.3.4.1.2 Flavanones

Retro–Diels–Alder (RDA) is the pathway fragmentation commonly used by flavanones. The fragment ions resulted from RDA fragments are more abundant than the loss of other radical ions, such as CH_3, CO, OH or H_2O[38]. Liquiritigenin (Compound **7**) was detected with [M-H]⁻ at *m/z* 255.0667. The identity was confirmed by comparing the data from a previous study, in which *Dalbergia odorifera* was characterized by LC-MS/MS, and based on the spectrum displayed of product ions at *m/z* 119.0495 ([M−H−$C_8H_8O_2$]⁻) and *m/z* 135.0083, corresponding to breaks of [^{1,3}A − H]⁻ and [^{1,3}B − H]⁻ fragments[39] . Four flavanones and derivates (compounds **8**, **9**, **10** and **11**) were tentatively identified in the optimized BRP extract as naringenin, Pinocembrin, 5,6-Dihydroxy-3',4'-dimethoxyflavanon and 6-Hydroxyflavanone,

according to the precursor ions [M−H]− at *m/z* 271.0619, 255.0668, 315.0882 and 239.0722, respectively. The identification of naringenin was confirmed by a product ion at *m/z* 119.0487[40]. Pinocembrin was identified by comparing our findings with those of a previous report, where this compound was found in leaf extracts of *Alpinia zerumbe,* yielding the *m/z* 255.0678 fragment[41]. Pinocembrin is an important marker in BRP, because it is also found in *Dalbergia ecastaphyllum*[42]. The compound 5,6-Dihydroxy-3',4'-dimethoxyflavanon (compound **10** with [M–H]− at *m/z* 315.0882), which was found for the first time in BRP, was tentatively identified based on a product ion at *m/z* 315.0881. Lastly, 6-Hydroxyflavanone displayed a product ion at *m/z* 239.0732 in the MS^2 spectra[38].

2.3.4.1.3 Chalcones

Isoliquiritigenin (compound **12** with [M–H]− at *m/z* 255.0676) was previously described in the literature and tentatively identified herein based on product ions at *m/z* 119.0496 and 135.0082[43]. The compound 2',4'-Dihydroxychalcone (compound **13**), detected with [M-H]− at *m/z* 239.0723, was identified based on fragment ions at *m/z* 239.0709, 197.0609 and 135.0085[44]. This compound was previously reported as an efficient antivirulence targing HlyU in *Vibrio vulnificus*[45]. Compound **14** was assigned as 7-hydroxyflavanone (*m/z* 239.0719), yielding the ion at *m/z* 197.0610 fragment[38]. Compound **15** detected with [M–H]− at *m/z* 271.0990 was tentatively identified as 2',6'-dihydroxy-4'-methoxydihydrochalcone based on ions products at *m/z* 254.0590, 135.0444 and 109.0287. Compound **16**, with [M–H]− at *m/z* 271.0990, was identified as 2'-Hydroxy-4'-methoxychalcone ($C_{16}H_{13}O_3$−), yielding the fragments ions *m/z* 237.0552, 255.0665 and 253.0872. This compound was previously found in orange-yellow resin from *Zuccagnia punctate*[19].

2.3.4.1.4 Isoflavonoids

The isoflavones aglycones daidzein (compound **17,** *m/z* 253.0511), formononetin (compound **22**, *m/z* 267.0666) and biochanin A (compound **25**, *m/z* 283.0619) were detected in the BRP extract. Daidzein yielded a product ion at *m/z* 253.0513 $[M\text{-}C_6H_{10}O_5]^-$ as result of a loss of glucoside and another product ion at *m/z* 135.0089 $[M\text{-}H\text{-}C_8H_6O]^-$[46]. The main fragment ions in the MS^2 spectra of Formononetin corresponded to successive losses such as CH_3, CHO, CO[39]. Its MS^2 spectra showed the fragments ions at *m/z* 252.0431 $[M–H–CH_3]^-$, 223.0404 ($C_{14}H_7O_3$)

[M − H-CH_3-CHO] ⁻ and 195.0456 [M − H-CH_3-CHO-CO] ⁻. A fragment ion at *m/z* 268.0389 [M–H–CH_3]⁻, which was produced due to the loss of a CH_3 group, and ions at *m/z* 239.0354[M-CO_2] and 211.0393 [M-CO_2-CO], were suggestive of Biochanin A fragmentation[35]. Compound **18,** with [M–H]− at *m/z* 283.0617 ($C1_6H_{11}O_5$), showed typical product ions at *m/z* 268.0353 ($C_{15}H_8O_5$), 211.0422, 224.0506 and 239.0313. Therefore, it was tentatively classified as Calycosin [47]. Dihydrobiochanin A (Compound **19** with [M–H]− at *m/z* 285.0776) and vestitone (Compound **20** with [M–H]− at *m/z* 285.0776) were characterized based on the fragment ions 270.0541 and 270.0535, respectively[48,49]. Vestitol (Compound **21**) with [M–H]− at *m/z* 285.0776 ($C_{16}H_{15}O_4$) displayed product ions at *m/z* 135.0450 ($C_8H_7O_2$), 109.0282 ($C_6H_5O_2$) and 149.0604[50]. Even though compound **22** ([M–H]− at *m/z* 271.0990) showed a similar fragment to vestitol, it was tentatively identified as neovestitol based on the main fragment ions at *m/z* 135.0360,197.0482 and 212.0707[5]. Vestitol and neovestitol have been previously isolated from BRP and were reported to have strong biological properties. Compound **24** ([M–H] − at *m/z* 255.0673) was tentatively identified as demethyl medicarpin based on fragment ions at *m/z* 255.0668, 151.0032, 107.0118 and 213.0532. Compound **25** was characterized as medicarpin according to the precursor ion at *m/z* 269.0827. In its MS^2 spectra, the following typical product ions were detected: 254.0594([M−H−CH_3•]•⁻), 225.0540, 105.0191, 121.0300 ($C_7H_5O_2$,$^{3,5}A^-$) and 133.0287[51]. Compound **27** (with [M–H]− at *m/z* 283.0619) and compound **28** (with [M–H] − at *m/z* 283.0988) were tentatively identified as 5,4′-Dihydroxy-7-methoxyisoflavone and 3,9-Dimethoxypterocarpan, respectively. In their MS^2 spectra, 5,4′-Dihydroxy-7-methoxyisoflavone displayed fragment ions at *m/z* 268.0383 [M-H-CH_3]**,** 211.0422 [M-CO_2-CO] and 223.0402 [M-H-CO-H_2O][35], whereas 3,9-Dimethoxypterocarpan showed fragment ions at *m/z* 253.0515, 225.0564 and 268.0754.

2.3.4.2 Flavonols, neoflavonoids, coumarins, and polyprenylated benzophenone derivates

Dalbergin (Compound **30** with [M–H]− at m/z 267.0667) yielded the product ions *m/z* 252.0465 and 224.0503, corresponding to the loss of a CO_2 and further loss of H_2O from the precursor ion[49]. Compound **29** ([M–H] − at m/z 285.0743) was tentatively identified as 7-Hydroxy-6-methoxydihydroflavonol based on fragment ions at *m/z* 270.0534. Guttiferone E (Compound **31)** displayed deprotonated molecular ion

at [M–H]− *m/z* 601.3571. Its MS^2 spectra showed fragment ions at *m/z* 109.0291[$C_6O_2H_5$]-, 108.0214, 202.9997, 177.0198[M-H-$C_{10}H_{16}O$]-, 335.1285[52]. Lastly, compound **32** ([M–H]− at m/z 601.3670) was tentatively identified as oblongifolin B, yielding the fragment ion at m/z 109.0292.

In addition to comparing the tentative compounds with the literature, we further compared the data against the electronic database available from metadata-centric approaches, such as Mass Bank of North America (MoNA) and Mass Bank. The LC-QTOF-ESI-MS/MS analysis in negative mode enabled the identification for the first time in BRP of the following phenolic compounds: flavones (tricin, genkwanin, hispidulin and 8-Hydroxy-5-methoxyflavanone), flavanones (5,6-Dihydroxy-3',4'-dimethoxyflavanone and 6-Hydroxyflavanone), chalcones (2',4'-Dihydroxychalcone and 2',6'-dihydroxy-4'-methoxydihydrochalcone), isoflavonoids (dihydrobiochanin A, demethyl medicarpin, 5,4′-Dihydroxy-7-methoxyisoflavone and 3,9-Dimethoxypterocarpan) and flavanols (7-Hydroxy-6-methoxydihydroflavonol).

2.4 Conclusion

The optimization of conditions for extraction of antioxidant compounds from BRP was successfully performed using the Response Surface Methodology. The optimal extraction conditions for stronger antioxidant activity and higher phenolic content were 90% ethanol at 80 °C for 30 min. Thirty-two phenolic compounds were tentatively identified by LC-QTOF-ESI-MS/MS, of which thirteen were found for the first time in BRP. Our study may guide further research in the field, since this is the first study that reported the optimization of the extraction of phenolic compounds from BRP samples. Collectively, our results highlight the importance of BRP as a source of a wide variety of phenolic compounds with significant antioxidant properties.

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3. TRANSPORT OF PHENOLIC COMPOUNDS FROM BRAZILIAN RED PROPOLIS IN CACO-2 CELL MODEL AND THEIR BIOGUIDED ANTIOXIDANT ACTIVITY

Abstract

Phenolic compounds extracted from natural products have been shown to be effective against ROS/RNS. In our study, we determined the antioxidant capacity of flavonoids from Brazilian Red Propolis (BRP) before and after *in vitro* gastrointestinal digestion and after transport across Caco-2 cells. Eleven compounds were tentatively identified in the digested BRP by LC-QTOF-ESI-MS/MS, including typical chemical makers of red propolis. The recovery rates (%) after gastrointestinal digestion were as follows: isoliquiritigenin (24.46%) > liquitirigenin (18.81%) > daidzein (13.57%) > vestitol (4.66%) > neovestitol (4.66%) > formononetin (2.13 %). The antioxidant activity of the extract significantly decreased throughout the digestive tract ($P < 0.05$). The bioactive compounds from BRP were able to cross Caco-2 cell monolayers after digestion, with the following absorption rates: isoliquiritigenin (113%) > formononetin (79%) > vestitol (72 %) > neovestitol (72%) > liquiritigenin (62%) > daidzein. Thus, even at lower levels, BRP extract can be absorbed into the gastrointestinal tract and maintain its ROS/RNS scavenging capacity.

Keywords: Isoflavones aglycones. ROS/RNS. Propolis. Polyphenol absorption.

3.1 Introduction

Brazilian red propolis (BRP) is a natural product widely consumed as a functional food. Some of its bioactive compounds such as vestitol, neovestitol, isoliquiritigenin, medicarpin and formononetin, were reported to be potentially effective for the prevention and control of human diseases due to their anti-inflammatory, antimicrobial and antioxidant effects[1–4].

In recent years, there has been a great effort to understand the pathophysiological mechanisms of reactive oxygen species (ROS) and reactive nitrogen species (RNS). When overproduced, these free radicals may have a severe impact on health and trigger the onset of chronic neurodegenerative diseases, cancer,

as well as respiratory and cardiovascular disorders[5]. Therefore, identifying potential natural antioxidants able to scavenge ROS and RNS is much needed.

The health benefits of most daily consumed natural products are attributed to their chemical composition and biological properties. However, for a better understanding of their actual impact on health outcomes, it is critical to determine the effective amount of compounds that are absorbed and assimilated throughout the human body[6]. For that reason, an increase in studies related to in vitro digestion has been observed in the past years. Several parameters affecting the phenolic composition and antioxidant activity in the gastrointestinal tract should be considered, including the food matrix, particle size and the extraction method, as well as physiological conditions such as pH and enzyme activity[7,8].

The *in vitro* digestion/Caco-2 cell culture model was used in our study to determine the bioaccessibility of BRP compounds. This model is recommended to study absorption, transport, and metabolism of chemical substances based on the morphological and functional similarities between Caco-2 cells and enterocytes[9–11]. Furthermore, these cells have been a good alternative to animal studies, which are accepted by both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) to investigate the intestinal permeability of drugs and foods[12].

While the phenolics present in BRP samples have been extensively described, mainly isoflavonoids, their antioxidant efficiency after gastrointestinal digestion remains largely unknown. Thus, this study aimed to determine the ROS/RNS scavenging capacity of the BRP extract and phenolic composition (LC-QTOF-ESI-MS/MS) before and after *in vitro* gastrointestinal digestion and after transport across Caco-2 cells.

3.2. Material and methods

3.2.1 Chemicals

The following chemicals were used in this study: Folin–Ciocalteau reagent (Dinamica Quimica Contemporanea, Diadema, SP, Brazil); sodium carbonate, potassium chloride, ethanol; monobasic and dibasic potassium phosphate, the standards ({±})-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, potassium peroxydisulfate, fluorescein sodium salt and 2,20-azobis(2-methylpropionamidine) dihydrochloride (AAPH), nitrotetrazolium blue

chloride (NBT), sodium hypochlorite solution (NaOCl), phenazine methosulfate (PMS), rhodamine 123, diaminofluorescein-2 (DAF-2), sodium nitroprusside, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of analytical grade.

3.2.2 Gastrointestinal digestion *in vitro*

The *in vitro* digestion was performed as previously described by Minekus et al.[13] All experiment conditions, such as stock solutions, enzyme concentrations, pH, gastric fluid as well as the intestinal fluid, were prepared according to a established protocol[13]. In the final digestion mixtures, Pepsin (P7000 from porcine gastric mucosa, Sigma-Aldrich, St. Louis, US), pancreatin (P1750 from porcine pancreas, Sigma-Aldrich, St. Louis, US) and bile salts (B8631 bile from porcine, Sigma-Aldrich, St. Louis, US) were used at concentrations of 2000 U/mL, 100 U/mL (based on trypsin activity) and 10 mM. The experiment was carried out using 50 mL centrifuge tubes placed in an incubator (Ethik, 410-TDR) under rocking (175 rpm) and 37 °C. Briefly, 2 mL of BRP extract (100 mg mL^{-1}) were mixed with 4 mL of simulated gastric fluid (SGF) containing $CaCl_2$ and pepsin. The pH of the samples was adjusted to 3.0 with 1 M HCL. The samples were incubated at 37 °C for 2 h. For the intestinal phase, 8 mL of simulated intestinal fluid (SIF) containing pancreatin and bile salts was added, and the pH was adjusted to 6.9 with 1 M NaOH. Then, samples remained in the incubator for 2 h at 37 °C, centrifuged at 10,000 rpm at 4°C for 10 min and kept at −20 °C until analysis.

3.2.3 Caco-2 Experiments

Caco-2 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 1 mg/mL streptomycin, 1% nonessential amino acids, and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, USA) and incubated at 37 °C in 5% CO_2 incubator. The cytotoxic effect was determined using based on MTT assay[14]. Briefly, Caco-2 cells were seeded onto 96-well plates at 1×10^5 cells/well and incubated in 5% CO_2 at 37 °C for 24 h. Next, the spent media was removed, and Caco-2 cells were treated with different concentrations of the digested BRP extract for 4 h. The media was discarded and the cells were incubated in the presence of MTT

for 3 h. The content of the wells was once again discarded and an aliquot of 150 µL of DMSO was added to each well. The absorbance was measured at 570 nm in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Transport assays were performed as previously described[15]. Caco-2 cells were seeded onto six-well Transwell plates with 0.4 µm pores (Corning Incorporated; NY, USA) at a density of 3×10^5 cells per insert. The culture media was replaced every two days along ~ 28 days. Caco-2 monolayers were used when transepithelial electrical resistance (TEER) values were larger than 250 Ω cm^2. Apical and basolateral compartments were washed once and incubated with Hanks' Balanced Salt Solution (HBSS) (1.5 and 2.6 mL). After that, apical compartments were treated with the digested BRP extract and diluted in HBSS (only HBSS in the basolateral compartment). The plates were incubated at 37 °C for 4 h. The integrity of the Caco-2 monolayer was measured by TEER before and after the experiment. Lastly, apical and basolateral samples were freeze-dried and stored at −20 °C until the analysis

3.2.4 Total phenolic compounds

The analysis of the total phenolic content (TPC) was performed according to the Folin–Ciocalteau method, with some modifications. Briefly, 20 µL of the BRP and digested extracts and 100 µL of the Folin–Ciocalteau solution (10% in water) were pipetted into the wells of a microplate. After 5 min, 75 µL of a 7.5% sodium carbonate aqueous solution were added to each well. The absorbance was measured at 740 nm after 40 min. Gallic acid was used as a standard to determine the linear regression and calculate the TPC. The results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract[16]. All samples were analysed in triplicate.

3.2.5 Reactive oxygen and nitrogen species (ROS and RNS)

3.2.5.1 Superoxide anion ($O_2^{\bullet-}$)

The capacity of the BRP and digested extracts to scavenge $O_2^{\bullet-}$ generated by the NADH/PMS system was determined as previously described[17]. Briefly, 100 µL of NADH, 50 µL of NBT, 100 µL of the samples (at different concentrations) and 50 µL of PMS were mixed in a microplate. The assay was performed at 25 °C and the absorbance was measured at 560 nm after 5 min in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). A control was prepared replacing the sample with the buffer, and a blank was prepared for each sample dilution replacing PMS and

NADH with the buffer. The results were expressed as IC_{50}, that is, the mean concentration (µg/mL) of the BRP extract required to quench 50% of the superoxide radicals[17] .

3.2.5.2 Peroxyl radical ($ROO^{\bullet}$)

Briefly, 30 µL of BRP or digested extracts plus 60 µL of fluorescein and 110 µL of an AAPH solution were transferred to a microplate. The reaction was performed at 37 °C and absorbance was measured every minute for 2 h at 485 nm (excitation) and 528 nm (emission) using in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Trolox standard was used at concentrations ranging from 12.5 to 400 µM. The results were expressed as µmol/Trolox equivalents (TE) per g of sample[17].

3.2.5.3 Hypochlorous acid (HOCl)

The HOCl scavenging activity of BRP and digested extracts was measured by monitoring their effects on HOCl-induced oxidation of dihydrorhodamine 123 (DHR) to rhodamine 123, with modifications. HOCl was prepared using a 1% NaOCl solution, and the pH was adjusted to 6.2. The reaction mixture contained the sample at different concentrations, as well as phosphate buffer (pH 7.4), DHR, and HOCl, in a final volume of 300 µL. The assay was carried out at 37 °C in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA), and fluorescence was measured immediately at 528 ± 20 nm (emission) and 485 ± 20 nm (excitation). The results were expressed as IC_{50} (µg/mL) of the sample[17].

3.2.5.4 Nitric oxide ($NO^{\bullet}$)

The nitric oxide ($NO^{\bullet}$) activity of the BRP and digested extracts was determined using diaminofluorescein-2 (DAF-2) as a $NO^{\bullet}$ probe. Briefly, 50 µL of the BRP extract plus 50 µL of SNP solution, 50 µL of buffer and 50 µL of DAF solution were added to the wells of a 96-well plate. Changes in fluorescence (excitation = 495 nm, emission = 515 nm) were measured in a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) over a 120-min period at 5-min intervals. The results were expressed as IC_{50} (µg/mL) of the sample[17].

3.2.6 High-resolution mass spectrometry analysis (LC-ESI-QTOF-MS/MS) of cell culture sample

A liquid chromatography analysis was carried out using a chromatograph (Shimadzu Co., Tokyo) with a LC-30AD quaternary pump and SPD-20A photodiode array detector (PDA). Reversed phase chromatography was performed using Phenomenex Luna C18 column (4.6 x 250 mm x 5 µm). The high-resolution mass spectrometer MAXIS 3G –Bruker Daltonics (Bruker Daltonics, Bremen, Germany) was equipped with a Z-electrospray (ESI) interface operating in negative ion mode with a nominal resolution of 60.000 m/z. Twenty microliters of the apical sample were injected into the liquid chromatography system under the following analytical conditions: nebulizer at 2 Bar; dry gas at 8 L/min; temperature at 200 °C and HV at 4,500 V. The mobile phase consisted of two solvents: (A) water/acetic acid (99.5/0.5, v/v) and (B) methanol. The flow rate was 1 mL/min, and the gradient was initiated with 30% B, increasing to 40% B (15 min), 50% B (30 min), 60% B (45 min), 75% B (65 min), 75% B (85 min), 90% B (95 min), decreasing to 30% B (105 min). The run was complete after 114 min. An external calibration was carried out using the software MAXIS 3G – Bruker Daltonics 4.3 to check for mass precision and data analysis. The compounds were tentatively identified by comparing their exact mass (*m/z*) and MS^2 spectra in negative mode to the database available in the literature and commercial standards. A Trolox standard (expressed as µM) was used to quantify the compounds detected in the BRP extract.

3.2.7 Statistical analysis

All assays were carried out in triplicate and the values were expressed as mean ± standard deviation. Multigroup comparisons were carried out using one-way Analysis of Variance (ANOVA) with Tukey's post-hoc test. Statistical significance was accepted at $P < 0.05$.

3.3 Results and discussion

3.3.1 Identification of digested/transported polyphenols of BRP across Caco-2 cell monolayers

Eleven polyphenols were tentatively detected by LC-QTOF-ESI-MS/MS in the digested and basolateral BRP fractions after transepithelial transport across Caco-2 cell monolayers (Table1). Among them, six are major flavonoids commonly found in

BRP (liquiritigenin, daidzein, isoliquiritigenin, vestitol, neovestitol and formononetin). Therefore, these compounds were chosen for follow-up studies. The compounds were tentatively identified by comparing their mass spectra against the literature data and metadata-centric approaches [*e.g.*, Mass Bank of North America (MoNA) and Mass Bank].

Liquiritigenin (Compound **1** with [M − H]− at *m/z* 255.0667), was identified based on the product ions at *m/z* 119.0495 ([M − H − $C_8H_8O_2$]$^-$) and 135.0083, as a result of breaks on the [^{1,3}A − H]$^-$ and [^{1,3}B − H]$^-$ fragments[18]. Daidzein (Compound **2** with [M − H]− at *m/z* 253.0511) yielded fragment ions at *m/z* 253.0513 [M-$C_6H_{10}O_5$]$^-$, 135.0089 [M-H-C_8H_6O]$^-$ and 119.0488[19]. Product ions at *m/z* 270.0534, 268.0383, 78.9984 and 123.0078 corresponded to 7-Hydroxy-6-methoxydihydroflavonol fragmentation (compound **3** with [M − H]− at *m/z* 285.0743).

Naringenin (compound **4** with [M−H]− at *m/z* 271.0619) was tentatively identified based on a product ion at *m/z* 119.0487[20]. Genkwanin (compound **5** with ([M-H]− at *m/z* 283.0624) displayed product ions at *m/z* 268.0360, 283.0583 and 269.0397[21]. Isoliquiritigenin (compound **6** with [M–H]− at *m/z* 255.0676) was detected based on product ions at *m/z* 119.0496 and 135.0082[22]. Compounds **7**, **8** and **10** were identified as vestitol, neovestitol and formononetin, according to precursor ions [M − H]− at *m/z* 285.0776 ($C_{16}H_{15}O_4$), 271.0990 and 267.0666, respectively. Vestitol (Figure 1A) was confirmed by product ions at *m/z* 135.0450 ($C_8H_7O_2$), 109.0282 ($C_6H_5O_2$) and 149.0604[23] Neovestitol (Figure 1B) was tentatively identified based on product ions at *m/z* 135.0360,197.0482 and 212.0707[24]. Formononetin displayed fragment ions at *m/z* 252.0431 [M–H–CH_3] $^-$, 223.0404 ($C_{14}H_7O_3$) [M − H-CH_3-CHO] $^-$ and 195.0456 [M − H-CH_3-CHO-CO] $^-$[18].

Compounds **9** and **11** were tentatively identified as 2',4'-Dihydroxychalcone ([M-H]− at *m/z* 239.0723)[25] and 8-Hydroxy-5-methoxyflavanone ([M-H]− at *m/z* 269.0831) based on fragment ions at *m/z* 239.0709 and *m/z* 254.0589, respectively.

Table 1 - Phenolic compounds tentatively identified by LC-ESI-QTOF-MS/MS in Brazilian red propolis extract after *in vitro* digestion (Caco-2 cell model)

No.	Compound	RT (min)	Molecular formula	[M-H]⁻	MS fragments (*m/z*)
1	Liquiritigenin	23.1	$C_{15}H_{12}O_4$	255.0667	**119.0495**; 135.0083; 255.0656; 120.0526
2	Daidzein	26.4	$C_{15}H_{10}O_4$	253.0511	**253.0513**; 208.0523; 119.0488; 135.0089
3	7-Hydroxy-6-methoxydihydroflavonol	29.5	$C_{16}H_{14}O5$	285.0743	**270.0534**; 268.0383; 78.9984; 123.0078
4	Naringenin	30.4	$C_{15}H_{12}O_5$	271.0619	**119.0487**; 151.0029; 254.0596; 271.0609; 165.0207
5	Genkwanin	35.2	$C_{16}H_{12}O_5$	283.0624	**268.0360**; 283.0583; 269.0397
6	Isoliquiritigenin	39.4	$C_{15}H_{12}O_4$	255.0676	**119.0496**; 135.0082; 120.0531; 151.0384; 255.0665
7	Vestitol	40.9	$C_{16}H_{16}O_4$	271.0987	**135.0450**; 109.0282; 149.0604; 147.0452; 271.0986; 256.0747
8	Neovestitol	41.0	$C_{16}H_{16}O_4$	271.0990	**135.0360**; 109.0217;256.0555; 197.0482; 212.0707
9	2',4'-Dihydroxychalcone	42.3	$C_{15}H_{12}O_3$	239.0723	**239.0709**; 197.0609; 135.0085; 198.0667
10	Formononetin	43.4	$C_{16}H_{12}O_4$	267.0666	**252.0431**; 254.0594; 223.0404; 195.0456; 253.0483
11	8-Hydroxy-5-methoxyflavanone	44.2	$C_{16}H_{14}O_4$	269.0831	**254.0589**; 252.0437; 195.0451; 210.0685

Bold values indicate the main fragments; RT = retention time; [M-H]⁻ (negative ionization mode) experimental mass of compound.

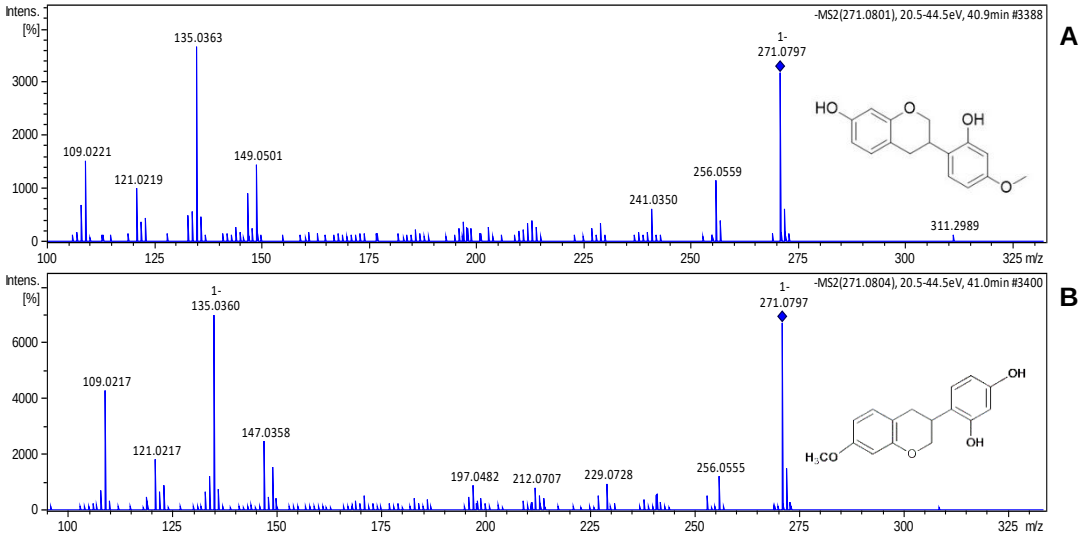

Figure 1 - Chemical structure and product ion mass spectra of (A) vestitol and (B) neovestitol

3.3.2 Stability of phenolic compounds during *in vitro* digestion

Propolis is a natural product widely known for its intrinsic biological properties, which are mostly attributed to its bioactive compounds (*e.g*., isoflavones). Here, we determined the effects of gastrointestinal digestion on the phenolic composition of BRP extract (Figure 2). Our findings indicate that the TPC was significantly reduced throughout gastrointestinal digestion. The amount of TPC recovered in the gastric and intestinal phases was 65 % and 71% lower than that of the undigested sample, respectively. Gómez-Maqueo et al.[26] observed losses of TPC between 27.4 % and 47.1 % after digestion in prickly pear fruits. During evaluation of several Brown propolis samples, Yen et al.[27] observed that the TPC of several brown propolis samples decreased as compared to the undigested extract. Bilušić et al.[28] observed a significant ($r = 0.6926$, $P = 0.0125$) and extremely significant ($r = 0.9170$, $P < 0.0001$) correlation between the stability of carotenoids under acidic and alkaline conditions, respectively. This suggests that the amount of recovered TPC could be pH related. Thus, indicating that the amount of TPC could be associated to pH. In opposite to those results, Ozdal et al.[29] observed that the levels of phenolic compounds in Turkish propolis increased throughout the gastric and intestinal phases. However, the authors used a crude material in their experiments, which may have affected the amount of TPC. Hence, as suggested by the authors, using propolis extracts rather than raw propolis might be more appropriate for this purpose.

Several variables may affect the efficiency of absorption of polyphenolic compounds along the gastrointestinal tract, including the composition of the digested matrix, physicochemical properties (*e.g*., pH, temperature and molecular weight) and compound hydrophilicity[30–32]. It is important to consider that the Folin-Ciocalteu reagent is non-specific to phenolic compounds so that it can be reduced by many non-phenolic compounds (*e.g*., aromatic amines, sulfur dioxide, ascorbic acid, Cu(I), Fe(II) etc.) as well as by proteins and small peptides that are formed during digestion[33]. There is not a single factor driving TPC availability during *in vitro* digestion. Hence, all these aspects should be considered and also the complexity of the phenolic source. More importantly, a higher content of phenolic compounds does not necessarily translate into greater bioavailability[34].

As shown in Figure 3, TPC recovery rates (%) were significantly reduced along the digestion process *in vitro*, except for liquiritigenin in the gastric phase. At this phase, the recovery rates were ranked as follow: liquitirigenin > (35.69 %) > daidzein

(25.15%) > vestitol (14.82%) > neovestitol (13.27%) > isoliquiritigenin (9.79%) > formononetin (7.45%). As for the intestinal phase, the recovery rates (%) were as follows: isoliquiritigenin (24.46%) > liquitirigenin (18.81%) > daidzein (13.57%) > vestitol (4.66%) > neovestitol (4.66%) > formononetin (2.13 %). Interestingly, isoliquiritigenin was the only compound whose recovery rate increased during the intestinal phase.

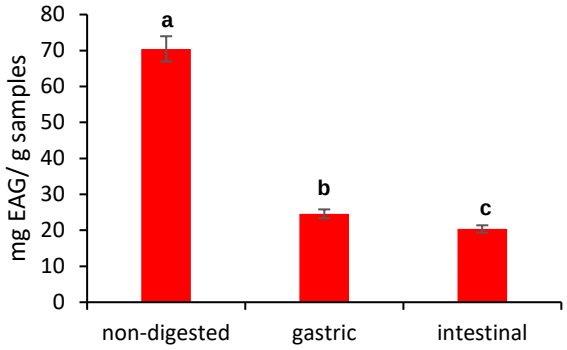

Figure 2 – Total phenolic compounds in extracts of Brazilian red propolis extract during gastrointestinal digestion. Values represent the mean ± SD (n=3). Different letters indicate significant differences in the TPC between digestive phases ($p < 0.05$)

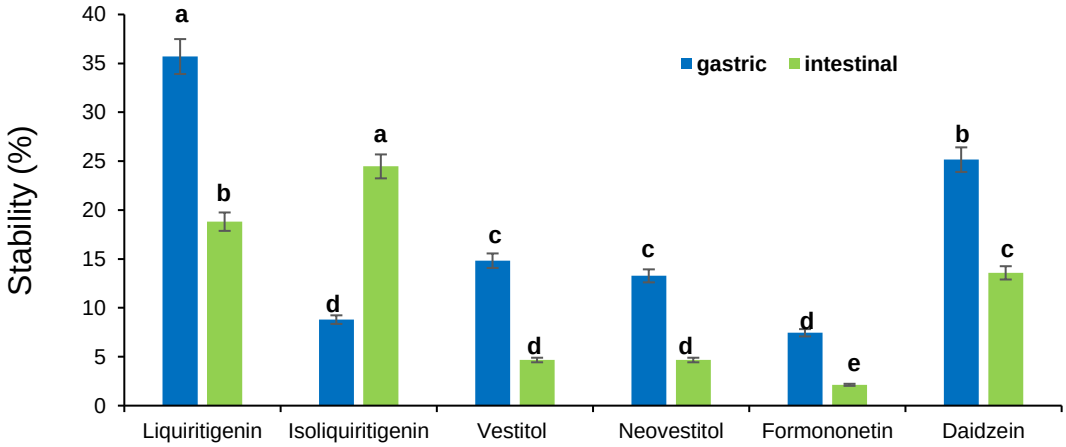

Figure 3 – Effect of *in vitro* digestion on phenolic concentration of extracts of Brazilian red propolis extract. Values represent mean ± SD (n=3). Different letters indicate significant differences in phenolic compounds between digestive phases ($p < 0.05$)

The effects of the digestion process on the stability of isoflavone aglycones is not completely understood, as these compounds are less frequently found in natural products. In addition, in most studies, isoflavone aglycones are extracted from soybeans, which make it difficult to compare the findings with those of other materials, given that soybeans are rich in carbohydrates and proteins.

In our study, the recovery of isoflavone aglycones in the intestinal phase was significantly lower than that in the gastric phase. We assumed this can be attributed to the absence of colonic fermentation in our *in vitro* digestion model. Some authors reported that the colonic microbiome can maximize the recovery of isoflavone aglycones. In a soybean meal aqueous extract, for instance, the content of daidzein was up to seven-fold higher than that of undigested samples[35]. Luo et al.[36] observed that formononetin and its glycoside ononin had a better absorption in the large intestine segments than in other parts of the small intestine, which is explained by the site-specific distribution of these isoflavones in the gut tract. Therefore, the lack of *in vitro* colonic microbiome in our study model might be the major contributor to the poor recovery of such isoflavones.

3.3.3 ROS/RNS scavenging of the BRP extract during *in vitro* digestion

Mounting evidence has shown that the development of several chronic and degenerative diseases is associated with overproduction of free radicals[5]. This has increased the interest in natural products as new sources of bioactive compounds with potential antioxidant capacity. However, most of the studies thus far have examined the antioxidant properties of extracts or crude materials, while little is known about their effectiveness once consumed by humans.

The ROS/RNS scavenging capacity of the BRP extract was determined before and after *in vitro* digestion (Table 2). The results showed that the scavenging capacity of the extract was significantly reduced throughout the digestive tract. In all tested models, the antioxidant activity of the undigested sample was higher than that of the digested fractions. However, a slight increase in the peroxyl radical ($ROO^{\bullet}$) and hypochlorous acid (HOCl) scavenging activity was observed from the gastric to the intestinal phase. Interestingly, although none of digested fractions were more efficient than the original extract, their HOCl scavenging effects in the intestinal fraction (IC_{50} 47.97 ± 4.13 μg/mL) were more effective than those of ascorbic acid

(IC_{50}: 198.42 ± 10.71 µg/mL), *p*-coumaric acid (IC_{50}: 74.25µg/mL) and 5-caffeoylquinic acid (IC_{50}: 70.91µg/mL)[37,38].

Table 2 – ROS and RNS scavenging capacity of Brazilian red propolis extract submitted to gastrointestinal digestion

Sample	µmol TE/g	IC_{50} (µg/mL)		
	$ROO^{\bullet}$	$O_2^{\bullet-}$	HOCl	$NO^{\bullet}$
Non-digested	2538.67± 67.07[a]	318.95 ± 2.82[a]	29.14 ± 4.16[a]	1658.51 ± 368.15[b]
Gastric digestion	493.51± 41.60[c]	955.26 ± 48.02[b]	75.34 ± 10.35[c]	769.037 ± 141.31[a]
Intestinal digestion	601.36± 35.71[b]	1823.46 ± 47.34[c]	47.97 ± 4.13[b]	2096.32 ± 482.70[c]

The results were expressed as mean ± SD (n=3). Different letters in the column indicate statistical differences ($p < 0.05$).

Intriguingly, the gastric fraction of the BRP extract presented superoxide and nitric oxide scavenging effects two-fold greater than those of the intestinal fraction. According to previous reports, isoflavones are the main bioactive compounds in BRP[39,40]. Hence, we assume that the low antioxidant capacity of the intestinal fractions is a result of the reduced concentration of isoflavones throughout the gastrointestinal tract. Consistent with our findings, Wyspiańska et al.[41] observed that the antioxidant activity of isoflavone-enriched isotonic drinks in the intestinal phase was 25 % lower than that of undigested beverages. Ribeiro et al.[42] reported that despite the higher amount of polyphenol content observed in the intestinal fraction of a juçara-containing smoothie, its antioxidant potential was reduced due to the alkaline pH of the intestine. In addition, *Physalis peruviana* L. extract had its antioxidant activity decreased from 36 % in the gastric phase to 10 % when reaching the intestine[43]. Hence, on the basis of these previous reports and our findings, we assume that the pH can significantly affect the antioxidant activity of polyphenols both under *in vitro* and *in vivo* conditions[8]. Thus, the ROS/RNS scavenging capacity of metabolites in acidic/alkaline environments can be different than under neutral conditions.

3.3.4 Caco-2 cell transepithelial transportation

The Caco-2 cell model may provide insights into the mechanisms of absorption of phenolic compounds from different sources in the gastrointestinal system. In our study, we used this model to determine the transepithelial transport of the digested BRP extract. As shown in Figure 4, the phenolic compounds of the extract were capable of crossing the Caco-2 cell monolayer, with significant differences in their absorption rates though. The isoflavones formononetin, vestitol and neovestitol,

showed the highest recovery rates, which corresponded to 79 %, 72% and 72%, respectively. In line with this, Luo et al.[36] found a recovery rate of formononetin of 77.8 % after transport across Caco-2 cells. These authors also detected the presence of the compound in the plasma of rats, suggesting it has a high systemic distribution. A recovery rate of 55% was observed for daidzein whereas the flavone liquiritigenin had a recovery rate of 62 %, even though it was found in high concentrations throughout the gastrointestinal tract. Previous studies reported that isoflavones can permeate Caco-2 monolayers better than flavones, chalcones and flavonols[44]. Fang et al.[27] investigated the transport of 30 flavonoids across Caco-2 cells and reported that the permeability ratio of formononetin and daidzein was higher than 1.00, suggesting greater accumulation of these compounds on the basolateral side. The easy transport of these isoflavones across the epithelial barrier can be attributed to their lipophilicity, as aglycones are generally more liposoluble than glycosides.

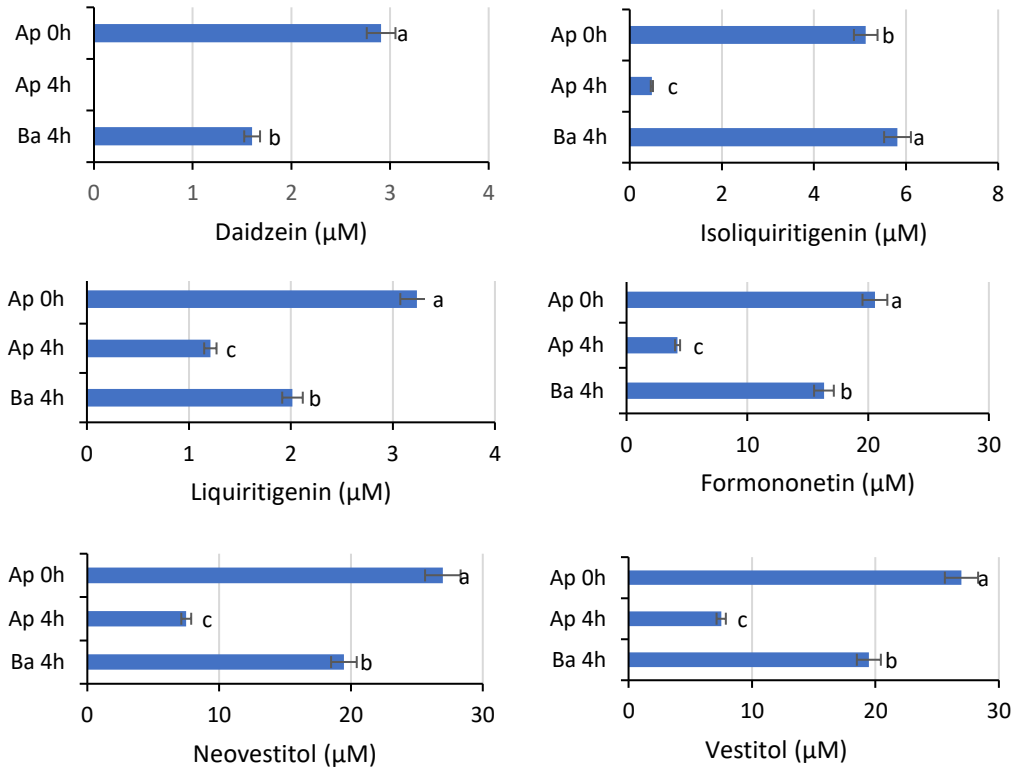

Figure 4 – Phenolic compound concentration in digested Brazilian red propolis extract after transport across Caco-2 cells. Ap 0h: apical (before incubation); Ap 4h: apical after 4 hrs of incubation; Ba: basolateral after 4 hrs of incubation. Values correspond to the mean ± SD (n = 3). Values bearing the different letters were significantly different ($p < 0.05$)

In our study, isoliquiritigenin showed the highest recovery rate (113%). According to Tin et al.[44], the permeation of lipophilic flavonoids, such as isoliquiritigenin, is driven by the concentration gradient. Therefore, passive diffusion is suggested as a major transport mechanism. However, it is important to point out that aside from the factors that can affect permeability, such as lipophilicity and interaction with the membrane, the absorption rate of target compounds also depends on their source. Wang et al.[45] examined the transport of isoflavones from red clover products and found that even when biochanin A was used at the same loading concentration, there were differences in percent absorption and the extent of metabolism, which were attributed by the authors to the food matrix. Here, the antioxidant activity of the digested/transported fractions was evaluated based on their peroxyl radical scavenging capacity (Figure 5). The ORAC value of the basolateral fraction (1.12 ± 0.11) was 2.66-fold higher than that of the apical fraction (0.42 ± 0.08), indicating that the BRP extract can maintain its bioactivity after transport across the epithelial barrier.

To the best of our knowledge, this is the first study in the literature reporting on the transepithelial transport of vestitol and neovestitol. Even though these compounds were poorly recovered after cell transport, we reasoned that they may remain biologically active, since a higher gut permeability does not always correlate with greater bioavailability[36].

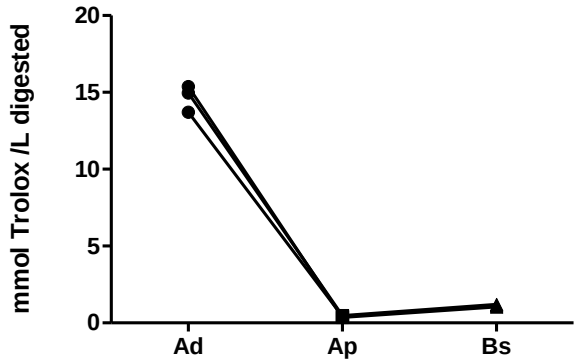

Figure 4 – Antioxidant activity (ORAC) of Brazilian red propolis extract after transport across Caco-2 cell. Ad: after digestion; Ap: apical after 4 hrs of incubation; Ba: basolateral after 4 hrs of incubation

3.4 Conclusion

In summary, the phenolic concentration of BRP extract was significantly reduced along the digestion process *in vitro*. However, some isoflavones could be detected after transport across Caco-2 cell monolayers, the bioactivity was affected. Our findings may guide further research to understand the bioavailability of phenolic compounds from Brazilian red propolis.

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4. FINAL CONSIDERATIONS

The first phase of our study consisted of optimizing the conditions for extraction of phenolic compounds from BRP. While this is a critical procedure to maximize the bioactivity of natural products, it is commonly neglected in most studies. Besides the influence of environmental conditions on the chemical composition of naturally-occurring products, the extraction method also plays a significant role in their bioactivity.

Studying the bioactivity of natural products throughout gastrointestinal digestion is a challenging task, as the human tract is a very complex system and because there is no other system able to perfectly emulate the physiological absorption ratios of the human body. In our study, the *in vitro* digestion/Caco-2 cell model was used to investigate the absorption rate of BRP compounds as well as their ROS/RNS scavenging capacity. Even though the model has important limitations to consider, we could describe for the first time the absorption rates of the main isoflavones present in BRP.

Collectively, our results may contribute to understanding the effects of human digestion on the biological properties of phenolic compounds and support future research regarding their bioavailability. Further studies should consider microbiome models to determine precisely the interactive mechanisms of BRP phenolics throughout the human body.