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THAIANALY LEITE ABREU

**ESTUDO DOS COMPOSTOS FENÓLICOS DA CASCA DE CAFÉ ORGÂNICO:
BIOACESSIBILIDADE, BIOATIVIDADES, INFLUÊNCIA DE METABÓLITOS NA
MICROBIOTA HUMANA E PROTEÇÃO DE CÉLULAS CACO-2**

JOÃO PESSOA – PB

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Orientadora: Prof^a. Dr.^a Taliana Kênia Alencar Bezerra

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Prof^a. Dr.^a Taliana Kênia Alencar Bezerra – PPGCTA/CT/UFPB
Presidente da Banca Examinadora

Prof^a Dr.^a Marta Suely Madruga – PPGCTA/CT/UFPB
Examinador Interno

Prof. Dr. Fábio Anderson Pereira da Silva - UFPB
Examinador Interno

Prof^a. Dr.^a Maria Teresa Bertoldo Pacheco- ITAL
Examinador Externo

Prof. Dr. Mário Roberto Maróstica Júnior – DEPAN/FEA/UNICAMP
Examinador Externo

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RESUMO

A casca de café é o subproduto mais expressivo da indústria cafeeira, e é geralmente descartado ou usado como adubo. O aproveitamento da casca de café pode gerar benefícios ambientais, sociais e aliado a produção orgânica de cultivo mais sustentável esses impactos são mais atenuados. Este subproduto tem sido cada vez mais estudado e utilizado devido às suas propriedades antioxidantes, anti-inflamatórias, anticoagulante, antimicrobiano e potencial prebiótico. Tais efeitos estão relacionados a compostos bioativos, como os fenólicos e macroantioxidantes vinculados as fibras, que são moléculas importantes capazes de reduzir o estresse oxidativo, regular a expressão de genes, proteger as células do corpo contra os danos de ROS e modular o microbioma intestinal de indivíduos acometidos de diabetes. Diante do exposto objetivou-se através desse estudo avaliar a casca de café orgânico quanto sua composição de compostos fenólicos e macroantioxidantes, capacidade antioxidante *in vitro* (DPPH, ABTS, ORAC e FRAP), digestão simulada *in vitro*, bioacessibilidade, efeito anticoagulante, efeitos citoprotetores/citotóxicos sobre células Caco-2 (MTT, e DCF-DA), fermentação colônica *in vitro* com inóculo fecal de diabéticos além citometria de fluxo e o índice prebiótico. No primeiro estudo baseou-se na realização de três extratos, nomeadamente polifenóis extraíveis (EP), polifenóis não extraíveis hidrolisáveis (HNEP) e polifenóis não extraíveis (NEP), que foram caracterizados e avaliados por suas propriedades bioativas após digestão gastrointestinal simulada. Os resultados mostram que o processo de extração afeta a ocorrência de polifenóis das cascas de café, principalmente para ácido cafeico, ácido gálico e ácido clorogênico. Os polifenóis livres e condensados encontrados nos extratos e na casca não digerida além de propriedades antioxidantes contra os radicais ABTS, FRAP, DPPH e ORAC também tiveram notável biodisponibilidade e potencial anticoagulante. Os resultados do segundo estudo demonstraram bom potencial da casca, com atividade antioxidante que manteve consideravelmente o efeito contra todos os radicais em condições gástricas. A amostra também apresentou viabilidade celular e resultou na inibição de ROS em todas as concentrações testadas. Sobre a fermentação fecal, houve correlação positiva da presença da casca na liberação de compostos fenólicos. Já o índice prebiótico positivo foi encontrado para CH, enquanto índice prebiótico negativo foi encontrado para NC, indicando uma sobreposição de bactérias benéficas em detrimento de bactérias indesejáveis durante a fermentação colônica. Tais resultados indicam o grande potencial da casca de café como fonte de compostos bioativos relacionados a efeitos biológicos, celulares e metabólicos importantes na manutenção da microbiota de indivíduos acometidos de diabetes, uma vez que possuem comprometimento da barreira intestinal, e o subproduto pode auxiliar em novas perspectivas para ciência e tecnologia de alimentos como um potencial nutracêutico.

Palavras-chave: subprodutos; café orgânico; fenólicos; bioatividades; microbiota intestinal

ABSTRACT

Coffee husks are the main by-product of the coffee industry and are generally discarded or used as fertilizer. The use of coffee husks can have environmental and social benefits, and when combined with the organic production of a more sustainable crop, these impacts are mitigated. This byproduct is increasingly being studied and used for its antioxidant, anti-inflammatory, anticoagulant, and antimicrobial properties, as well as its prebiotic potential. These effects are related to bioactive compounds such as phenols and fiber-bound macroantioxidants, which are able to reduce oxidative stress, regulate gene expression, protect body cells from ROS damage, and modulate the gut microbiome of people with diabetes. In view of the above, the aim of this study was to evaluate organic coffee husks in terms of their composition of phenolic compounds and macroantioxidants, their antioxidant capacity (DPPH, ABTS, ORAC and FRAP), their in vitro simulated digestion, bioavailability, anticoagulant effect, cytoprotective effects/cytotoxicity on Caco-2 cells (MTT and DCF-DA), in vitro intestinal fermentation with fecal inoculum of diabetic patients, in addition to flow cytometry and prebiotic index. The first study was based on the realization of three extracts, namely extractable polyphenols (EP), non-extractable hydrolyzable polyphenols (HNEP) and non-extractable polyphenols (NEP), which were characterized and evaluated for their bioactive properties after simulated gastrointestinal digestion. The results show that the transfer process affects the occurrence of polyphenols in coffee peels, especially for caffeic acid, gallic acid, and chlorogenic acid. The free and condensed polyphenols found in the extracts and undigested bark exhibited remarkable bioavailability and anticoagulant potential in addition to antioxidant properties against ABTS, FRAP, DPPH and ORAC radicals. The results of the second study showed good potential of the peel with antioxidant activity that significantly maintained the effect against all radicals under gastric conditions. The sample also showed cell vitality and developed in ROS in all studies. As for fecal fermentation, there was a positive change in the release of phenolic compounds in the presence of the bark. The positive prebiotic index was found for CH, while the negative prebiotic index was found for NC, indicating an overlap of bacteria to the detriment of bacteria during colonic fermentation. These results indicate the great potential of coffee husks as a source of bioactive compounds associated with important biological, cellular, and metabolic effects in maintaining the microbiota of individuals with diabetes because they have a disrupted intestinal barrier, and the byproduct may open new perspectives for food science and technology as a potential nutraceutical.

Keywords: by-products; organic coffee; phenolics; bioactivities; gut microbiota

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1 INTRODUÇÃO

O Brasil destaca-se como o principal produtor e exportador mundial de café, apresentando forte impacto na economia do país. De acordo com a *International Coffee* o país foi responsável por 39,4% da produção internacional e 78,2% da produção do continente sul-americano (ICO 2021). No entanto, alguns estados do Nordeste têm se destacado na produção de café, como Bahia, que é o maior produtor da região, produzindo cerca de 2 milhões de sacas de 60kg em 2020 e Pernambuco, que também tem uma produção significativa de café arábica de alta qualidade e destaca-se com o cultivo orgânico sombreado que implica em uma série de melhorias para a qualidade, produção ecológica, social e econômica do café (QUEIROGA et al., 2021).

Os consumidores de café mudaram seus hábitos e passaram a valorizar a produção com redução de impactos ambientais e segurança alimentar. Por isso, o processamento do café orgânico e o aproveitamento de seus subprodutos, como a casca, é incentivado para agregar valor e reduzir desperdícios, mantendo as propriedades nutricionais e bioativas. Estudos mostram que a casca de café é uma fonte acessível de compostos bioativos com potencial para reduzir doenças cardiovasculares, câncer, diabetes e outros benefícios para o metabolismo humano (DAS; RAMANATHAN, 2023; SILVA et al., 2020).

Nos últimos anos estudos avaliam a composição química da casca de café, com ênfase nos compostos bioativos, atividade antioxidante, assim como seu metabolismo, bioacessibilidade, propriedades probióticas e biológicas resultantes do elevado teor de compostos fenólicos e macroantioxidantes vinculados as fibras dietéticas (JANISSEN; HUYNH, 2018; SILVA et al., 2020). Os macroantioxidantes representam uma fração emergente e ignorada de antioxidantes alimentares pouco estimados, visto que correspondem a uma quantidade significativa que permanece nos resíduos das extrações convencionais de fenólicos e não são recuperados, e comparados aos polifenóis extraíveis possuem características nutricionais e fisiológicas diferenciadas (PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018).

Avaliar a bioacessibilidade dos compostos fenólicos da casca de café é o fator primordial a ser levado em consideração. Choi e Kim (2017) e Nam, Park e Nam (2020) alegam que componentes majoritários associados aos subprodutos de café (ácido clorogênico e ácido cafeico) desempenham importante papel no efeito da atividade antitrombótica *in vitro*, atividade de coagulação, efeito na função plaquetária e viabilidade celular, havendo assim a

possibilidade de aprofundamento em novas pesquisas que incentivem tais efeitos e propriedades biológicas advindas da matriz (IRIONDO-DEHOND et al., 2019).

Existem lacunas científicas sobre a metabolização intestinal bacteriana e o potencial antioxidante de compostos fenólicos provenientes de subprodutos de café. Estudos com modelo celular têm mostrado resultados promissores em relação aos efeitos de compostos fenólicos na barreira intestinal, incluindo a inibição do estresse oxidativo e redução de inflamação, que se aproximam da fisiologia humana. (DAS; RAMANATHAN, 2023; GURUNG et al., 2020)

As células Caco-2 são frequentemente usadas em estudos *in vitro* para simular a absorção de compostos presentes no café no intestino humano. Essas células têm características semelhantes às células do intestino delgado e são capazes de transportar nutrientes e compostos bioativos do intestino para a circulação sistêmica. Estudos têm revelado que extratos de café possuem atividade antioxidante significativa, que aumenta com o tempo de exposição das células Caco-2 a soluções extraídas do grão. Além disso, pesquisas indicam que os ácidos fenólicos presentes nos subprodutos do café podem ajudar na absorção de ferro pelas células Caco-2, o que sugere benefícios na prevenção de deficiências desse composto (DE SÁ et al., 2015; DE PAULA et al., 2019).

A compreensão da metabolização microbiana de compostos fenólicos é um indicativo importante para esclarecer respostas fisiológicas. Estudos recentes têm mostrado que algumas bactérias intestinais como *Lactobacillus acidophilus* e *Bifidobacterium* são capazes de metabolizar compostos presentes no café e seus subprodutos, como a cafeína e o ácido clorogênico. Isso pode resultar em compostos mais simples e biologicamente ativos, capazes de trazer benefícios para a saúde, incluindo uma melhora na regulação da glicemia e um menor risco de desenvolvimento de diabetes tipo 2 (DAS; RAMANATHAN et al., 2023).

Nesse aspecto, o presente estudo objetiva-se em realizar a prospecção de compostos bioativos dos subprodutos oriundos do beneficiamento do café orgânico, do ponto de vista nutricional, biológico e metabólico, visando a agregação de valor e redução da geração de resíduos com potencialidades inexploradas.

2 REVISÃO DA LITERATURA

A revisão da literatura está apresentada sob a forma de artigo de revisão, em atendimento a Norma Complementar nº 03/2011 do PPGCTA. O artigo foi submetido ao periódico Food Bioscience, sob o título “Coffee Husks: A Global Bibliometric Overview of Technological Potential and Bioactivity”.

COFFEE HUSKS: A GLOBAL BIBLIOMETRIC OVERVIEW OF TECHNOLOGICAL POTENTIAL AND BIOACTIVITY

ABSTRACT

Coffee husks have been widely studied in the last years, mainly because they have a great potential of being bioprocessed in several areas, they also stand out as a by-product that can be used as a natural source to obtain bioactive molecules, which can be transformed into new ingredients for application in the food and in the pharmaceutical industries. The objective was to conduct a mixed methodology review (literature review and bibliometric analysis) on composition, bioactive and technological potentialities, and applications of coffee husks, in order to list the main countries, institutions, authors, areas, keywords, and journals that explore the possible use of this by-product, correlating this information to future applications, mainly focusing on the area of Food Science and Technology. The scientific data collection, in recent years, has evidenced coffee husks has a notorious potential in diverse areas, and research has contributed to mitigate environmental impacts through the reuse of agro-industrial by-products, making the production chain more sustainable and economically strengthening this commodity (coffee).

Keywords: agro-industrial by-product, *coffea arabica*, *coffea canephora*, sustainable use, bibliometric indicator.

Introduction

Brazil stands out as the world's leading producer and exporter of coffee, strongly impacting its economy. According to the International Coffee Organization (ICO, 2021a), in the 2020/2021 crop, which comprises the period from April 2020 to March 2021, the country was responsible for 39.4% of the international production and for 78.2% of the production in South America, producing a total of approximately 69 million 60 kg bags, of which 64.3% were destined for domestic consumption (ICO, 2021b).

Coffee bean processing results in a high quantity of by-products. Among the by-products, primarily, there are the skin, pulp, mucilage, and parchment, however, all of them are stuck to the coffee husk. According to Arya et al. (ARYA et al., 2021) in the coffee bean processing, the percentage of husk comprises approximately 50% of the production, that is, the weight of coffee processed is approximately equal to the weight of the husk by-product generated (skin, pulp, mucilage, and parchment). Thus, considering the production destined to domestic consumption, it is estimated that Brazil generated 22,183.5 tons of coffee husks in the 2020/2021 crop, and the production destined to be exported generated 12,316.5 tons.

Coffee husks can be used in various technological innovations adding economic value to the by-product in addition to mitigating environmental impacts. According to Murthy & Naidu (MURTHY; NAIDU, 2012), coffee by-products are rich in nutrients such as carbohydrates, proteins, and dietary fibers, besides being a source of bioactive compounds. During the 1990s and early 2000s, research with coffee husk emphasized its use for citric acid production (SHANKARANAND; LONSANE, 1994), microorganism cultivation (ROUSSOS et al., 1995), and fermentation (Brand et al, 2000). However, due to the coffee husk's potential, mainly the bioactive one, in recent years, studies have evaluated its chemical composition, highlighting bioactive compounds with antioxidant activity resulting from the high content of phenolic compounds (CASTALDO et al., 2020; JANISSEN; HUYNH, 2018b; SANTOS et al., 2021; SILVA et al., 2020).

Considering the high generation of by-products from the coffee processing and, consequently, the great concern with their destination, it is essential to map additional information about the by-product aiming to summarize applications addressed in publications related to the subject. Through statistical analysis, consistent data which can contribute to the advancement of science, and consequently, to economy, can be obtained. Within a particular approach, (RODRÍGUEZ-ROJAS et al., 2019) consider that bibliometrics is a statistical

method used by researchers to evaluate research results, such as the importance and influence of authors, institutions, journals, among others. In this way, it is possible to align scientific backgrounds with trends and feasible applications.

In view of this perspective, this research proposes the evaluation of publication trends involving the coffee husk theme and its properties, specifying main countries, institutions, authors, areas, keywords, and journals that explore the potential use of the main coffee by-product, summarizing the most influential studies within the theme and serving as a basis for future studies, with a greater focus on Food Science and Technology.

Literature review

History and current coffee market

The history of coffee began in Ethiopia, when the effects of its consumption (stimulating and energetic) were observed. However, Arabia was responsible for mastering the initial technique of planting and preparing the product around the year 575, when coffee crossed the Red Sea from Ethiopia to the Arabian Peninsula (Martins, 2017).

According to Hejna (HEJNA, 2021), coffee is the most important food commodity; around 60 tropical and subtropical countries produce coffee extensively and, for some of these countries, this raw material is the main agricultural export product.

The coffee drink is world widely consumed thanks to its pleasant flavor attributes, in addition to its stimulating effect. The largest coffee beverage consumption markets are the United States, Brazil, and Germany, with Brazil being the second largest consumer of the product (AYSELI; KELEBEK; SELLI, 2021).

Its world production in the 2020/2021 crop reached about 175.347 million 60 kg bags, with Brazil accounting for approximately 39.4% of the world's coffee production, 69 million of these bags (ICO, 2021a). According to ABIC (2021), in 2020, 82% of the coffee production sector in Brazil was composed of micro and small companies.

Coffee beans can be produced and processed by both units of agricultural cooperatives and by large industries (DEBASTIANI et al., 2021). According to Consonni et al. (CONSONNI; POLLA; CAGLIANI, 2018a), the conventional coffee cultivation is still the most used in Brazilian crops, in this system chemicals are used, such as fertilizers, both for fertilization and pest management, in order to obtain a high productivity.

However, there is a tendency to replace this form of cultivation with new techniques, such as the use of the organic system. Currently, organic agriculture has stood out for the benefits it offers for food security and sustainability, due to the absence of chemical fertilizers and pesticides (BEDOYA et al., 2017).

There are more than 100 coffee species distributed around the world, however, only two have economic importance, *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta), responsible for 60% and 40% of the global production, respectively, commercially known as Arabica coffee and Robusta coffee (Esquivel & Jimenez, 2012; ICO, 2021a). The coffee bean composition may vary according to the species used, and Arabica coffee is the most appreciated by consumers (BAQUETA et al., 2017; CHENG et al., 2016).

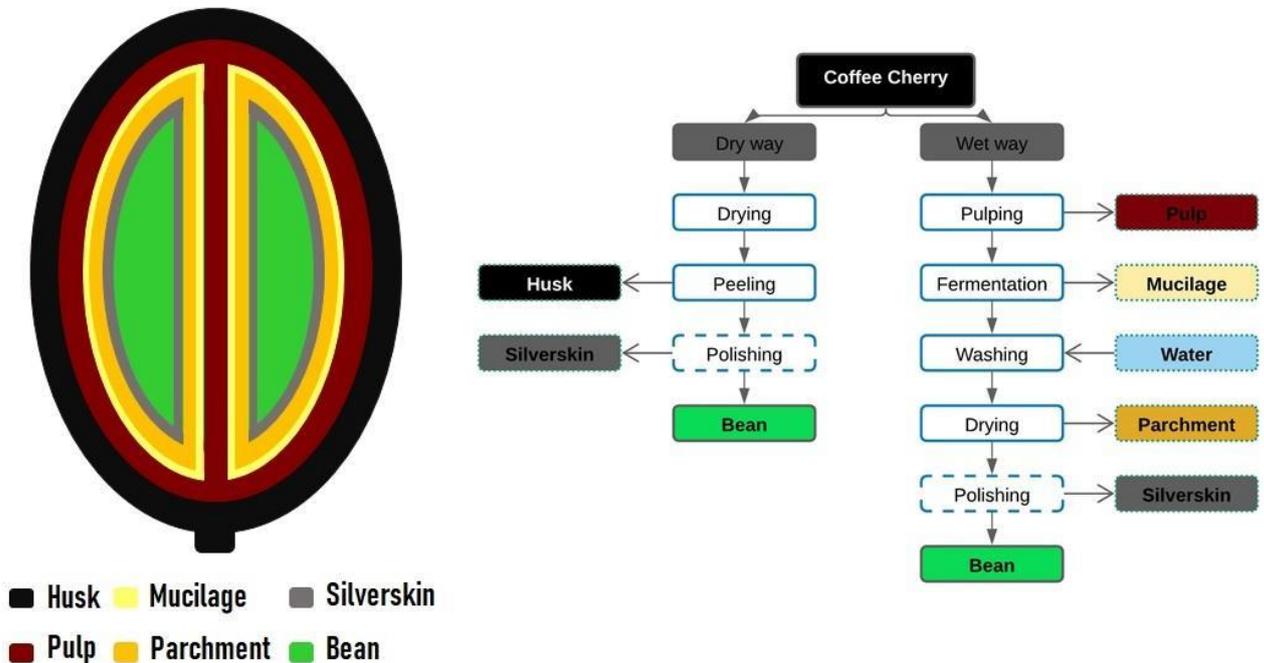
According to the Council of Coffee Exporters of Brazil (CECAFÉ, 2021), in the period from May 2020 to April 2021, the total quantity of exported coffee was almost 45.9 million bags, with a revenue greater than R\$ 31.345 billion. Among the varieties, Arabica coffee had participated in more than 79.92% of exports.

Furthermore, according to CECAFÉ (2021), from January to April 2021 the main destinations of Brazilian coffee were: United States, with over 2.852 million bags of coffee; Germany, with almost 2.68 million; Italy, with more than 1.121 million; Belgium, with almost 1.094 million; Japan, with more than 792,000; Colombia, with more than 431,000; Russia, with almost 416,200; Turkey, with more than 328,000; France, with almost 313,000; and Spain with almost 298,900 bags.

Coffee and processing

The coffee fruit is developed as a 10-15 mm long cherry, containing two seeds, that is, two coffee beans. The grains are covered in layers of pericarp, which is formed by external layers of exocarp (skin), mesocarp (pulp and mucilage), fruit endocarp (parchment), and silver skin (Figure 1) (GEMECHU, 2020).

Figure 1. Coffee fruit. and processing.



Source: Author, 2021.

The coffee fruit presents primary and secondary metabolites that accumulate unevenly in all parts of the fruit (GEMECHU, 2020). Similar to many plant foods, coffee beans are composed of polysaccharides, such as cellulose and hemicellulose, also containing significant amount of soluble carbohydrates, proteins, free amino acids, and minerals (ESQUIVEL; JIMÉNEZ, 2012).

The secondary metabolites, found to the greatest extent in the coffee fruit, are caffeine (abundant in the coffee drink and associated with stimulating effects) and antioxidant polyphenols, more specifically chlorogenic acids, widely disseminated as health promoters (AÇIKALIN; SANLIER, 2021; CLIFFORD et al., 2017; HU et al., 2019). Due to the chemical composition and wide consumption, coffee and its by-products have been the object of study of researchers (CORDOBA et al., 2020).

Several factors can influence the quality of a beverage, including processing, the main activity of the coffee industry, which can be dry or wet (Figure 1); and depending on the method, the by-products generated have different terminologies: pulp or husk (Gemechu, 2020). The dry method, commonly employed for Robusta, is technologically simpler and cheaper compared to the wet method, commonly employed for Arabica. In the dry method, after

harvesting, the coffee cherries are dried until they reach a moisture content of 10-11%, in which the grains are separated, and the husk (skin, pulp, mucilage, and parchment) is removed in a peeling machine (Gemechu, 2020).

In the wet method, after harvesting, the grains are immersed in water to separate the unripe from the ripe (they float and sink, respectively), then the pulps are mechanically removed with a pulping machine (SANTOS et al., 2021) and the grains are fermented for 12-48 hours for the mucilage removal, followed by a wash to completely remove the mucilage remnants. Finally, the grains are dried to remove the parchment. The silver skin remains, and its removal is optional to obtain a premium coffee through a mechanical polishing (Esquível & Jiménez, 2012).

Coffee by-products

Rice husk, sugarcane bagasse, and coffee husk are the main agricultural by-products of agribusiness in Brazil (CAMPOS-VEGA et al., 2015). The by-products generated in the processing of coffee, such as skin, pulp, mucilage, parchment, silver skin, and unripe and defective coffee beans, are a source of essential nutrients, but are also a good source of secondary metabolites, such as bioactive compounds, which are strongly associated with multiple benefits to human health (ESQUIVEL; JIMÉNEZ, 2012; HEEGER et al., 2017).

The nutritional composition of coffee by-products has the following constituents: carbohydrates (44-82%), proteins (4-19%), total dietary fibers (18-91%), lipids (0.7%), and minerals (0.5-10%); it is important to note that the difference in the contents are related to the processing and the type of by-product generated (Gemechu, 2020).

Alike many plant matrices, carbohydrates are found in great abundance, totaling 58-85% (GOUVEA et al., 2009). Carbohydrates are composed mainly of cellulose and hemicellulose and the combined content of these compounds in the by-products of coffee, husk and parchment, is 56 and 74%, respectively (BEKALO; REINHARDT, 2010). Fermentable sugars are found in coffee husk and pulp, and they are widely used as a substrate for the production of fermented and distilled alcohol (WOLDESENBET; WOLDEYES; CHANDRAVANSI, 2016).

The main phytochemicals present in coffee and its by-products are phenolic compounds, mainly chlorogenic acid and its metabolites, and alkaloids, with a focus on caffeine (GALALI; OMAR; SAJADI, 2020). The quantitative determination of these compounds is already widely described in the scientific literature, however, there are differences in the content

of these compounds between each study, and this is due to the geographical origin of coffee, variety, and analytical and extraction methods (CLIFFORD et al., 2017; HU et al., 2019).

Thus, coffee by-products are potential agro-industrial resources that can be reused because they contain relevant nutritional ingredients and phytochemicals of high potential for application in several areas, such as the food and the pharmaceutical industries, in addition to the fact that this reuse is of environmental importance.

Coffee husks

Coffee husks are the main by-product generated during dry coffee processing. The chemical composition of coffee husks may vary according to their variety, cultivation conditions, and processing (BAQUETA et al., 2017; BLINOVÁ et al., 2017). According to Janissen & Huynh (JANISSEN; HUYNH, 2018b), the chemical composition of coffee husks on dry basis presents: proteins (8-11%), carbohydrates (58-85%), cellulose (43%), hemicellulose (7%), lignin (9%), lipids (0.5-3%), minerals (3–7%), fibers (24–30.8%), in addition to the presence of bioactive compounds.

According to Castaldo et al. (CASTALDO et al., 2018) the phenolic compound found in the largest amount in coffee husks is 5-caffeoylquinic acid; there is also significant amounts of trigonelline (1.83 mg/g), caffeine (1.33 mg/g), theophylline (15.04 mg/kg), and theobromine (3.33 mg/kg).

Lately, studies using coffee husks have focused on the production of composting, biofuels, ethanol, and biogas production, and although this material is sought to be used in the production of different value-added compounds, there are studies on their bioprocessing potential (FELIPE; OLIVEIRA; BICAS, 2017; GARCÍA; DEL BIANCHI, 2015; SILVA et al., 2020).

Thus, studies related to the reuse of coffee husk are of great interest and importance and the results of some studies prove the potential of its use (GEMECHU, 2020; HOSEINI et al., 2021a; SANTOS et al., 2021).

Bibliometric analysis

Using coffee husks as the term for the search in the Web of Science database, 363 publications were located from 1994 to 2020 (Table S1 of the supplementary material), which can be seen in Figure 2 (A). Regarding the idiomatic distribution (Table S2 of the

supplementary material), the predominance was the English language with 90.9% of the publications, which corroborates with what is expected, since English is considered the global scientific language and provides a greater content coverage. Then, Portuguese (8%), presenting a significant value in relation to the other two languages, Spanish (0.8%), and French (0.3%).

As can be seen in Figure 2 (A), although the first publication is dated to 1994, only from 2018 was there a significant increase in the number of publications on coffee husks, compared to previous years. This can be explained by the gradual increase in production (28.46%), consumption (23.98%), and worldwide import and export (26.75%) of coffee in relation to the 2007/2008 and 2017/2018 crops (EMBRAPA, 2019), because it implies a greater generation of by-products, with emphasis on the husk. This results in an increased concern and interest related to the destination and/or reuse, both to mitigate environmental impacts and to add economic value.

In addition to the number of annual publications, it is of great importance to know the origin of researchers and groups who have published in the area of the object of study, and therefore, an analysis to understand the countries and institutions that hold the largest number of publications in this field was also performed. Thus, it is noticed that among the 24 countries that research the most on coffee husks (Table 1), Brazil, India, Spain, France, and Colombia stood out in the scientific dissemination of the subject.

Table 1 - Countries and organizations that publish the most

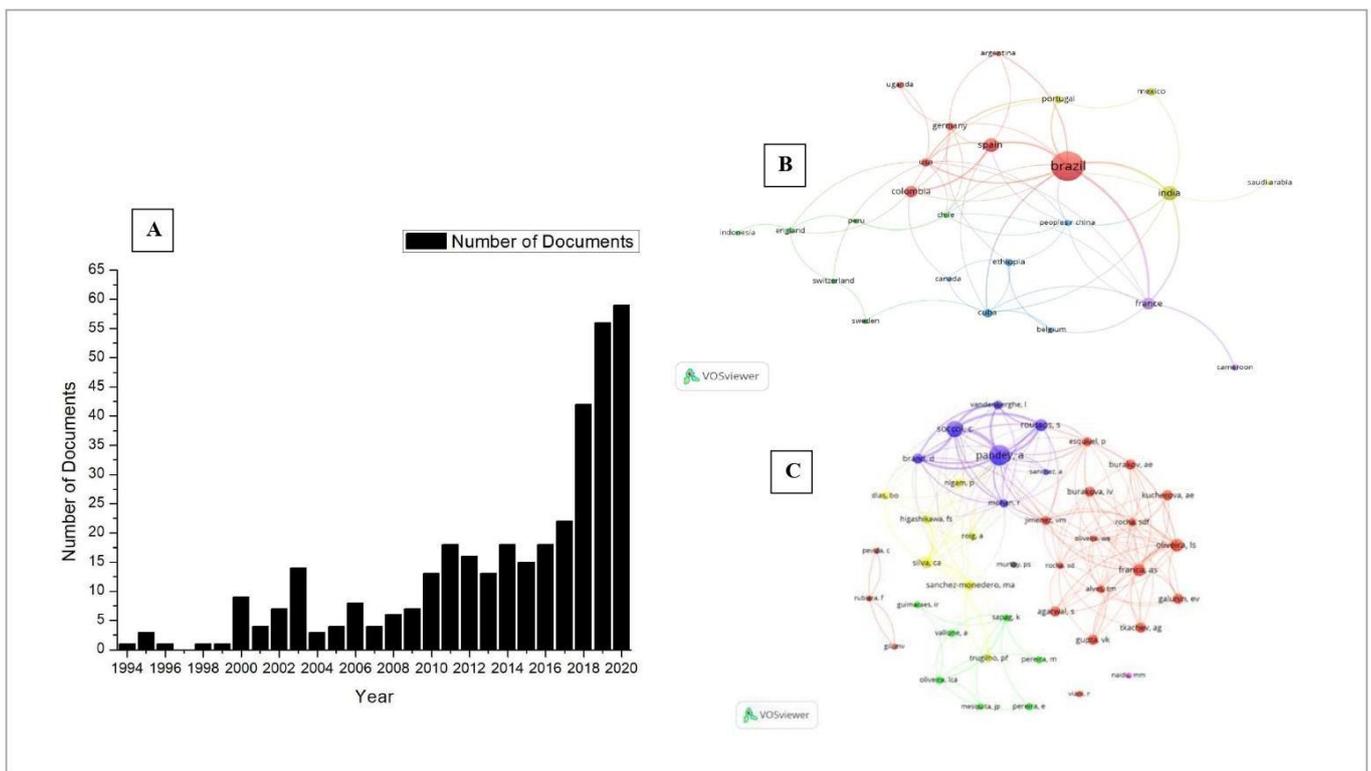
Ranking	Countries	NP	NC	Ranking	Countries	NP	NC
1	Brazil	165	382	13	Uganda	8	104
			3				
2	India	41	175	14	China	8	99
			5				
3	Spain	36	103	15	England	7	48
			7				
4	France	29	945	16	Argentina	6	227
5	Colombia	26	347	17	Sweden	6	129
6	Cuba	16	176	18	Peru	6	113
7	EUA	14	300	19	Saudi Arabia	6	60
8	Mexico	13	244	20	Indonesia	6	1
9	Portugal	12	347	21	Switzerland	5	191

10	Germany	12	262	22	Chile	5	116
11	Etiópia	12	198	23	Belgium	5	109
12	Cameroon	9	80	24	Canada	5	50
Ranking	Organizations			Countries			NP
1	Federal University of Lavras			Brazil			46
2	Federal University of Paraná			Brazil			21
3	Federal University of Minas Gerais			Brazil			19
4	Federal University of Viçosa			Brazil			18
5	Industrial Scientific Research Council			India			17
6	Autonomous University of Barcelona			Spain			15
7	Development Research Institute			France			15
8	University Aix Marseille			France			12
9	Campinas State University			Brazil			12
10	National Institute of Interdisciplinary Science and Technology			India			11
11	Superior Council for Scientific Investigations			Spain			9
12	University of Sao Paulo			Brazil			9
13	Paulista State University			Brazil			9
14	Brazilian Agricultural Research Company – Embrapa Coffee			Brazil			8
15	Makerere University			Uganda			8
16	National University of Colombia			Colombia			8
17	Federal University of Espírito Santo			Brazil			8
18	Dschang University			Cameroon			6
19	University of the East Santiago de Cuba			Cuba			6
20	Polytechnic University of Valencia			Spain			6
21	University of the Andes Colombia			Colombia			6

NP – Number of Publications; NC – Number of Citation

Brazil is a leader in coffee husk research and as previously mentioned, it is the main producer and exporter of raw materials, therefore it is believed that the largest number of publications in the country is directly related to its large production. Among the organizations representing the authors and co-authors, 21 institutions published 6 or more papers on coffee husks, of which 9 are Brazilian. In addition, it is important to highlight that with the exception of Embrapa Café, which is located in the Federal District, the other Brazilian institutions are concentrated in the states that produce and consume coffee the most in the country: Minas Gerais, Espirito Santo, Sao Paulo, and Parana (ICO, 2020a), which makes coffee husk more accessible to researchers and of interest for studies, especially to economic value aggregation.

Figure 2. **A** Registration of publications over the years, with the coffee husk as an object of study; **B** Collaboration among countries according to co-authorship (Network visualization map); **C** Most Influential Authors.



Source: Author, 2021

In relation to the other 12 institutions, 3 are from Spain, 2 are from India, 2 are from France, 2 are from Colombia, 1 is from Cuba, 1 is from Cameroon, and 1 is from Uganda. Another important point in this field of research is that there is an interaction between research groups belonging to different institutions and countries, which can be observed in Figure 2 (B) that

demonstrates a social network map of research collaborations. The size of the node represents the number of publications, and the colors indicate the cluster to which the country belongs.

Brazil (red cluster) was the most dominant country with regard to international collaborations, the country is present in a cluster with 7 other countries (Spain, Colombia, United States, Germany, Uganda, and Argentina) and it is also observed that there is a strong research collaboration between Brazil, Spain, and Colombia, which can be noticed by the thickness of the line that connects them (DOMINGUES et al., 2020; MATOSO et al., 2019, 2020). In this cluster, some of the studies on coffee husks are focused on the area of Food Science and Technology, especially on the extraction of volatile compounds Conesa et al. (CONESA et al., 2016), and on the characterization of cellulose and fibers present in coffee husks (COLLAZO-BIGLIARDI; ORTEGA-TORO; CHIRALT, 2019; COLLAZO-BIGLIARDI; ORTEGA-TORO; CHIRALT BOIX, 2018).

It is also worth mentioning that, despite not belonging to the same cluster of India (yellow), Brazil has a strong collaboration with this country presenting some works in partnership (MACHADO et al., 2002, 2004; MEDEIROS et al., 2006), and these are the countries that publish the most about coffee husks. This cooperation involves institutions such as the Federal University of Parana and the University of Campinas (Brazil), and the CSIR National Institute of Interdisciplinary Science and Technology and the Indian Institute of Toxicological Research (India). It is also noteworthy that Spain (red cluster) conducted research with 6 other countries, with greater cooperation with Brazil and Colombia, as mentioned earlier. Cameroon (violet cluster) has only research with France, while Cuba (blue cluster) collaborated with 8 out of the 24 countries.

Although Brazil is the country with the highest number of publications on coffee husks, other countries have also contributed to the progress of research in this area. Hejna (HEJNA, 2021) reports that coffee is the most important food commodity in the world, about 60 countries produce coffee extensively, and consequently generate a high amount of coffee husks.

Figure 2 (C) depicts the most cited authors in publications related to the search (coffee husks), the size of the circles expresses the intensity of citations.

The authors of the same cluster are linked according to publications carried out in partnership, with greater intensity. In addition, authors of distinct clusters may have conducted research together, but with less intensity; this relationship is indicated by the lines that connect them. With the exception of clusters composed only of one author, all clusters present Brazilian authors, which is explained by the fact that the country is the largest producer in the world and has greater availability of coffee and its by-products, creating interest of authors from other

countries to partnership in research. In addition, there are authors who migrate from institutions or countries when completing an academic background and initiating another, which also favors the growth of publication by authors in different countries, such as Shilpi Agarwal (Agarwal, s) and Vinod Kumar Gupta (Gupta, vk) of the red cluster that researched in India, Saudi Arabia, and South Africa.

In Figure 2 (C) it can be observed that from the blue cluster, the authors Ashok Pandey, (Pandey, a), Carlos Ricardo Soccol (Soccol, c), Sevastianos Roussos (Roussos, s), and Débora Brand (Brand, d) were represented by the largest circles, that is, the most cited authors worldwide with publications that emphasize coffee husk (1615, 1017, 564. and 436, respectively). It can also be seen that the lines connecting them are thicker compared to the other authors of other clusters, which indicates the recurring partnership in research, of which "Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system," "Packed Bed Column Fermenter and Kinetic Modeling for Upgrading the Nutritional Quality of Coffee Husk in Solid-State Fermentation," "Development of a bionematicide with *Paecilomyces lilacinus* to control *Meloidogyne incognita*," "Relationship between coffee husk caffeine degradation and respiration of *Aspergillus* sp. LPBx in solid-state fermentation," and "Biotechnological potential of coffee pulp and coffee husk for bioprocesses" are papers in which all authors participated and these papers are related to the authors' main areas of common research: Engineering, Fermentation, Chemistry, and Biotechnology.

Regarding the countries of the authors from the blue cluster, Sevastianos Roussos (Roussos, s) is from France and Ashok Pandey (Pandey, a) from India, who has worked professionally in several countries such as France, United Kingdom, Switzerland, Malaysia, Thailand, among others (including Brazil, where he works to this day). From Brazil there is Carlos Ricardo Soccol (Soccol, c), Deborah Brand (Brand, d), Luciana Porto de Souza Vandenberghe (Vandenberghe, l), and also Radjiskumar Mohan (Mohan, r) who is born in Suriname, but with academic training and professional activity carried out in Brazil.

In general terms, it is noteworthy that the researchers with longer working time also have a greater number of publications, and consequently accumulate citations due to the exposure of the papers. And in contrast, researchers who are early in their careers have few papers recently published, but may present content of greater interest to science, however they will present fewer citations due to the short publication time. Therefore, it is necessary to normalize these values considering the number of citations received by each publication (Table S3 of the supplementary material).

From the red cluster, though the authors Shilpi Agarwal (Agarwal, s), Alexander E. Burakov (Burakov, ae), Irina V. Burakova (Burakova, iv), Evgeny V. Galunin (Galunin, ev), Vinod Kumar Gupta (Gupta, vk), Anastassia E. Kucherova (Kucherova, ae), and Alexey G. Tkachev (Tkachev, ag) were less cited than the main authors of the blue cluster (indicated by the cluster size in Figure 5), they received 434 citations in a single paper they wrote together in 2018 (Adsorption of heavy metals on conventional and nanostructured materials for wastewater treatment purposes: a review). It is worth mentioning that the most cited author, Ashok Pandey, had 1,615 citations in his 18 works on coffee husks, that is, he had approximately 90 citations per paper.

Therefore, it is believed that if the authors of the red cluster continue to research on coffee husks, they might be the most cited in the future, showing the importance and influence of the research conducted by this group, besides explaining, and emphasizing why normalization is necessary. In addition to Brazil, the countries of the authors from the red cluster are mainly India, Russia, South Africa, Saudi Arabia, and Costa Rica, and they published primarily in the areas of Chemistry, Engineering, Agronomy, and Medicine.

The yellow and green clusters are composed mostly by authors from Brazil who are mainly distributed in the first three institutions that published the most on coffee husks (Federal University of Lavras, Federal University of Parana, and Federal University of Minas Gerais), and researched mainly in the areas of Chemistry, Engineering, Agronomy, and Food Science and Technology.

The most cited papers are highlighted in Table 2; a total of 15 are technical papers and 5 are review papers. These review papers discuss the use of coffee husks as a substrate in solid state fermentation (PANDEY, 2003; SOCCOL; VANDENBERGHE, 2003), information on the adsorption of heavy metal ions using coffee husks as adsorbents (BURAKOV et al., 2018) and information on the functional properties of coffee husks (ESQUIVEL; JIMÉNEZ, 2012), showing that there is a wide scientific application on the subject.

It is interesting to highlight that the most cited papers correspond to the adsorption areas (AHMAD; RAHMAN, 2011; BURAKOV et al., 2018), bio coal/fuel (DIAS et al., 2010; DOMINGUES et al., 2017; GIL et al., 2010; OLIVEIRA et al., 2009, 2008a, 2008b; SAENGER et al., 2001; VÉLEZ et al., 2009), fermentation (BRAND et al., 2000; MARTÍNEZ-AVILA et al., 2019; MURTHY; NAIDU, 2012; PANDEY, 2003; SOCCOL; VANDENBERGHE, 2003), enzymes (BATESTIN; MACEDO, 2007), functional properties (ANDRADE et al., 2012; ESQUIVEL; JIMÉNEZ, 2012; IRIONDO-DEHOND et al., 2019b; PANDEY et al., 2000), and chromatography (PITTET et al., 1996).

The study with the most citations (657) was published in the “Biochemical Engineering Journal” by Pandey (2003), and it reported on the reuse of agro-industrial by-products for use in solid state fermentation process. In addition, other studies are related to the adsorption process through the use of coffee husks and describe the treatment of wastewater, elimination of heavy metals and dyes and the production of hydro coal and activated carbon that aid the process.

The most recent studies with coffee husks are emphasizing the functional properties and application in the food industry. The studies report that coffee husks have classes of bioactive compounds, especially phenolics, which strongly present associations with multiple benefits to the consumer’s health. Examples of these potentialities are antioxidant, anti-inflammatory, antimicrobial, anti-aging, anticancer, anti-cellulitis activities Santos et al. (SANTOS et al., 2021), functional foods generated from coffee by-products Gemechu (GEMECHU, 2020), and prebiotic production (RATNADEWI et al., 2020a).

However, the authors report the need for a better exploration of the potentialities of coffee husks, since the functionalities of the coffee drink have already been elucidated and there is a need to use the large volume of husks that is generated, therefore, it is of great importance to search for alternatives and technologies that allow the use of these by-products (GEMECHU, 2020; SETTER et al., 2020).

With regard to the most relevant publications in the area, it is important to highlight that, as already mentioned, the quantity of years since the paper was published will directly influence the number of its citations, therefore, older papers have an advantage over the most recent ones. With this, a normalization of these results was used to assist in the evaluation of the annual mean of citations received by each document, thus making it possible to break the existing time barrier. In this perspective, Table 3 shows the 20 papers with the highest mean of citation per year.

According to Table 3, it can be observed that Burakov et al. (BURAKOV et al., 2018) presented approximately 145 citations per year, while Pandey (2003) presented 36. Hypothetically, if they continue to receive this same mean of annual citations and do not publish any more research, in 3 years, they will have 868 and 766 total citations, respectively, which in the future leads to Burakov et al. (2018) being the most cited authors.

Results showed that 212 journals published papers related to coffee husk. Table 3 shows the 20 most relevant journals in publication numbers in this area. The results reveal that the 3 most cited journals are "Bioresource Technology", "Science and Agrotechnology", and "Renewable Energy", and they presented 12, 7, and 6 documents, respectively. It is noteworthy

that the main areas of publication are Agricultural Sciences, Food Science and Technology, Agribusiness, Biofuels, Bioprocesses, and Biotechnology.

Regarding the impact factor, 50% of the journals represented in Table 3 presented a value greater than 3.0 (in 2020). Such journals are relevant within their respective areas, since this index corresponds to the level of credibility of their publications, according to the number of citations obtained in papers published in the respective journal.

As can be observed in Table 3, the journal with the most publications has an impact factor of 9.642 and an H-index of 294. Table 2 shows that Bioresource Technology published 4 out of the 20 papers with the highest number of citations (BATTESTIN; MACEDO, 2007; DIAS et al., 2010; GIL et al., 2010; VANDENBERGHE et al., 2000), and after normalization, only the paper published by Dias et al. (2010) presented an annual mean of 21.55 citations (Table 3).

Table 2 - Ranking of the most cited articles represented by the author and journal in which it was published

	Documents	NC	Periodics
1	Pandey (2003)	657	Biochemical Engineering Journal
2	Burakov et al., (2018)	434	Ecotoxicology and Environmental Safety
3	Esquivel e Jiménez (2012)	311	Food Research International
4	Pandey et al., (2000)	250	Biochemical Engineering Journal
5	Dias et al., (2010)	237	Bioresource Technology
6	Oliveira et al., (2008)	230	Journal of Hazardous Materials
7	Oliveira et al., (2009)	181	Journal of Hazardous Materials
8	Oliveira et al., (2008)	167	Journal of Hazardous Materials
9	Murthy e Naidu (2012)	159	Food and Bioprocess Technology
10	Domingues et al., (2017)	146	Plos One
11	Ahmad e Rahman (2011)	143	Chemical Engineering Journal
12	Soccol e Vandenberghe (2003)	141	Biochemical Engineering Journal
13	Gil et al., (2010)	133	Bioresource Technology
14	Pittet et al., (1996)	124	Journal of Agricultural and Food Chemistry
15	Vélez et al., (2009)	119	Fuel

16	Vandenberghe et al., (2000)	110	Bioresource Technology
17	Brand et al., (2000)	103	Enzyme and Microbial Technology
18	Battestin e Macedo (2007)	92	Bioresource Technology
19	Andrade et al., (2012)	87	Talanta
20	Saenger et al., (2001)	84	Renewable Energy

NC – Number of Citations

Analysis of the 20 most cited articles (Table 2) and journals (Table 3) shows that there is no correlation between them. Although the Journal Biochemistry Engineering is responsible for publishing the most cited article, it ranks 9^o among the most cited journals.

Table 3 - Ranking of the most cited articles by normalization and ranking of the most cited journals without normalization

	Documents	Periodics	NC	N Y	MC
1	Burakov et al., (2018)	Ecotoxicology and Environmental Safety	434	3	144,67
2	Pandey (2003)	Biochemical Engineering Journal	657	18	36,50
3	Domingues et al. (2017)	Plos One	146	4	36,50
4	Esquivel e Jiménez (2012)	Food Research International	311	9	34,56
5	Dias et al., (2010)	Bioresource Technology	237	11	21,55
6	Tran et al., (2020)	Science of The Total Environment	21	1	21,00
7	Quispe et al., (2017)	Waste Management	83	4	20,75
8	Callazo-Bigliardi et al., (2018)	Carbohydrate Polymers	62	3	20,67
9	Iriondo-DeHond et al., (2019)	Innovative Food Science & Emerging Technologies	38	2	19,00
10	Setter et al., (2020)	Fuel	19	1	19,00
11	Oliveira et al., (2008)	Journal of Hazardous Materials	230	13	17,69
12	Murthy e Naidu (2012)	Food and Bioprocess Technology	159	9	17,67
13	Murthy et al., (2019)	Microchemical Journal	34	2	17,00
14	Pereira (2009)	Journal of Hazardous Materials	181	12	15,08

15	Girelli; Astolffi; Scuto (2020)	Chemosphere	15	1	15,00
16	Ahmad e Rahman (2011)	Chemical Engineering Journal	143	10	14,30
17	Rodríguez (2018)	Journal of Environmental Chemical Engineering	41	3	13,67
18	García et al. (2019)	Fuel	26	2	13,00
19	Morais et al.,(2019)	Food Chemistry	26	2	13,00
20	Oliveira (2008)	Journal of Hazardous Materials	167	13	12,85
Periodics			ND	Impact factor*	h-Index*
1	Bioresource Technology		12	9.642	294
2	Ciencia e Agrotecnologia		7	1.390	30
3	Renewable Energy		6	8.001	191
4	Journal of Environmental Chemical Engineering		6	5.909	72
5	Revista Brasileira de Ciência do Solo		6	1,2	51
6	Waste and Biomass Valorization		6	3.703	41
7	Química Nova		5	NR	NR
8	Semina-Ciências Agrárias		5	NR	NR
9	Biochemical Engineering Journal		4	3.978	124
10	Process Biochemistry		4	3.757	157
11	Fuel		4	6.609	213
12	LWT-Food Science and Technology		4	4.952	133
13	Food Chemistry		4	7.514	262
14	Brazilian Archives of Biology and Technology		4	0.797	49
15	Brazilian Journal of Microbiology		4	2.476	64
16	Energy & Fuels		4	3.605	186
17	Brazilian Journal of Animal Science		4	0.967	53
18	Cuban Journal of Agricultural Science		4	NR	NR
19	Environmental Science and Pollution Research		4	4.223	113
20	Desalination and Water Treatment		4	1.254	60

NY: Number of years; NC: Number of Citations; ND: Number of Documents; MC: Annual Average Citation; *: in the year 2020; NR: Impact factor does not reported to 2020.

Table 4 - Publications and records areas

Research areas	Records	% of 363
Agriculture	83	22.8
Engineering	80	22.0
Chemistry	59	16.2
Fuel and energy	59	16.2
Biotechnology applied to microbiology	49	13.4
Environmental Science Ecology	48	13.2
Food Science and Technology	43	11.8

Source: Author, 2021

In general, the keywords with the highest occurrences are indicated by the annual mean, as can be seen in the scale of Figure 3. It is understood that the first records of the literature influence the initial trends and the recent words with higher occurrences are possibly highlighted at the end of the scale. In 2008, there were only 8 scientific papers published. The papers with more citations are those from Oliveira et al. (OLIVEIRA et al., 2008a) and W.E. Oliveira et al., (OLIVEIRA et al., 2008b) each paper was cited 232 and 168 times, respectively, and both address the themes of "biosorption" and point out that the most relevant research on coffee husk reports its use as a bio adsorbent in the treatment and removal of contaminants in aqueous effluents.

The term "pyrolysis" is also linked to this same theme according to W.E. Oliveira et al. (2008) in the study of ferric chloride as a new activating agent to obtain activated carbons from agro-industrial by-products (coffee husks). Franca et al. (FRANCA et al., 2008) reported the production of ethanol from coffee husks and later, the use of the by-products generated to obtain adsorbents with various application possibilities. Overall, the influence of chemical, agriculture, biofuels, and biotechnology as precursors in studies on coffee husks is strongly perceived. Currently, the literature continues to present several studies related to these same themes with emphasis on adsorption, fuels, and biotransformation of by-products.

According to Figure 3, the term "solid state fermentation" has great relevance in several periods that include the mean number of publications. As previously stated in Table 5, the records of scientific production are constituted based on a wide range of publications and the greater the interconnection between themes and related areas the greater the representativeness of the study (SOARES et al., 2016).

Currently, research with coffee husks is gaining prominence in the area of Food Science and Technology, indicated by the keywords: enzymes, caffeine, cellulosic fibers, agro-industrial by-products, and antioxidant activity, among others, shown in (Chart 1 of the supplementary material).

The scale trend in Figure 3, regarding the use of these terms, constitutes the mean of the most recent studies, indicating the potential of this area in some reported papers. The by-products of coffee processing present antioxidant activity in addition to nutritional quality and they are potentially rich in dietary fibers, comprising a natural source of bioactive substances for application in the food industry (IRIONDO-DEHOND et al., 2019a). Studies indicate that husks of some vegetables usually have a substance with higher bioactive potential than their own pulp (DE SOUZA et al., 2018; MEYBECK; GITZ, 2017).

In view of the above, the exploration of bioactive compounds from by-products of the coffee processing emerges to encourage the use of these by-products from a nutritional and technological point of view. This use aims to add value and to reduce environmental impacts. This subject has been recently studied and it creates interest in all areas, and this can be proven in Figure 3 by the appearance of the term "life cycle assessment," a technique for evaluating and quantifying possible environmental impacts associated with a product or process.

Bioactive potentialities

Strategies for the use of by-products from coffee processing challenge the food industry to new perspectives and better use of these by-products aiming at strengthening of a sustainable agro-industrial system (Castillo et al., 2013). One consequence of the large production of coffee and advances in the processing industry is the high generation of by-products, as already reported. In addition to the nutritional and antioxidant activity, the by-products are potentially rich in dietary fibers, corresponding to a natural source of bioactive substances for application in the food industry.

Bioactive compounds exert positive effects on human health due to their properties that add effects on the biological properties of the food matrix; these effects may be associated with

the prevention of diseases such as cancer, cardiovascular diseases, among other benefits, directly influencing the organism's physiological response (CARNEIRO; OLIVEIRA; ALVES, 2021; MURADOR et al., 2019). Studies have been conducted to investigate the nutritional value and the potential of bioactive compounds in coffee by-products, demonstrating the presence of phenolic compounds, polyphenols, carotenoids, minerals, vitamins, and antioxidant dietary fibers (Iriondo-DeHond et al., 2019; Gemechu, 2020; Hejna, 2021).

Other studies reveal that coffee by-products are responsible for anti-inflammatory, antioxidant, antimicrobial, anti-aging, and anticholesterolemic effects (Castaldo et al., 2018; Collazo-Bigliardi et al., 2019; Fitria et al., 2020; Santos et al., 2021). In approaches to health-directed effects, Rebollo-Hernanz et al. (REBOLLO-HERNANZ et al., 2019) reported the inhibiting potential of phenolic compounds from coffee husks in adipogenesis, inflammations related to obesity, mitochondrial dysfunction, and insulin resistance.

Choi & Kim (CHOI; KIM, 2017) claimed that chlorogenic acid plays an important role in the effect of in vitro antithrombotic activity, coagulation activity, effect on platelet function and cell viability. It is observed that the same compound is responsible for the inhibition capacity and induced platelet aggregation. Chlorogenic acid, in a specific dose, is able to significantly decrease platelet inflammatory mediators, and it may possibly help protect the cardiovascular system (FUENTES et al., 2014).

In this aspect, research confirms a majority occurrence of chlorogenic acid associated with by-products of coffee husks, creating the possibility of further research that encourages the evaluation of such effects and biological properties from the matrix (Iriondo-DeHond et al., 2019). To meet these effects, bioactive compounds must be bio accessible, that is, available for absorption from the intestine and through blood circulation until they reach the target organ (LIANG et al., 2012).

In general, the concentration of bioactive compounds and antioxidant activity present in coffee husks suggest that this by-product can be used as a raw material for different preparations of functional foods because the results show that these compounds are similar or superior to those found in other fruits and beverages commercially available (Hejna, 2021; Hoseine, 2021).

Technological potentialities

Studies which use coffee husks are of great interest and importance for technological application. Some studies emphasize the extraction of compounds with antioxidant potential

for application in the food industry, for example in the meat industry, helping to reduce lipid and protein oxidation reactions of meat products (Silva et al. 2021).

Some studies also emphasize the use of coffee husk as a source of xylooligosaccharides (XOS), characterizing it as a viable potential substrate for the cultivation of the *Lactobacillus Casei* strain (Ratnadewi et al., 2020).

The area of flavor reveals itself as a very promising field, and when linked to obtaining aromatic compounds from natural sources, it gains great potential with regard to the safety of the developed product (Hoseini et al., 2021), on the other hand, few studies addressed the use of the by-product coffee husks. There is a record of a beverage formulated with coffee husks (cascara) and evaluated for its antioxidant activity and aromatic compounds (PUA et al., 2021). Coffee husks can be a source of essential oils, but this process requires attention in its direct use in food due to the presence of antinutritional factors of caffeine, undesirable products of the Maillard reaction, in addition to tannins and other toxic substances (DELGADO; ARBELAEZ; ROJANO, 2019; HOSEINI et al., 2021a).

Dietary fibers from coffee husks have great technological potential, with good water and oil retention capacities and, when exposed to emulsification-assisted enzymatic treatment, they provide a high yield of soluble dietary fiber (BALLESTEROS; TEIXEIRA; MUSSATTO, 2014; DONG et al., 2020).

Other studies reveal that vegetable by-products are a source of non-extractable polyphenols that are associated with dietary fibers, called macro antioxidants (PÉREZ-JIMÉNEZ; ARRANZ; SAURA-CALIXTO, 2009; PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018). Non-extractable polyphenols associated with fibers represent an emerging and ignored fraction of food antioxidants, and when compared with extractable polyphenols, they present differentiated nutritional and physiological characteristics. The effect of these antioxidant dietary fibers or macro antioxidants, associated with coffee by-products, has been reported in some studies that prove the efficacy of the matrix as an alternative source of such compounds, responsible for maintaining the digestive system and the intestinal microbiota balance (Janissen & Huynh, 2018; Iriondo-DeHond et al. 2019; Dong et al, 2020).

In view of the above, the prospection of compounds from the coffee by-product is necessary to encourage its use from a nutritional and technological point of view, aiming to add value and to reduce waste generation.

Toxicology

The concepts of Food Safety and Food Security are recurrent, although some have disagreement. The first is associated with the practice of preventive measures through the control of physical, chemical, or biological agents that can contaminate food, aiming to not endanger the health or physical integrity of the consumer. Food Security, on the other hand, is related to the implementation of projects to ensure citizens the access to food with nutritional quality and sufficient quantities for a healthy and active life.

In coffee, the excessive presence of certain stable, non-degradable, persistent, and cumulative metal elements can be toxic to consumers and cause harmful acute, or chronic effects on the body. Due to the process of formation, environmental conditions, and technological practices, these elements can be present in the soil, easily absorbed by plants, and accumulated in grains (da Silva, 2015).

However, specifically on coffee husks, the literature is still scarce of studies investigating the toxicology of the by-product. Delfiol et al. (DELFIOL et al., 2012) concluded that coffee husk as animal feed was toxic to horses due to the high levels of caffeine present in the composition, therefore, they observed the following clinical signs: excitability, restlessness, involuntary muscle tremors, chewing movements and constant tremors of the lips and tongue, excessive sweating, and increased breathing and heart rate.

Prospecting and applications

Food products

Coffee husks can be used in the production of flavor and aroma compounds through solid state fermentation (Felipe et al. 2017). In the study conducted by Soares et al. (2000), the authors used coffee husk as a substrate for fermentation of *Candida fimbriata* and found a strong pineapple aroma (supplemented with 20 and 35% of glucose) and a strong banana aroma (supplemented with 46% of glucose).

In addition, coffee husk can be used in the production of enzymes through the use of fermentative processes. Gusmão & Leal (GUSMÃO; LEAL, 2014) evaluated 3 fungal strains of the genus *Aspergillus* spp. in the production of amylase, endoglucanase (CMCase), exoglycosidase (avicelase), and pectinase under solid state fermentation, containing coffee husk as the only carbon source. Results showed that both fungal isolates were able to produce those enzymes and they presented activities of 178.36 nKat.g⁻¹ (amylase), 63.34 nKat.g⁻¹ (CMCase), 36.67 nKat.g⁻¹ (avicelase), and 103.35 nKat.g⁻¹ (pectinase).

Gluglielmetti et al. (GUGLIELMETTI et al., 2019) used a coffee husk extract in the formulation of gluten-free bread. The formulations demonstrated high nutritional and sensory quality for celiacs, in addition to a potential of reducing the risk of gastrointestinal diseases related to oxidative stress, attributed to the content of phenolic compounds present in coffee husks. The product presented antioxidant capacity of 721 mg CGA eq/g, compared with a commercial formulation of bread that is approximately 574 mg CGA eq/g; the formulation containing coffee husk extract offers additional health benefits to consumers.

Silva et al. (2021), studying different methods of extracting phenolic compounds from organic coffee husks, found compounds such as chlorogenic acid (337.07 $\mu\text{g/g}$), gallic acid (20.81 $\mu\text{g/g}$), and caffeic acid (6.15 $\mu\text{g/g}$), in addition to having found levels of total flavonoids (4.53 mg EC/g) and condensed tannins (79.71 mg EC/g). The authors found extracts with high antioxidant activity, through DPPH radical inhibition methods (84.95%) and ferric reducing antioxidant power assay (FRAP) (3136.4 $\mu\text{mol TE/g}$).

The results highlight that coffee husks are efficient as a raw material for obtaining extracts with high antioxidant potential and that these extracts can be used by the food industry in the form of powder, facilitating the insertion in processed products, such as meat products or packaging, acting as a natural antioxidant capable of inhibiting lipid and protein oxidation reactions.

Pharmaceutical products

Some studies have also highlighted the anti-inflammatory, anti-aging, sunscreen, and anticellulite effects of coffee by-products due to the presence of compounds such as chlorogenic acid and its metabolites, caffeine, and flavonoids. These studies may give more support to the use of matrices such as coffee husk, which is presented as a natural source for extraction of the previously mentioned compounds (ANDRADE et al., 2012; CASTALDO et al., 2018; DAS NEVES et al., 2019; GARCÍA; ROSSANA; DEL BIANCHI, 2015; HOSEINI et al., 2021a; SILVA et al., 2020).

As an example of application in cosmetics, Castillo et al. (2013) studied the emulsion of olive oil in water, adding 0.4% of a coffee husk powder extract. The results showed that the formulation had a phenolic compound content of 11 mg Trolox/100 mL, and an antioxidant activity of 118 mg/100 mL of lotion. However, the study did not demonstrate any investigation in cosmetic activity, in addition to the analyzes mentioned.

Although there are still many studies with coffee husks for application in the pharmaceutical industry and considering the presence of bioactive compounds in this residue, coffee husk is a promising option of use (Santos et al., 2021). Therefore, future research in this field is necessary to incorporate the by-product coffee husks in pharmaceutical and cosmetic formulations. The use of this by-product, besides causing a lower environmental impact, generates new sources of income and adds value to the commodity.

Conclusion

Based on the survey of scientific data produced in recent years, the bibliometric review showed that coffee husks have a visible potential in diverse areas. Though the by-product has a great potential for reuse in industry and in food systems, studies related to the area of Food Science and Technology are still scarce. A limiting factor may be associated with a lack of studies addressing the toxicological safety of coffee husks, which possibly emerges as a barrier in the large-scale use of this by-product in the development of new functional foods.

The reuse of by-products is one of the largest investment sectors in developed countries, where research has contributed to the reduction of environmental impacts, making the production chain more sustainable and adding value to the coffee agribusiness scenario, economically influencing the strengthening of this commodity.

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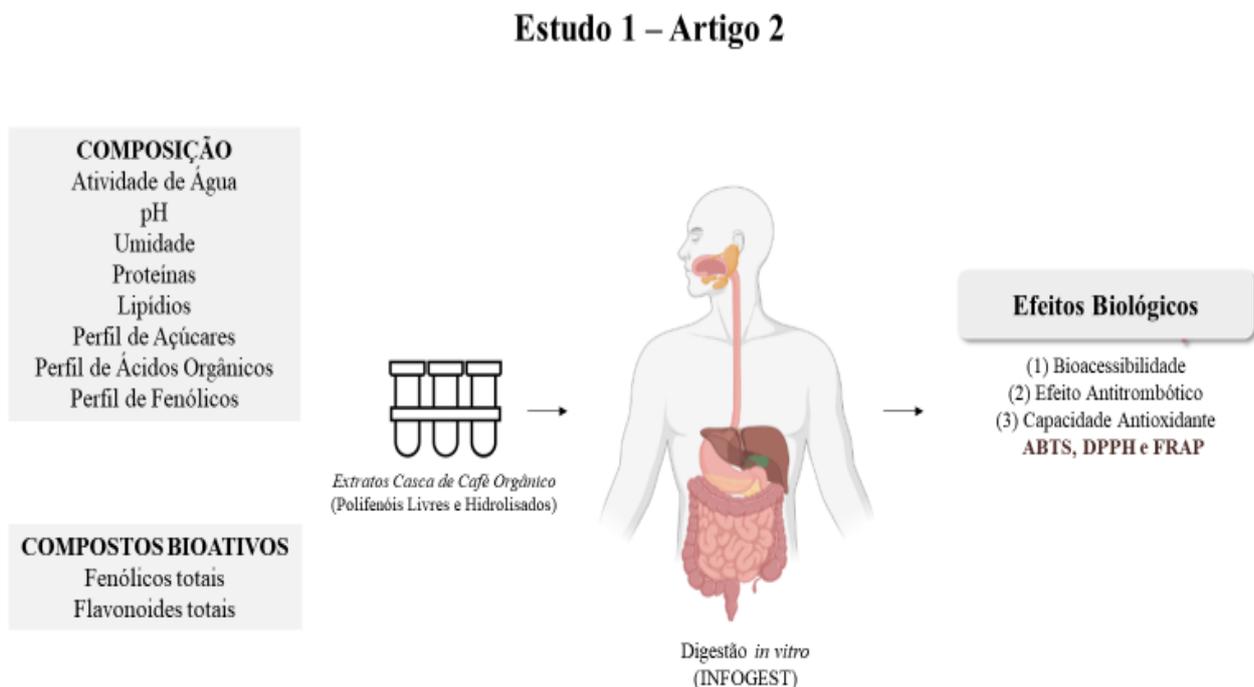
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3 MATERIAL E MÉTODOS

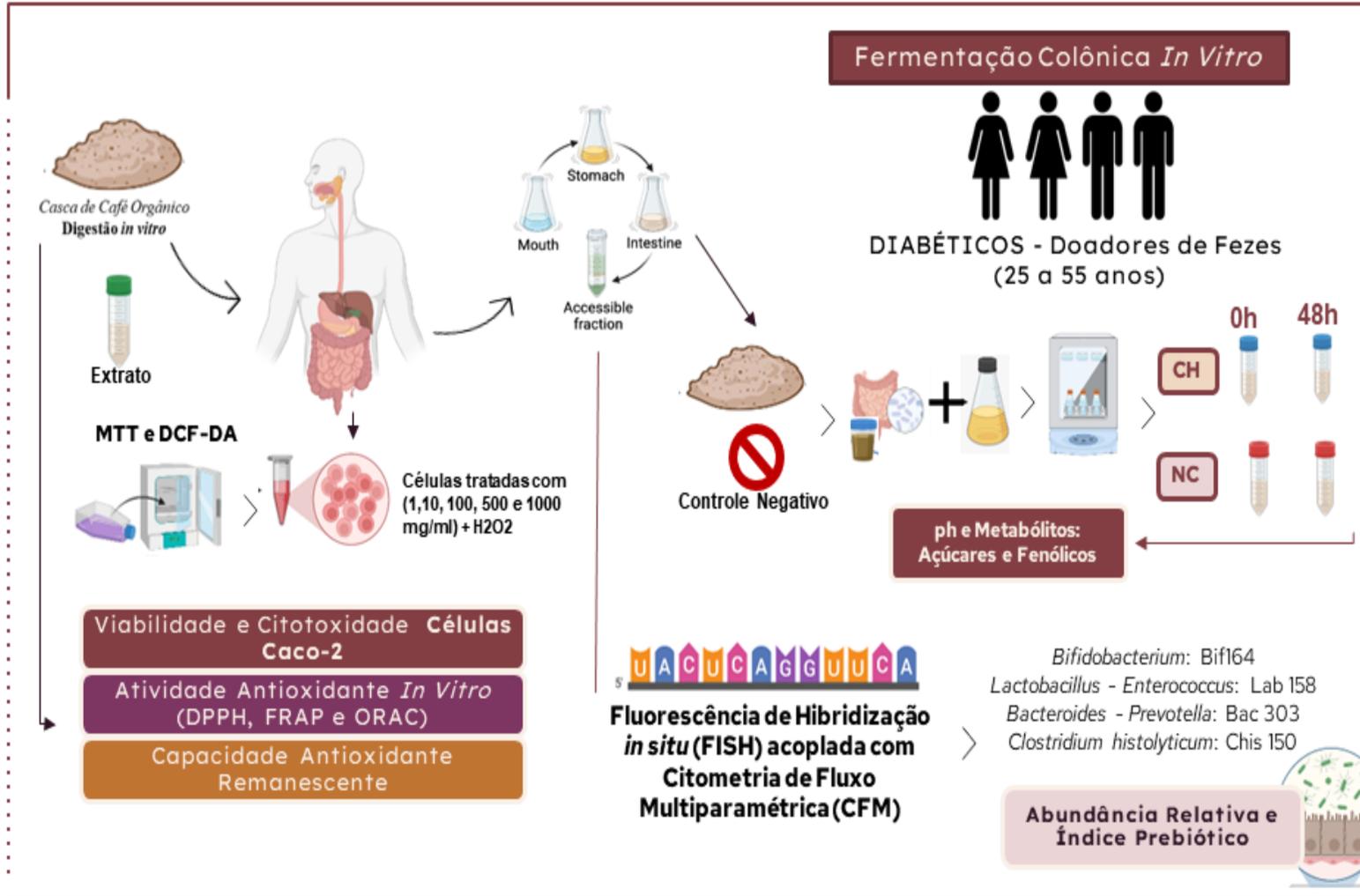
3.1 MATERIAL

As cascas de café orgânico (100% arábica e typica) foram coletadas na cidade de Taquaritinga do Norte, Pernambuco, Brasil (Latitude: -7.88809, 36° 5' 33" Oeste). Os cafés orgânicos são produzidos com a técnica de sombreamento e foram colhidos manualmente. Após o processamento úmido do café, as cascas foram separadas dos grãos e congeladas até a realização do estudo.

Figura 4 - Delineamento Experimental



Estudo 2 – Artigo 3



3.2 PREPARAÇÃO DAS AMOSTRAS^b

Inicialmente foi realizado o processo de secagem das cascas de café, em estufa de circulação forçada a 40 °C por 48 horas. As cascas secas foram trituradas em moinho de facas do tipo *Wiley* à 2mm, até apresentarem aspecto de farelo fino e condicionado em embalagens vedadas hermeticamente, sob proteção da luz e em temperatura de refrigeração, até a execução das análises.



Figura 5 - Casca de café *in natura* e desidratada Fonte: Acervo Autor.

Fonte: Autor (2022)

3.3 CARACTERIZAÇÃO FÍSICA E QUÍMICA DA FARINHA DA CASCA DO CAFÉ ORGÂNICO

- **Rendimento**

Foi realizada a determinação do rendimento da farinha da casca de café orgânico das farinhas obtidas, o resultado foi calculado em porcentagem a partir da relação do peso da amostra seca e da amostra *in natura*, como demonstrado na Equação 1.

$$\text{Rendimento (\%)} = \frac{\text{Peso amostra seca}}{\text{Peso da amostra in natura}} \times 100$$

Os parâmetros de pH e cor instrumental foram avaliados de acordo com os métodos a seguir:

- **pH**

O pH foi determinado com auxílio de um equipamento medidor de pH, previamente calibrado. Onde 10g da amostra foram diluídas em 100 mL de água e em seguida realizada a leitura.

- **Cor**

A análise de cor das amostras foi feita, utilizando o espectrofotômetro MiniScan HunterLab XE Plus, no sistema de cor Cielab. Determinando-se os parâmetros de L*: luminosidade; a*: transição da cor verde (-a*) para o vermelho (+a*) e b*: transição da cor azul (-b*) para a cor amarela (+b*).

As análises físico-químicas de umidade e proteínas foram realizadas, em triplicata, seguindo a metodologia descrita pela *Analytical Chemistry - AOAC* (2010).

- **Proteínas**

A determinação de protídeos foi realizada de acordo com o método de digestão *Kjeldahl*, fundamentada no conteúdo de nitrogênio das amostras. Adotou-se o fator de correção utilizado para farinha e derivados de trigo 5,70 bonde os resultados foram expressos em percentual, de acordo com a equação a seguir:

$$Proteínas\ totais\ (g/100g) = \frac{(VA-VB) \times fa \times F \times 0,14}{Peso\ da\ amostra} \quad (2)$$

Onde: VA – volume de HCl 0,1M gasto na titulação da amostra; VB – volume de HCl 0,1M gasto na titulação do branco; fa – fator de correção da solução de ácido clorídrico; F – fator de correspondência de nitrogênio – proteína.

- **Umidade**

Método padrão de dessecação em estufa a 105°C, seguindo metodologia descrita no manual de normas analíticas da AOAC (2010).

- **Lipídeos**

A análise de lipídeos das amostras foi realizada seguindo os procedimentos de Folch, Lees, Stanley (1957).

3.3.1 Perfil de açúcares

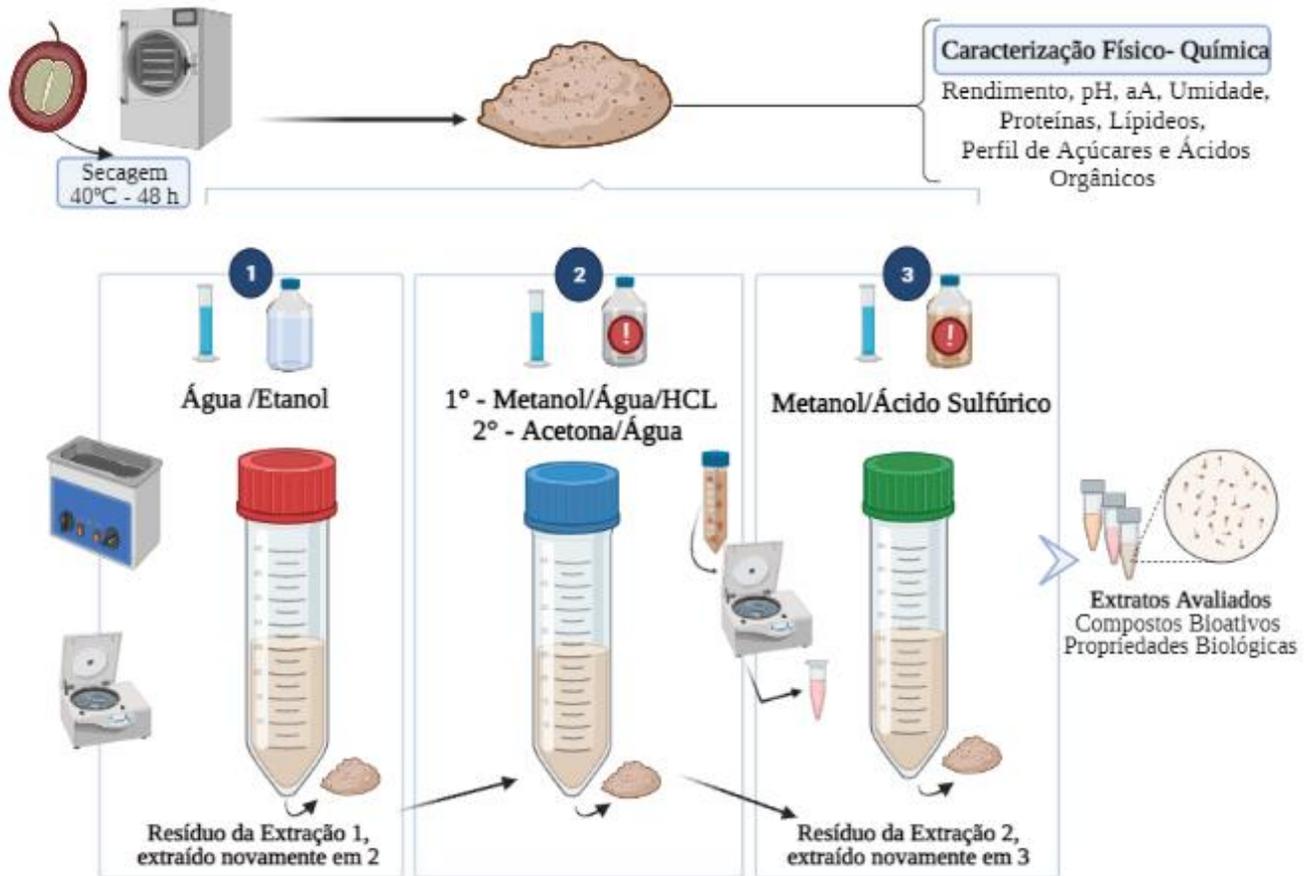
O perfil de açúcares foi determinado de acordo com a metodologia descrita por Zeppa, Conterno e Gerbi (2001). Para tanto, foram usados 2g de amostra, seguido de diluição em 10 mL de água ultrapura, centrifugação ($6000 \times g$, $4^\circ C$) por 10 minutos, e filtração em filtro de celulose de $0,45 \mu m$. A quantificação do perfil de açúcares foi realizada usando um sistema de Cromatografia Líquida de Alta Eficiência, da Agilent, modelo 1260 Infinity LC (VARIAN, Waters, Califórnia, USA), acoplado com uma coluna Agilent Hi Plex Ca ($7,7 \times 300 \text{ mm}$, 8μ), a uma temperatura de $85^\circ C$ e detector de índice de refração (VARIAN), amostrador manual com alça de $20 \mu L$. A vazão aplicada foi de $0,6 \text{ mL/min}$, com tempo de execução de 30 minutos. A fase móvel utilizada foi a água ultrapura. Soluções padrão foram injetadas para obter o tempo de retenção de cada composto. Os resultados são expressos em g de açúcares /100 g.

3.3.2 Perfil de ácidos orgânicos

O perfil de ácidos orgânicos foi determinado de acordo com a mesma metodologia descrita por Zeppa, Conterno e Gerbi (2001) em perfil de açúcares. A fase móvel utilizada foi a água ultrapura levemente acidificada. Soluções padrão foram injetadas para obter o tempo de retenção de cada ácido. Os resultados são expressos em g do ácido/ 100g.

3.4 PREPARO DOS EXTRATOS DA CASCA DE CAFÉ ORGÂNICO

Figura 6 – Procedimentos de obtenção de extrato



Fonte: Autor (2022)

3.4.1 Extrato de Polifenóis Extraíveis (PE)

A farinha das cascas de café orgânico foi pesada diretamente em tubo falcon, homogeneizada manualmente por 5 minutos com a solução extratora contendo água e etanol (1:1). A proporção da farinha e solução extratora foi de 1:10 (p:v). Posteriormente a mistura resultante foi submetida a incubação em banho-maria a 60°C/60 min. Por fim, centrifugada a 3.500 x g por 20 min a 10 °C em centrífuga universal refrigerada e recolhido o sobrenadante e filtrado em papel de filtro, seguindo a metodologia proposta por Silva et al. (2020). O extrato obtido foi rotaevaporado utilizando temperatura máxima de 60°C por 30 minutos. Posteriormente, completou com água ultra-pura, atingindo o volume inicial do extrato.

3.4.2 Extrato de Polifenóis Não Extraíveis Hidrolisáveis (PNE-H)

Inicialmente foi pesado 0,5g da farinha em 20 mL de metanol/água/HCL (50:50, v/v) que será levado a uma extração em banho de ultrassom (UNIQUE, modelo USC-1800, Brasil) a 40 KHz durante 30 min a 25°C. A amostra foi centrifugada a 1372 G por 15 min usando centrifuga, modelo SL-701 (Solab, São Paulo, Brasil). O resíduo retido na primeira fase do extrato (sobrenadante), foi novamente extraído com 20 mL de acetona/água (70:30, v/v), passando novamente pelos mesmos procedimentos de ultrassom e centrifugação. Os extratos metanólicos e acetônicos foram combinados em um balão volumétrico de 25 mL e o volume foi completado com água deionizada (RUFINO et al., 2010). O extrato foi concentrado utilizando um evaporador rotativo (Fisatom 802, São Paulo, Brasil) até o volume final de 5 mL. Foi realizado a filtração de uma alíquota, para a obtenção de polifenóis não extraíveis.

3.4.3 Extrato Polifenóis Não Extraíveis (PNE)

O sobrenadante da extração do Polifenóis Extraíveis foi hidrolisado com metanol e ácido sulfúrico (80:20 v/v) por 20h a 85°C, posteriormente o extrato foi centrifugado nas mesmas condições de (PE), o pH foi ajustado com NaOH para 5,5 e finalizando com a filtração o extrato com filtro qualitativo. Após a filtração o extrato foi reconstituído 2 vezes, primeiramente com metanol (5mL) e metanol a 50% (5mL), posteriormente a primeira lavagem 1 mL da amostra foi diluído novamente com metanol (1mL) e metanol a 80% (1mL). Por fim o conteúdo foi combinado e rotaevaporado nas mesmas condições de (PE) (HARTZFELD et al., 2002).

3.5 ENSAIO DA DIGESTÃO GASTROINTESTINAL *in vitro*

O estudo de liberação de compostos bioativos presentes nas partículas utilizando fluídos gástrico e intestinal simulados, foi realizado de acordo com adaptações da metodologia utilizada por Minekus et al. (2014). Uma alíquota de cada extrato: PE- etanol, PNE-H e PNE, foi preparada para determinação do efeito das condições do trato gastrointestinal simulado. De acordo com o consenso internacional relatado pela INFOGEST, foram preparadas soluções estoques de eletrólitos e enzimas que compreendem as fases bucal, gástrica e intestinal. Todos

os ensaios foram realizados em tubos Falcon estéreis de 50 mL, mantidos em um Shaker termostático agitado a 120 rpm a 37°C, para simulação das fases da digestão.

Tabela 5- Preparo de soluções estoque utilizadas na digestão *in-vitro*

Sais	Solução estoque (Concentração)		Solução salivar (pH 7,0)	Solução gástrica (pH 3,0)	Solução intestinal (pH 7,0)
	Solução estoque para preparar 500 mL				
	g.L ⁻¹	mol.L ⁻¹	Vol (mL)	Vol (mL)	Vol (mL)
KCl	37,3	0,5	15,1	6,9	6,8
KH₂PO₄	68	0,5	3,7	0,9	0,8
NaHCO₃	84	1,0	6,8	12,5	42,5
NaCl	117	2,0	-	11,8	9,6
MgCl₂(H₂O)₆	30,5	0,15	0,5	0,4	1,1
(NH₄)₂CO₃	48	0,5	0,06	0,5	-
HCl	-	6,0	0,09	1,3	0,7

Na fase oral, 5 g de amostra foram misturadas a 4 mL de fluido salivar (Tabela 5), 25 µL de CaCl₂ 0,3 M, 0,5 mL de solução aquosa de amilase 75 U.mL⁻¹ e 0,475 mL de água grau Milli-Q, mantendo-se a mistura incubada por 2 min. Após este período, iniciou-se a etapa gástrica da digestão. Para tal, foram adicionados 8 mL de solução gástrica, 1 mL de solução aquosa de pepsina 2000 U.mL⁻¹ e 0,5 µL de CaCl₂ 0,3 M. Após a mistura das soluções, o pH foi corrigido para 3,0 com auxílio de HCl 1M e o volume final (20 mL) foi alcançado pela adição de água grau Milli-Q. As amostras foram, então, incubadas por 2 h. Ao fim da fase gástrica, a ação enzimática foi interrompida em banho de gelo por 10 minutos. As amostras foram centrifugadas a 4°C e 5000 g por 15 minutos, sendo os sobrenadantes separados e

armazenados sob congelamento até a realização das análises. A bioacessibilidade foi expressa em porcentagem e determinada de acordo com a Equação 4 (DE ASSIS et al., 2021).

$$\text{Bioacessibilidade (\%)} = (\text{CF digerido} / \text{CF não digerido}) \times 100 \quad (\text{Eq. 4})$$

Onde: CF digerido corresponde à concentração do composto analisado das cascas de café digerida (fase intestinal) e CF não digerido a concentração do composto analisado das cascas de café não digeridas (extrato inicial).

3.6 ANÁLISE DOS COMPOSTOS BIOATIVOS DOS EXTRATOS DA CASCA DE CAFÉ ORGÂNICO E FLUIDOS DA DIGESTÃO GASTROINTESTINAL *IN VITRO*

3.6.1 Determinação de Compostos Fenólicos Totais

O teor fenólico total (TPC) dos extratos foi determinado pelo método de Folin-Ciocalteu proposto por Andrade et al. (2012) com adaptações. Onde homogeneizou-se alíquotas de 100 μL do padrão com 7.900 μL de água destilada, 500 μL do reagente Folin Ciocalteu (2 M) e 1.500 μL de carbonato de sódio à 20%. Em seguida, os frascos foram agitados e mantidos em repouso, no escuro, por 2 horas, seguidos de leitura em espectrofotômetro UV-VIS (Quimis, Q798U, São Paulo, Brasil), a 765 nm. Os resultados foram calculados com o auxílio de uma curva padrão de ácido clorogênico (10 à 50 $\mu\text{g}\cdot\text{mL}^{-1}$) e o conteúdo de compostos fenólicos totais foi expresso em miligramas de ácido clorogênico equivalente (CAE) por 100 gramas de amostra (mg CAE/100g).

3.6.2 Determinação de Flavonoides Totais

O teor de flavonoides totais foi mensurado de acordo com a metodologia proposta por Zhishen, Mengcheng e Jianming (1999), com algumas modificações. Uma alíquota de 100 μL do extrato foi homogeneizado com 150 μL de NaNO_2 5% (m:v), aguardando reagir por 5 minutos. Em seguida, foi adicionado 150 μL de AlCl_3 10% (m:v), aguardando reagir por 6 minutos. Por fim, foi adicionado 1000 μL de NaOH 1M e 1200 μL de água destilada. A absorbância da mistura foi comparada a um branco (solvente) e medido a 510 nm. Uma curva padrão foi construída com catequina (20 - 200 $\mu\text{g}/\text{mL}$) e o conteúdo de flavonoides totais foi expresso como miligrama de catequina equivalente por grama de amostra (mg CE/ g-1).

3.6.3 Perfil de Compostos Fenólicos

O perfil dos compostos fenólicos dos extratos da casca de café orgânico foi determinado através de cromatografia líquida de alta eficiência em fase reversa (CLAE), usando uma coluna C18 Shim-pack (com diâmetro interno de 4,6 mm e comprimento de 250 mm). Uma alíquota de 20 μL de cada amostra foi injetada na coluna termostatizada a 40°C, utilizando a fase móvel composta de acetonitrila/ácido fórmico 0,1% (15:85, v/v) escoando com vazão de 0,8 mL/min. A determinação quantitativa foi baseada no método de padrão externo por comparação com o tempo de retenção de padrões de compostos

fenólicos puros. Para todas as amostras, a concentração final dos compostos será determinada pela média dos resultados de duas injeções consecutivas (ANDRADE et al., 2012).

3.7 AVALIAÇÃO DAS BIOATIVIDADES DOS EXTRATOS DAS CASCAS DE CAFÉ ORGÂNICO E FLUÍDOS DA DIGESTÃO GASTROINTESTINAL *IN VITRO*

3.7.1 Atividade antioxidante pela capacidade de sequestrar o radical 1,1-difenil-2-picrilhidrazil – DPPH

Inicialmente, os extratos e fluídos digeridos foram adicionados para reação com o radical estável DPPH (1,1-difenil-2-picrilhidrazil) em uma solução de metanol. A redução do radical do DPPH foi medida através da leitura da absorbância a 515 nm em 30 min de reação. Uma curva padrão com soluções de Trolox (6-hidroxi-2,5,7,8-tetrametilcromo-2-ácido carboxílico) foi estabelecida e os resultados foram expressos em atividade antioxidante equivalente de trolox por grama de amostra (BRAND-WILLIAMS; CUVELIER; BERSET, 1995).

3.7.2 Atividade antioxidante através da redução do ferro – FRAP (*Ferric Reducing Antioxidant Power*)

Foi utilizada a metodologia descrita por (BENZIE; STRAIN, 1999), onde para o preparo do reagente FRAP foi misturado 25 mL de tampão acetato 0,3 mol L⁻¹ (pH 3,6), 2,5 mL de uma solução de TPTZ 10 mM (preparada com HCl 40 mM) e 2,5 mL de uma solução aquosa de cloreto férrico 20 mM. As amostras foram diluídas em água destilada, seguindo diluições adequadas para a leitura no espectrofotômetro. Uma alíquota de 90 µL do extrato diluído foi transferida para tubos de ensaio, juntamente com 270 µL de água destilada, 2,7 mL do reagente FRAP recém preparado. Esta mistura foi homogeneizada e mantida em banho-maria a 37°C. Após 30 min, realiza-se a leitura a 595 nm em espectrofotômetro. Para a quantificação da capacidade antioxidante foi construída uma curva padrão de Trolox. A capacidade antioxidante foi expressa em µmol Trolox equivalente/g de extrato seco.

3.7.3 Atividade Sequestradora do Radical 2,2- azino-bis(3-etilbezotiazolina)-6-ácido sulfônico (ABTS^{•+})

A capacidade sequestradora do radical $ABTS^{\bullet+}$ foi determinada seguindo a metodologia proposta por Re et al. (1999). A solução de $ABTS^{\bullet+}$ foi preparada usando 5 mL de uma solução de 2,2'-Azinobis-[3-ethylbenzothiazoline-6-suphonic acid)-diammonium salt] – (ABTS) 7,0 mM e 88 μ L de uma solução de persulfato de potássio ($K_2S_2O_8$) 140 mM. O sistema foi mantido em repouso de 12 a 16 horas a temperatura ambiente e na ausência de luz. Uma vez formado o radical $ABTS^{\bullet+}$, o mesmo foi diluído em etanol para o ajuste da absorbância de $0,70 \pm 0,02$ a 734 nm. Em seguida, uma alíquota de 30 μ L da amostra foi adicionada à 3000 μ L do radical $ABTS^{\bullet+}$. Após 6 minutos de incubação à temperatura ambiente e no escuro, a absorbância foi medida a 734 nm contra um branco (etanol). Em seguida, a atividade de eliminação do radical de acordo com a capacidade antioxidante dos extratos foi verificada a 734 nm em espectrofotômetro UV-VIS (Quimis, Q798U, São Paulo, Brasil). Os resultados foram expressos em porcentagem de inibição do radical $ABTS^{\bullet+}$ em relação ao potencial antioxidante das amostras.

3.7.4 Ensaio da capacidade de absorção dos radicais oxigenados Orac (Oxygen Radical Absorbance Capacity).

A capacidade antioxidante da casca de café e fluídos da digestão foi determinada utilizando-se o método capacidade de absorção do radical oxigênio (ORAC), baseado no método descrito por Chisté et al. (2011). Os extratos (20 μ L) e a solução de fluoresceína (120 μ L) foram pipetados 32 em microplaca de 96 poços. A mistura foi pré-incubada durante 15 min a 37 °C. Solução de AAPH (2,2'-azobis (2amidino-propano) dicloridrato) (60 μ L) foi adicionada e a reação ocorreu num volume final de 200 μ L. A microplaca foi lida num leitor de microplacas BMG Fluostar Omega (BMG Labtech, ALE) e a fluorescência registrada a cada 2 minutos durante 100 minutos, com agitação automática antes de cada leitura. Oito concentrações de solução de Trolox (8-840 μ M) foram usadas para a construção da curva padrão e um controle positivo feito com a solução extratora. Todas as misturas de reação foram preparadas em triplicata, com três ensaios independentes para cada amostra. Os resultados foram com base na área sob a curva (AUC) para o tempo de declínio da fluorescência e os resultados expressos em mol equivalente de Trolox (ET) por unidade de massa de amostra (g).

3.7.5 Ensaio de coagulação

Determinação do tempo de tromboplastina parcial ativada (TTPa) e tempo de protrombina (PT)

As duas determinações foram realizadas de acordo com metodologia descrita por Silva et al. (2020), utilizando um coagulômetro semi-automático BF II (Dade Behring). O plasma, para utilização nos ensaios, foi obtido a partir de sangue coletado de três voluntários saudáveis, logo após suspensos em citrato trissódico 3,8 % (p/v) (1/10) e centrifugado 1726 x g por 15 minutos (25°C).

Para o TTPa foi usado 50 µL das substâncias de estudo (concentração ótica (DO) de 0.025 em 595nm); 50 µL de plasma e 50 µL do reagente para TTPa (Dade actin activated cephaloplastin Dade proveniente da Behring, Marburg, Alemanha) com incubação por 120 segundos e após foi adicionado 50 µL de cloreto de cálcio 0,025 M.

Para a determinação de PT, 50 µL de plasma foram incubados com 50 µL de concentrações crescentes dos extratos e fases digeridas (0,075-0,4 mg), NaCl 0,15 M ou heparina ($5 \cdot 10^{-3}$ UFH) para 60 seg seguido pela adição de 100 µL de liofilizado tromboplastina cerebral de coelho. Todas as atividades anticoagulantes foram definidas com base na concentração necessária para prolongar plasma humano coagulando até duas vezes o tempo de controle. Os ensaios foram realizados em duplicata e os resultados expressos em minutos.

3.8 ENSAIOS DE CITOTOXIDADE/CITOPROTEÇÃO

3.8.1 Cultura de Células CACO-2

As células foram semeadas em frascos de 75 cm² e cultivadas em DMEM suplementado com 10% de soro fetal bovino, 1% de penicilina/estreptomicina, 8,4 mM de HEPES, 1% de piruvato de sódio, 1% de aminoácidos não essenciais e 1% de L-glutamina. O meio foi substituído a cada 2-3 dias. Células em crescimento exponencial foram destacadas dos frascos de cultura usando 0,05% de tripsina/ácido etilenodiaminotetraacético (EDTA) e semeadas em placas de 96 poços com 2x10⁴ células por poço. Após atingir a confluência, o meio de cultivo foi removido e as células foram pré-tratadas com controle ou diferentes concentrações de CF (1, 10, 100, 500 e 1000 mg/ml) durante 3 h. Em seguida, as células foram estimuladas com controle ou peróxido de hidrogênio na concentração de 1mM por mais 2 horas. As células do grupo controle foram tratadas com meio livre de FBS. Ensaio de viabilidade celular e avaliação da produção de ROS foi avaliado.

3.8.2 Avaliação da viabilidade celular com ensaio de redução de MTT

O método é baseado na captação celular de brometo de 3-(4,5-dimetiltiazol-2il)-2,5-difenil tetrazolina ou MTT (M5655, Sigma-Aldrich St. Louis, MO, EUA) que é endocitado e mitocondrial desidrogenases de células viáveis clivam o anel de tetrazólio, produzindo cristais roxos de formazan que são insolúveis em soluções aquosas. As células foram incubadas com 50 µg/MTT durante os últimos 30 minutos de tratamento a 37 °C. Em seguida, o meio foi removido e os cristais de MTT foram dissolvidos em DMSO. Os valores de absorvância foram medidos em 560 e 650 nm. A redução do MTT foi calculada como $(\text{abs a } 560 \text{ nm}) - (\text{abs a } 650 \text{ nm})$ e expressa como uma porcentagem do valor basal.

3.8.3 Avaliação da produção de ROS com ensaio DCF-DA

DCFDA é um corante fluorogênico que mede a atividade das espécies reativas de oxigênio (ROS) dentro da célula. O ensaio DCFDA é baseado na difusão de DCFDA na célula, desacetilação por esterases celulares e oxidação por ROS em 2', 7' - diclorofluoresceína (DCF). Nos últimos 30 min de pré-tratamento, as células foram incubadas com 20 µM de DCF-DA. Após, o meio de pré-tratamento foi removido e as células foram lavadas com PBS para remover o DCF-DA não internalizado. As células foram tratadas com meio (grupo controle), ou concentração de CF ou estímulo mais concentração de CF e a fluorescência foi medida imediatamente durante 120 min. A AUC foi calculada e a diferença entre a AUC da amostra e a AUC do controle foi usada como base seletiva do tempo de co-incubação de DCFH-DA e tratamento puro com células Caco-2.

3.9 INÓCULO FECAL HUMANO E SISTEMA DE FERMENTAÇÃO COLÔNICA

O procedimento foi realizado conforme a metodologia adaptada descrita por Medeiros et al. (2021). Amostras fecais de 4 voluntários adultos diabéticos (dois homens e duas mulheres, com idade entre 25 e 55 anos) foram utilizadas após aprovação do Comitê de Ética em Pesquisa com Seres Humanos (Universidade Federal da Paraíba, João Pessoa, Paraíba, Brasil). Os critérios de inclusão compreendem a pessoas que não sofressem de nenhuma doença gastrointestinal ou colônica, seguindo uma dieta onívora regular sem usar alimentos probióticos, probióticos concentrados ou prebióticos nas últimas 48h e sem antibióticos durante os seis meses anteriores ao estudo.

Os doadores receberam instruções específicas além de um kit de coleta adequado. O kit de coleta de fezes incluiu tubos coletores esterilizados, espátulas

descartáveis, instruções para coleta/envio da amostra, luvas descartáveis e álcool 70%. Além disso, as instruções contidas no kit descreveram as etapas de higienização das mãos e manuseio asséptico das amostras. As fezes foram coletadas em tubos estéreis. As amostras foram dispostas em uma jarra com um sistema gerador de anaerobiose (AnaeroGen, Oxoid, Basingstoke, Inglaterra) e transportados para o laboratório. Os experimentos começaram imediatamente após a chegada das fezes para garantir uma microbiota fecal fresca, e as fezes serão manipuladas em ambiente sob anaerobiose. Para os experimentos, a mesma quantidade de fezes de cada doador foi reunida e misturada com solução salina tamponada com fosfato estéril (PBS; 0,1 M; pH 7,4; 1:10, p/v) sob agitação (200 rpm) por 2 min.

Cada lote foi composto por 40% do meio de fermentação (v/v), 40% de inóculo fecal humano (v/v) e 20% do farelo de casca de café digerida (p/v). A fermentação será realizada a 37°C por 0 e 48h em condições anaeróbicas (AnaeroGen). O lote sem adição de farelo de casca de café corresponde ao controle negativo. Os sais e reagentes utilizados no meio de fermentação foram adquiridos da Sigma-Aldrich (St. Louis, EUA).

3.10 DETERMINAÇÃO DE pH E CONTEÚDOS DE COMPOSTOS FENÓLICOS E AÇÚCARES DURANTE A FERMENTAÇÃO COLÔNICA

3.10.1 pH

Os valores de pH (método 981.12) foram determinados com um potenciômetro digital (Quimis, Diadema, SP, Brasil) (AOAC, 2016).

3.10.2 Perfil de Compostos Fenólicos

Os teores de compostos fenólicos foram determinados em CH liofilizado antes e após a exposição à digestão gastrointestinal simulada (fração não dialisada). Extratos metanólicos de JB expostos e não expostos à digestão gastrointestinal simulada foram preparados para determinar os teores de compostos fenólicos usando procedimentos previamente descritos (Massa et al., 2020).

A separação e quantificação dos compostos fenólicos (ácido caftárico, ácido gálico, ácido siríngico, cianidina 3-glicosídeo, delphinidina 3-glicosídeo, catequina, galato de epigalocatequina, epicatequina, galato de epicatequina, procianidina A2, procianidina B1, procianidina B2, hesperidina, kaempferol 3-glicosídeo, quercetina 3-glicosídeo,

rutina e cis-resveratrol) foram realizados com cromatografia líquida de alta performance (HPLC) usando um cromatógrafo Agilent (modelo 1260 Infinity LC, Agilent Technologies, St. Clara, CA, EUA) e análises condições descritas anteriormente (Massa et al., 2020). Uma pré-coluna Zorbax C18 (12,6 × 4,6 mm, 5 µm; Agilent Technologies) e uma coluna Zorbax Eclipse Plus RP-C18 (100 × 4,6 mm, 5 µm; Agilent Technologies) foram usadas para a determinação de compostos fenólicos. Os dados foram processados com o software OpenLAB CDS ChemStation Edition™ (Agilent Technologies). Os picos dos compostos fenólicos foram identificados por comparação de seus tempos de retenção com os de padrões externos (Sigma-Aldrich). A quantificação dos compostos fenólicos foi feita com curvas de calibração dos padrões externos (R^2 de $\geq 0,998$) (Menezes et al., 2021).

3.10.3 Perfil de Açúcares

O perfil de açúcar foi determinado de acordo com a metodologia descrita por Zeppa et al. (2001). Para tanto, foram utilizados 2 g da amostra, diluída em 10 mL de água ultrapura, centrifugada por 10 minutos (6000×g, 4°C) e filtrada em filtro de celulose de 0,45 µm. O perfil de açúcares foi quantificado usando um sistema de cromatografia líquida de alta performance Agilent, modelo 1260 Infinity LC (VARIAN, Waters, Califórnia, EUA), acoplado a uma coluna Agilent Hi Plex Ca (7,7 × 300 mm, 8 µ), a uma temperatura de 85°C, com um detector de índice de refração (VARIAN) e um amostrador de loop manual de 20 µL. Os resultados foram expressos em g de açúcar/100g.

3.11 FLUORESCÊNCIA DE HIBRIDIZAÇÃO *IN SITU* (FISH) ACOPLADA COM CITOMETRIA DE FLUXO MULTIPARAMÉTRICA (CFM)

As modificações promovidas pelo farelo de casca de café orgânico digerida nos grupos da microbiota fecal durante a fermentação foram avaliadas por FISH acoplado a CFM. As análises de FISH foram realizadas com cinco sondas marcadas com Cy3 fluorescentes (oligonucleotídeos) capazes de hibridizar a região específica do gene 16S rRNA de grupos bacterianos selecionados, como segue: *Bifidobacterium*: Bif164 (BIF, estritamente anaeróbios), *Lactobacillus - Enterococcus*: Lab 158 (LAB, anaeróbios facultativos), *Bacteroides - Prevotella*: Bac 303 (BAC, estritamente anaeróbios) e *Clostridium histolyticum*: Chis 150 (CHIS, estritamente anaeróbios) (Sigma-Aldrich, St. Louis, EUA). A população bacteriana total foi quantificada usando SYBR Green (Invitrogen, Carlsbad, CA, EUA) (CONTERNO et al., 2019). Os grupos bacterianos

foram escolhidos porque representam membros da microbiota fecal que, uma vez modulados, são associados a respostas metabólicas positivas e negativas. Essa mensuração foi realizada através de um citômetro de fluxo BD Accuri C6 (New Jersey, EUA) usando excitação de 488 nm de um laser de estado sólido azul. Os sinais de células individuais através da zona de laser foram coletados sinais logarítmicos. Os sinais de fluorescência serão registrados nos canais FL1 (SYBR Green) e FL2 (Lab 158, Bif 164, Bac 303, Chis 150) e serão expressos como citogramas pelo BD Accuri C6 Software (CONTERNO et. al, 2019).

3.12 ANÁLISE ESTATÍSTICA

Todo o ensaio experimental foi realizado em triplicata, onde as diferenças entre o conteúdo dos compostos fenólicos e atividade antioxidante dos extratos das cascas de café orgânicos foram avaliados utilizando a análise de variância (ANOVA) e média (Tukey, $p \leq 0.05$). Todas as análises estatísticas foram realizadas com *Minitab*® 19 *Statistical*. O teste de correlação de Pearson avaliou a relação entre os valores dos compostos fenólicos. O software de cálculo *GraphPad Prism* 6.0 (GraphPad Software, La Jolla, CA, EUA) foi usado para as análises relacionadas aos dados da Citometria de Fluxo.

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4 RESULTADOS E DISCUSSÃO

Os resultados obtidos nesta pesquisa estão apresentados no formato de artigo de acordo com as normas estabelecidas pelo Programa de Pós-graduação em Ciência e Tecnologia de Alimento da UFPB (Norma complementar nº 03/2011).

Artigo 2: Unveiling the Bioactivities and Bioaccessibility of Phenolic Compounds from Organic Coffee Husks Using an In Vitro Digestion Model

Artigo 3: Organic arabica coffee husk has antioxidant and cell protective effects and induce beneficial changes in intestinal microbiota of diabetic adults during in vitro colonic fermentation

Artigo 2

Unveiling the Bioactivities and Bioaccessibility of Phenolic Compounds from Organic Coffee Husks Using an *In Vitro* Digestion Model

Thaianaly Leite Abreu^a, Mario Estévez^{b*}, Leila Moreira de Carvalho^a, Lorena Lucena de Medeiros^a, Valquíria Cardoso da Silva Pereira^a, Bruno Ramos Salu^c, Maria Luiza Vilela Oliva^c, Marta Suely Madruga^a, Taliana Kênia Alencar Bezerra^a

^a Post-Graduate Program in Food Science and Technology, Department of Food Engineering, Technology Centre, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil.

^bIPROCAR Research Institute, TECAL Research Group, Universidad de Extremadura, 10003, Cáceres, Spain. E-mail: mariovet@unex.es*

^cDepartment of Biochemistry, Federal University of São Paulo, São Paulo, SP, Brazil

E-mails: thaianaly.abreu@gmail.com, leilamdc@yahoo.com.br, lorenalucena@live.com, valquiriacsf@gmail.com, bruno_salu@hotmail.com, mlvoliva@unifesp.br, mismadruga@uol.com.br, taliana.kenia@hotmail.com

ABSTRACT

Background

The large amounts of by-products generated in the coffee industry are a problem for a sustainable agro-industry. Studies related to the biological potential of organic coffee husks are still limited. The aim of this work was to investigate the occurrence of phenolic compounds in organic coffee husks and to evaluate their potential as a source of bioactive dietary components.

Results

To achieve this objective, three extracts were prepared, namely extractable polyphenols (EP), hydrolyzable non-extractable polyphenols (HNEP) and non-extractable polyphenols (NEP). These extracts were characterized and evaluated for their bioactive properties after simulated gastrointestinal digestion. The results show that the extraction process affects the occurrence of phenols from coffee peels, especially for caffeic acid, gallic acid, and chlorogenic acid. The condensed phenols and polyphenols found in the extracts and digests not only showed antioxidant properties against ABTS, FRAP and DPPH radicals, but also had remarkable bioavailability and anticoagulant potential.

Conclusion

These results highlight the potential health benefits of phytochemicals from coffee husks and open new perspectives for the use of such compounds in dietary supplements.

Keywords: Polyphenols; Bioaccessibility; Antioxidant; Anticoagulant; Simulated Digestion; Circular economy.

1. INTRODUCTION

Coffee is the most consumed energy drink in the world, and Brazil stands out as the world's leading producer and exporter. Coffee has a strong impact on the country's economy ICO (ICO (INTERNATIONAL COFFEE ORGANIZATION), 2021b) and coffee bean processing leaves many by-products, approximately 50% of the total output.

The production of organic coffee aims to be environmentally sustainable by reducing the use of pesticides and fertilizers and optimizing production processes. One way to further minimize the environmental impact of organic coffee production is to reuse the husks as raw material in a circular economy system (CASTALDO *et al.*, 2018). There is growing interest in using waste materials from the agricultural processing industry in the food industry due to their potential content of "natural antioxidants" (ESTÉVEZ, 2021).

On the other hand, the bioactive compounds must be bioaccessible so that they can display the desired health effects in internal target organs (MCDADE *et al.*, 2021). Knowledge concerning phenolic intake, together with bioaccessibility/bioavailability throughout the gastrointestinal tract is fundamental to assess their biological significance for human health (SOARES *et al.*, 2021).

These byproducts have antioxidant properties, they also have anticoagulant and antihypertensive effects, and other benefits due to the presence of bioactive compounds. They are found in plants widely and can be extracted using organic solvents (6).

Non-extractable polyphenols are a type of polyphenol found in plant by-products that cannot be extracted using traditional methods. They are often associated with higher molecular weight compounds such as fibers, proteins, and melanoidins. Non-extractable polyphenols can be divided into three categories: hydrolyzable polyphenols, low molecular weight phenols, and non-extractable proanthocyanidins (GEMECHU, 2020;

PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018). These polyphenols have been shown to have antioxidant, anticoagulant, cardioprotective, antithrombotic, and anti-inflammatory effects, and may be useful as dietary supplements. Further research is needed to explore the potential health benefits of these compounds and to find ways to extract and utilize them (5,6).

The objective of this work was to investigate the phytochemical composition, *in vitro* bioaccessibility, and antioxidant and anticoagulant activities of polyphenolic extracts found in organic coffee processing by-products.

2. MATERIAL AND METHODS

2.1 MATERIAL

Organic coffee husks (100% Arabica and normal) were collected in the city of Taquaritinga do Norte, Pernambuco, Brazil (-7.88809 S, 36° 5' 33" W).

2.2 SAMPLE PREPARATION

The coffee husks were dried in a forced circulation oven at 40°C for 48 hours to obtain the phenolic compounds. The conditions used in this process are similar to those described in a previous study described by Silva et al. (SILVA *et al.*, 2020). The dried husks were then ground to a fine bran-like ground to 2 mm in a Wiley-type knife. They were then stored in antihhermetically sealed packages, protected from light, and refrigerated to -6°C until analysis was performed.

2.3 PHYSICAL AND CHEMICAL CHARACTERIZATION OF ORGANIC COFFEE FLOUR IN BULK

Physical and chemical characterization of organic coffee husk meal (in bulk) was performed. Lightness (L^*), red/green intensity (a^*), and yellow/blue intensity (b^*) were measured using a Konica Minolta colorimeter (model CR -400, Osaka, Japan), under specified conditions. The chemical analysis of the organic coffee husk meal (moisture, ash, proteins, lipids and minerals) was performed according to the methodology described by AOAC (10).

2.3.1 Sugar profile

The sugar profile was determined according to the methodology described by Zeppa et al. (ZEPPA; CONTERNO; GERBI, 2001). For this purpose, 2g of the sample was used, diluted in 10 mL of ultrapure water, centrifuged for 10 minutes ($6000\times g$, $4^\circ C$), and filtered through a $0.45\ \mu m$ cellulose filter. The sugar profile was quantified using an Agilent high-performance liquid chromatography system, model 1260 Infinity LC (VARIAN, Waters, California, USA), coupled to an Agilent Hi Plex Ca column ($7.7 \times 300\ mm$, $8\ \mu$), at a temperature of $85^\circ C$, with a refractive index detector (VARIAN) and a manual $20\ \mu L$ loop sampler. The results were expressed in g sugar/100g.

2.3.2 Organic acid profile

The profile of organic acids was determined according to the methodology described by Zeppa et al. (ZEPPA; CONTERNO; GERBI, 2001) using HPLC UV-VIS. Two g of the sample was used, diluted in 10 ml of ultrapure water, centrifuged for 10 min ($6000\times g$, at $4^\circ C$), and filtered through a $0.45\ \mu m$ cellulose filter. Quantification of the

organic acid profile was performed using methods similar to those described for the determination of the sugar profile. The results are expressed in g of acid/100g.

2.4 PREPARATION OF THE ORGANIC COFFEE HUSK EXTRACTS

2.4.1 Extractable Polyphenols (EP)

According to the method proposed by Silva et al. (SILVA *et al.*, 2020), the organic coffee husk flour was weighed directly into a falcon tube and homogenized manually for 5 min with an extraction solution of water and ethanol (1:1). The ratio of flour to extraction solution was 1:10 (p:v). The resulting mixture was incubated in a water bath at 60°C for 60 minutes and then centrifuged at 3,500×g for 20 minutes at 10°C. The supernatant was then collected and filtered through filter paper. The obtained extract was rota-evaporated at a temperature of 60°C for 5 consecutive hours (Fisatom 802, São Paulo, Brazil). Then, the extract was made up with ultrapure water to complete the original extract volume.

2.4.2 Hydrolysable Non-Extractable Polyphenols (H-NEP)

First, 0.5g of the flour was weighed in 20 mL of methanol/water/ HCL (50:50, v/v) and placed in an ultrasonic extraction bath (UNIQUE, model USC-1800, Brazil) at 40 KHz for 30 min at 25°C. The sample was centrifuged at 1372 x g for 15 min, centrifuge model SL -701 (Solab, São Paulo, Brazil). The residue (supernatant) remaining in the first phase of the extract was again extracted with 20 mL of acetone/water (70:30, v/v), again going through the same sonication and centrifugation procedures. The methanolic and acetone extracts were combined in a 25 mL volumetric flask and the volume was made up with deionized water (RUFINO *et al.*, 2010). The extract was concentrated to a final

volume of 5 mL using a rotary evaporator (Fisatom 802, São Paulo, Brazil). An aliquot was filtered to obtain non-extractable polyphenols.

2.4.3 Non-Extractable Polyphenols (NEP)

The supernatant of extractable polyphenols was hydrolyzed with methanol and sulfuric acid (80:20 v/v) for 20 hours at 85°C. The extract was then centrifuged under the same conditions as EP, the pH was adjusted to 5.5 with NaOH, and the extract was filtered with a quality filter. After filtration, the extract was reconstituted twice, first with methanol (5mL) and 50% methanol (50:50 v/v) (5mL), and after the first wash, 1 mL of the sample was diluted again with methanol (1mL) and 80% methanol (1mL). Finally, the contents were combined and roto-evaporated under the same conditions as for EP (13).

2.5 *IN VITRO* GASTROINTESTINAL DIGESTION ASSAY

The study of released bioactive compounds in the particles with simulated gastric and intestinal fluids was performed following the method used by Minekus et al. (MINEKUS *et al.*, 2014). Aliquots of each extract were prepared: EP, H- NEP, and NEP, to determine the effects of the simulated gastrointestinal conditions. Stock solutions of electrolytes and enzymes comprising the oral, gastric, and intestinal phases were prepared in accordance with international consensus as reported by INFOGEST. Bioaccessibility was expressed as a percentage and determined according to Equation 1 (15).

$$\text{Bioaccessibility (\%)} = (CF \text{ digested} / CF \text{ undigested}) \times 100 \quad (\text{Eq. 1})$$

Where: *CF digested* is the concentration of the analyzed compound from digested coffee husks (intestinal phase), and *CF undigested* is the concentration of analyzed compound from undigested coffee husks (initial extract).

2.6 *IN VITRO* ANALYSIS OF BIOACTIVE COMPOUNDS FROM ORGANIC COFFEE HUSK EXTRACT IN GASTROINTESTINAL DIGESTION FLUIDS

2.6.1 Determination of Total Phenolic Compounds

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method. Aliquots of the standard were mixed with distilled water, Folin-Ciocalteu reagent, and sodium carbonate, and the mixture was allowed to rest in the dark for 2 hours before being read in a UV-VIS spectrophotometer at 765 nm. Results were calculated using a standard curve for chlorogenic acid (10 to 50 $\mu\text{g} \cdot \text{mL}^{-1}$), and the content of total phenolic compounds was expressed in milligrams of chlorogenic acid equivalents (CAE) per 100 grams of sample (mg CAE/100g) (9).

2.6.2 Determination of Total Flavonoids

To measure the total flavonoid content of a sample, an aliquot of the extract is mixed with sodium nitrite and aluminum chloride to produce a colored complex. The absorbance of this complex is then measured at 510 nm using a UV-VIS spectrophotometer (Quimis, Q798U, São Paulo, Brazil), and the total flavonoid content is expressed in milligrams of catechin equivalent per gram of sample (mg EC /100g). A standard curve with catechin is used to determine the concentration of flavonoids in the sample (9).

2.6.3 Phenolic Compounds Profile

The profile of phenolic compounds in organic coffee husk extracts was determined using reversed-phase high-performance liquid chromatography (HPLC UV-VIS) and a C18 Shim-pack column (with an inner diameter of 4.6 mm and a length of 250 mm). A 20 μ L aliquot of each sample was injected into the thermostated column at 40°C, using a mobile phase composed of acetonitrile/0.1% formic acid (15:85, v/v) flowing at a rate of 0.8 mL/ min. For all samples, the final concentration of compounds was determined by the average of the results of two consecutive injections (16).

2.7 ASSESSMENT OF ORGANIC COFFEE HUSK EXTRACT DIGESTS BIOACTIVITY

2.7.1 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH•) Sequestering Activity

The ability of coffee husk extracts to scavenge the DPPH radical was determined using a method described by Brand-Williams et al. (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). The radical scavenging activity and antioxidant capacity of the coffee husk extracts were measured in triplicate using a UV-VIS spectrophotometer (Quimis, São Paulo, Brazil) 515 nm. The results were expressed in terms of equivalent Trolox antioxidant activity per gram of sample.

2.7.2 2,2-Azino-bis (3-Ethylbeothiazoline)-6-Sulphonic Acid Radical (ABTS•+) Sequestering Activity

The ability to sequester the ABTS•+ radical was determined according to the method proposed by Re et al. (18). The radical elimination activity as the antioxidant capacity of the coffee husk extracts was verified at 734 nm in a UV-VIS

spectrophotometer (Quimis, São Paulo, Brazil). Results were expressed as equivalent trolox antioxidant activity per gram of sample.

2.7.3 Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing capacity was evaluated using the ferric reducing antioxidant power (FRAP) method described by Benzie et al. (BENZIE; STRAIN, 1999). The antioxidant potential, or ability of the coffee husk extracts to reduce iron (Fe^{3+}) to the ferrous form (Fe^{2+}) was verified at 593 nm in a UV-VIS spectrophotometer (Quimis, São Paulo, Brazil). Based on the calibration curve prepared with different concentrations of Trolox (50-1000 μM), the results were expressed as equivalent of μmol of Trolox/g of sample.

2.7.4 Coagulation test: Determination of activated partial thromboplastin time (APTT) and Prothrombine time (PT)

Both determinations were performed in a dual-channel semiautomated coagulometer designed for low-volume laboratories, BF II Coagulometer (Dade Behring), according to the procedure described by Salu et al (20). Plasma for the assays was obtained from whole blood of three healthy volunteers, collected in 3.8% (w/v) trisodium citrate (1/10), and centrifuged at $1726 \times g$ for 15 minutes (25°C). Assays were performed in duplicate, and results were expressed in minutes.

2.8 STATISTICAL ANALYSIS

The entire experimental trial was performed in triplicate, where the differences between the phenolic compounds content and antioxidant activity of organic coffee husk

extracts were evaluated using analysis of variance (ANOVA) and mean (Tukey, $p \leq 0.05$). All statistical analyses were performed with *Statistica* Software 5.1.

3. RESULTS AND DISCUSSION

3.1 PHYSICAL AND CHEMICAL CHARACTERIZATION OF ORGANIC COFFEE HUSK FLOUR

In summary, coffee husks are a byproduct of coffee processing that can be dried and ground into flour (SILVA *et al.*, 2020). According to the results, a high yield of 20% is desirable for both economic and environmental reasons, as it can reduce waste and make better use of the raw material (8).

Coffee husks are naturally acidic and the pH can vary depending on factors such as soil, climate and processing conditions. The Codex Alimentarius (FAO, 2019) recommends a moisture content of 15.5% for flours. There are no specific guidelines for the moisture content of vegetable by-product flours, but the moisture content of (13.81% o, which is related to the low pH of 3.97) found in coffee husk flour is considered favorable to microbiological stability and safety.

The lightness (L^*) determined in this study (45.37) expresses a tendency towards a darker color, but approximately in the middle range of the scale. The variable a^* places the color on the green-red axis and the variable b^* on the blue-yellow axis. For the red intensity, the determined value of 5.13 shows the predominance of the red/brown color, characteristic of coffee samples. In total, b^* (9.03) was characterized as a pronounced yellowish tendency.

The chemical composition of coffee (beans and by-products) depends on the origin, variety, development, and harvest. In addition, due to organic farming, different chemical compounds may also be present (CONSONNI; POLLA; CAGLIANI, 2018).

As can be seen in Table 1, the results of the present study are consistent with data from the literature, as dry coffee husks are composed of approximately 80% carbohydrates, 8-11% proteins, and 0.5-2% lipids (GEMECHU, 2020; MOREIRA *et al.*, 2018).

Although the chemical composition of coffee processing by-products varies, the values found in the present study for proteins (9.41%) and lipids (0.50%) are similar to those reported by the above authors. Coffee husks contain chemical compounds with high potential for use in various biotechnological processes, but they also have environmental problems due to the toxicity of caffeine and tannins. These issues can be reduced by reusing coffee husks (IRIONDO-DEHOND *et al.*, 2019).

The Brazilian agribusiness industry is required to certify its management systems through organizations like NBR-ISO14001 in order to demonstrate to society and the market that it is reducing negative impacts on biodiversity (ABNT, 2015). Organic farming in the coffee industry offers various benefits, including environmental and nutritional advantages and improved food safety. It also contributes to the control and regulation of coffee production and can be internationally certified (CONSONNI; POLLA; CAGLIANI, 2018).

Coffee husks are a lignocellulosic byproduct that contains a high amount of dietary fiber and polyphenols, as well as polysaccharides that are attached to the cell wall. They have the potential to be converted into food ingredients and can be used as substrates for fermentation by microorganisms and bioconversion reactions due to their high organic content, low cost, and availability (BELMIRO *et al.*, 2021; PÉREZ-JIMÉNEZ; SAURACALIXTO, 2018).

The major sugars found in coffee husks were glucose, xylose, fructose, and arabinose. These sugars can be used by microorganisms in bioprocesses to produce bioethanol, xylitol, and arabitol, and they also have prebiotic potential that can be

exploited from coffee processing by-products (RATNADEWI *et al.*, 2020a). The concentrations of xylose and arabinose in the present coffee husks were 3.40 mg/mL and 6.44 mg/mL, respectively.

Shankar *et al.* (SHANKAR *et al.*, 2019) reports that bioconversion of shell by-products is a complex process that requires the use of pretreatments to break down polysaccharides and produce fermentable sugars. In the study on saccharification of pretreated corn, peanut, and coffee hulls with lignocellulolytic enzymes to produce bioethanol. However, it should be noted that the pretreatments of the husks for saccharification reported by these authors were optimized by the use of enzymes. Considering the data found in the present study and in the literature, we see the great biotechnological potential of coffee residues.

[Table 1 here]

In addition to carbohydrates, we detected nine organic acids (citric acid, tartaric acid, ascorbic acid, malic acid, pyruvic acid, succinic acid, lactic acid, formic acid, and propionic acid) in coffee husks. These results are shown in Table 2. Malic acid, succinic acid, and ascorbic acid were each found at higher concentrations ($5.05 > 3.46 > 2.09$ mg/100g). Bressani *et al.* (BRESSANI *et al.*, 2021) reported values for fermented coffee beans with concentrations of citric acid (2.78 to 4.65 mg.g⁻¹), succinic acid (0.58 and 2.40 mg.g⁻¹), and tartaric acid (0.02-0.93 mg.g⁻¹). Succinic acid had a higher concentration in the coffee husks. The differences between husk and grain, processing, and type of variety have a direct influence on the retention of these compounds. Phenolic compounds are generally found in conjugation with organic acids and sugars (MCDADE *et al.*, 2021), and the results found in the present study help to understand many aspects of coffee husk metabolism, although they remain to be elucidated because few data are available.

[Table 2 here]

3.2 BIOACCESSIBILITY OF PHENOLIC COMPOUNDS FROM POLYPHENOL EXTRACTS IN ORGANIC COFFEE HUSK

In this study, three organic coffee husk extracts were obtained using different extraction methods (extractable polyphenols: EP; hydrolyzable non-extractable polyphenols: H- NEP; non-extractable polyphenols NEP). To evaluate the stability and metabolism of the phenolic compounds in the extracts, each treatment was subjected to an in vitro gastrointestinal simulation that mimics human organism conditions. Studies with phenols generally underestimate the content of these compounds, since they include only soluble or extractable polyphenols (EP) in aqueous-organic solvents, low or medium molecular weight compounds quantified as total polyphenols (JAKOBEK, 2015).

A significant fraction of phenols remains in the residues of phenolic extractions, these so-called non-extractable polyphenols (NEP) or macro-antioxidants, which can be divided into several fractions: hydrolyzable polyphenols (H- NEP), low molecular weight phenols strongly associated with polysaccharides and proteins, non-extractable polyphenols (NEP) and non-extractable proanthocyanidins (PANE), which are high molecular weight structures (7).

After being subjected to simulated in vitro digestion, the extracts (EP, H- NEP and NEP) showed total phenolic concentrations ranging from of between 60.62 and 2430 mg/100g DW; with total flavonoids ranging from between 0.59 and 9.00 mg/100g DW (Table 3). However, a study by Silva et al. (SILVA *et al.*, 2020), which investigated different methods for the extraction of phenolic compounds and the antioxidant activity of coffee husks, found flavonoid levels ranging from 0.63 and 9.93 mg/g DW. The results

are similar to those of this study and are relevant to EP. However, they indicated that the extraction method for polyphenols/macroantioxidants does not affect the retention of total flavonoids present in coffee husk (MCDADE *et al.*, 2021). Although flavonoids are the most abundant phenolics in teas and some fruits, coffee is considered the better source of phenolic acids (5).

The retention of phenols was satisfactory and statistically different only in the NEP extracts, which had the lowest content (60.62 mg/100g DW). However, as described in the method of Hartzfeld *et al.* (HARTZFELD *et al.*, 2002), the other treatments showed good efficiency in polyphenol extraction, highlighting the H- NEP treatment, which was obtained by an acute chemical hydrolysis process that reduced the interaction between the polyphenols and the matrix, with a greater release due to bond cleavage (MCDADE *et al.*, 2021). In a study by Pérez-Jiménez *et al.* (PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018), significantly different results were obtained when two hydrolysis methods were used to extract macro-antioxidants in peels of different fruits. The EP contents for apple and pear peels were 1278 and 721 mg/100g DW, respectively. In the same study, based on EP extraction, the polyphenol content in banana peels was 1961.3 mg/100g DW, while for NEP it was 7667.2 mg/100g DW. In the present study, a similar behavior was observed, with the extraction being crucial for the retention of phenols in the H-NEP extracts. This result confirms that the selection of the extraction and processing procedure is crucial for the recovery of compounds with bioactive potential (MCDADE *et al.*, 2021; PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018).

There is a lot of information available in the literature about the presence of phenolic compounds in coffee husk by-products, but there are fewer studies on the bioavailability and bioaccessibility of these compounds and the presence of macro-antioxidants. To have an effect on the body, bioactive compounds must be able to be

absorbed by the intestine and remain in circulation until they reach their target organ (IRIONDO-DEHOND *et al.*, 2019). In terms of digestion/bioaccessibility, it was generally found that each extract (EP, H- NEP and NEP) had a significant decrease in concentration in the gastric phase of the *in vitro* simulation for both determinations (phenols and flavonoids). The NEP for total phenols was the only treatment that showed an increase in content.

In the intestinal phase, NEP showed the same behavior in both determinations, with a significant retention for the compounds, a 3-fold increase for phenols, and an approximately 2-fold increase for total flavonoids (SOARES *et al.*, 2021; ZENG *et al.*, 2018). It is important to emphasize that alkaline conditions and enzymatic hydrolysis of complex food components favor the release of phenolic acids from the food matrix and increase their concentration in the intestine through the formation of monomeric forms (MCDADE *et al.*, 2021).

The coffee husk extracts show a good stability of phenolic compounds during the digestive process and have potential as prebiotics that support the colonization and balance of the microbial flora in the colon. The extract NEP specifically has the potential to promote health if the bioactive compounds remain stable during metabolism in the colon and can circulate in the blood (PÉREZ-JIMÉNEZ; ARRANZ; SAURA-CALIXTO, 2009; PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018). For total phenolics, the observed bioaccessibility for each extract was NEP 297% > H- NEP 55.78% > EP 28.51%. Extraction differences were critical for the release and retention of phenols between phases. The flavonoids had bioaccessibilities of 184.7% for H- NEP, 68.10% for NEP, and 12.7% for EP.

As for the physiological effects attributed to phenolic compounds, the concentration of metabolites is the most important factor. Although NEP and H- NEP did

not contain remarkable amounts of flavonoids compared with the undigested extract (EP), it was generally observed that these compounds had good bioavailability (concentration reaching the intestinal phase) because, as previously reported, pH and hydrolysis conditions favored retention. Nieto-Figueroa et al. (NIETO-FIGUEROA *et al.*, 2020), research has shown that the highest concentration of total and individual phenols from cocoa shells occurs in the intestinal phase of digestion.

This may be due to the release of other phenols at the higher pH of the intestine, which can lead to the cleavage of other components and the production of phenolic acids as degradation products. Therefore, to better understand the retention and absorption of dietary polyphenols, it is necessary to study the properties of the matrix and the metabolism of the compound itself. Mcdade et al. (MCDADE *et al.*, 2021) noted that polyphenols must be available at a certain concentration in the target tissue to exert a health-related effect.

The properties of certain compounds, such as phenolic acids, are related to their interactions with other compounds and stages of metabolism. These interactions can include release from the food matrix through enzymatic hydrolysis or the bacterial microbiota, absorption, and biotransformations. To understand the impact of phenolic intake on human health, it is important to have knowledge about the levels of these compounds and their bioaccessibility and bioavailability throughout the gastrointestinal tract (29).

Table 3 shows the profile of the main phenolic compounds and individual bioavailability. The data show that the extracts and the resulting digestions had significant differences ($p < 0.05$). Another common behavior for all samples was the concentration of gallic acid and chlorogenic acid, as well as a decrease in phenolic content depending on the stage of digestion. New perspectives are emerging in the literature to identify

variations in phenolic composition between organic and conventional coffee samples, and some compounds are particularly associated with organic varieties (CONSONNI; POLLA; CAGLIANI, 2018). The differences between the content of main metabolites in the samples EP, H- NEP and NEP could be related to the differences in cultivation (organic). However, there is a need for further investigation because to date, there is no research specifically addressing the dietary macroantioxidant/polyphenol content or bioaccessibility of a matrix derived from an organic coffee byproduct. Syringic acid and quercetin made the lowest contribution to the profile, ranging from 0.06 $\mu\text{g/g-1}$ to 0.26 $\mu\text{g/g-1}$ and from 0.16 $\mu\text{g/g-1}$ to 1.19 $\mu\text{g/g-1}$, respectively. It is important to emphasize that the EP extract stood out with the highest phenolic retention. However, the other treatments, H- NEP and NEP, showed significant decreases before and after digestion, probably due to degradation under the more stringent processing conditions (temperature, solvent polarity, and pH).

Digestion had a positive effect on the bioaccessibility of the individual phenolic compounds, with the exception of syringic and caffeic acids, the other compounds were detected in all simulated gastric treatments. Chlorogenic acid showed higher bioaccessibility in H- NEP and NEP with a variation of 44.7% to 30.9%. Variations in bioactivity of phytochemicals are influenced by key interactions between compounds and/or digestive enzymes, variable pH, and other factors (SOARES *et al.*, 2021). Gallic acid exhibited bioaccessibility ranging from 10.96% to 5.76%. Gallic acid, a type of phenolic acid, was found to have high bioaccessibility (meaning it is easily absorbed by the body) after being digested, according to a study by Zeng *et al.* (ZENG *et al.*, 2018). The study also found that the most common chlorogenic acid in coffee byproducts is 5-O-caffeoylquinic acid (also known as 5-ACG or chlorogenic acid). During *in vitro* digestion, chlorogenic acid binds to pepsin in the gastric phase and forms a complex through Van

der Waals interactions and hydrogen bonding. It also decreases the rate of pancreatin hydrolysis in the intestinal phase. Caffeic acid is less stable after digestion by the pancreas and is retained more in the gastric phase compared to the intestinal phases (SOARES *et al.*, 2021).

The presence of high concentrations of phenolic compounds such as gallic acid, chlorogenic acid, and caffeic acid is a good indicator of bioavailability because these compounds remain stable during digestion. The concentration of these compounds can be reduced by digestion and metabolism (NIETO-FIGUEROA *et al.*, 2020). To understand the bioactivity of phenolic compounds isolated from their matrix of origin, it is necessary to observe the effects of digestion on their biological activity. The literature provides a significant contribution to studies on macro-antioxidants from coffee by-products. The *in vitro* model based on human physiology, which was applied in the present study, contributes to a better understanding of these aspects. However, to confirm these claims, it is necessary to explore new perspectives through *in vivo* and randomized clinical trials with the analyzed extracts (EP - H- NEP and NEP) as dietary supplements.

[Table 3 here]

3.3 ASSESSMENT OF BIOACTIVITY OF ORGANIC COFFEE HUSK EXTRACTS

3.3.1 Antioxidant activity

Antioxidant potential is the best known claim of polyphenols in food. Studies suggest that non-extractable polyphenols are important contributors to the total antioxidant activity of fruit peels and are equivalent or even superior to soluble antioxidants (PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018). Although there is no unified method to estimate the actual antioxidant activity of phenol-rich materials Estévez (ESTÉVEZ, 2021), three methods have been used complementarily to express the total

antioxidant capacity, taking into account different mechanisms of action (BRESSANI *et al.*, 2021). The 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and DPPH assays are used to determine the antiradical activity of phenolic compounds, while the reducing capacity of iron (III) (FRAP) is mainly used to determine their reducing capacity (RUFINO *et al.*, 2010).

The results of the application of the antioxidant capacity assays (DPPH, ABTS and FRAP) are shown in Table 4. Coffee husk extracts have been found to have high antioxidant capacity, particularly in the undigested form (known as the EP extract). This may be due to the presence of phytochemicals such as chlorogenic acid, which is known to have antioxidant properties (SILVA *et al.*, 2020). The extracts of coffee husks showed more efficient antioxidant activity in the ABTS inhibition assay when compared to other extracts, suggesting that the husks contain more antioxidant compounds and that these compounds may act as antioxidants through different mechanisms. However, it is important to note that the radical scavenging effects observed in laboratory tests may not necessarily be seen in the human body (ESTÉVEZ, 2021; PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018).

During digestion, complex polyphenols and other condensed compounds are naturally broken down and reach the intestine in reduced amounts (MCDADE *et al.*, 2021). When the extracts (PE, H- NEP and NEP) of coffee husks were digested *in vitro* and the concentrations of the compounds were diluted, a decrease in antiradical activity was observed. The presence of phenols such as gallic acid, caffeic acid, and chlorogenic acid likely contributes to the scavenging activity of these radicals. In addition to their ability to neutralize radicals, these phenolic compounds may also act as antioxidants by chelating metals involved in the formation of free radicals.

All extracts (PE, H- NEP and NEP) showed effective antioxidant activity in both ABTS and FRAP assays and bioavailability. Only EP showed activity against the DPPH radical. It is known that the main structural features of polyphenols are responsible for the effective antioxidant activity (3). The antioxidant capacity of the samples in the two fractions analyzed, with the exception of NEP, decreased significantly in the intestinal phase (1526.3 $\mu\text{M ET /g sample}$). However, the behavior toward the ABTS radical was unexpected and exceeded the potential of the undigested extract itself (1376.9 $\mu\text{M ET /g sample}$). Variations in the antioxidant activity of coffee husks may be caused by changes during digestion in the gastrointestinal tract. The high pH during digestion allows for reactions such as self-oxidation to occur in the intestinal lumen (MCDADE *et al.*, 2021). Inhibition of DPPH radicals was observed only with the extracts of EP and H- NEP. The other treatments did not show antioxidant activity. The antioxidant capacity of DPPH was affected by the extraction methods H- NEP and NEP ($p < 0.05$).

The antioxidant activity of caffeic acid was found to be lower than that of its derivatives, suggesting that its antioxidant potential decreases with increasing polarity of the environment. Factors such as the solvents used, the antioxidant determination method, and the samples after *in vitro* digestion may have contributed to the lower retention of caffeic acid in some samples (EP, H- NEP and NEP). The chemical and enzymatic reactions during digestion can also affect the integrity of caffeic acid and its effectiveness as an antioxidant (SOARES *et al.*, 2021).

Wang et al. (WANG *et al.*, 2021) reported that lipophilic reactions aimed at improving its hydrophobicity may also increase its antioxidant potential. In the extracts analyzed, a significant difference ($p < 0.05$) was found between the extracts and the phases after digestion, with the ability to scavenge ABTS radicals decreasing in the gastric phase and increasing in the intestinal phase. The increase in antioxidant content

and activity in the simulated intestinal phase could also be related to the interactions between the oxidizing factors of the pancreatic enzymes themselves (lipid oxidation and lipophilic enzymes) (MCDADE *et al.*, 2021; SOARES *et al.*, 2021), making the samples more susceptible to reactions.

In a study on fermented coffee, Bressani *et al.* (BRESSANI *et al.*, 2021) observed inactivation of DPPH radicals between 49.67-91.60 μM Trolox.g-1, ABTS 316.05-232.92 μM Trolox. g-1, and FRAP 106.00-232.54 μM FS.g -1 before fermentation. The antioxidant compounds present in beans (they are also present in the husk) are rich in soluble and non-extractable polyphenols that act against oxidative stress. However, the antioxidant activity of macromolecular compounds present in coffee husks needs to be measured by *in vivo* studies (MCDADE *et al.*, 2021; PÉREZ-JIMÉNEZ; SAURA-CALIXTO, 2018).

The extracts showed better bioaccessibility in the ABTS and FRAP determinations, with absorption greater than 50%. For DPPH, as previously reported, bioaccessibility was found only in the EP (10.28%), indicating that soluble antioxidants and their metabolites resist gastrointestinal tract conditions even at low concentrations. The extracts EP, H- NEP and NEP might play a crucial role in bioavailability in the digestive tract and body (MCDADE *et al.*, 2021). There is little information available on the fate of phenolic compounds from coffee husks during digestion, which makes it difficult to compare and equate absorption values between different matrices and methods. To determine the potential of food matrices as functional or nutraceutical ingredients, *in vitro* gastrointestinal digestion combined with bioacceptance is a reliable alternative (NIETO-FIGUEROA *et al.*, 2020). The antioxidant capacity of extracts can be correlated with the content of phenolic compounds and the retention of these compounds until the final stage of digestion (bioaccessibility).

[Table 4 here]

3.3.2 Coagulation assessment: APTT e PT

The anticoagulant effect of gastrointestinal digestive products from coffee husks was evaluated using APTT and PT assays, shown in Figure 1. APTT is an assessment of intrinsic clotting factors, while PT is used to characterize extrinsic clotting factors (20). The results show the anticoagulant properties of the coffee husk extracts obtained in the study before and after simulated gastrointestinal digestion in vitro. In general, all extracts showed prolonged antithrombotic activity compared with NaCl (0.15M), except for the EP gastric digestion samples. All intestinal phase samples showed significantly prolonged APTT.

With respect to PT, only the crude extract of H- NEP showed a significantly longer retention time, while the other extracts remained close to the retention time estimated in NaCl (20.3 seconds). In the gastric phase, a similar behavior to APTT is observed. On the other hand, in all samples (EP, H- NEP, and NEP), the intestinal phase was critical in increasing the ability of the digestive products to delay prothrombin action. In both determinations (APTT and PT), digestion phases had no effect on the anticoagulant properties of H- NEP.

Research has shown that many phenolic compounds, including flavonoids and polyphenols, have significant biological effects, including anticoagulant, antithrombotic, and antiplatelet activity. Chlorogenic acid present in coffee peel extracts has been found to have these effects in some studies, and it has also been tested in clinical trials with no cytotoxicity at safe doses between 0.5-1g, where it has shown neuroprotective,

cardiovascular, and antiplatelet effects. There are fewer studies on the homeostatic functions of coffee peels, but some research has been conducted in this area (6,9).

In a study investigating the enzymatic extract of decaffeinated coffee beans treated with chlorogenase, caffeic acid was reported to be the major blood clotting factor (CAI *et al.*, 2013).

The presence of phenolic acids and the differences between the profiles of the studied extracts indicated the influence of *in vitro* digestion on the anticoagulant activity of all extracts (EP, H- NEP and NEP). However, it is possible to observe a milder effect in gastric samples Bijak *et al.* (BIJAK *et al.*, 2011) explain that the binding of polyphenols to plasma proteins may delay the anticoagulant effect. This can be explained by the presence of pepsin, as an interaction between anticoagulants and proteins and/or digestive enzymes has been demonstrated (MORADI *et al.*, 2020).

The results of this research demonstrate the potential to obtain anticoagulant compounds from coffee byproducts. The bioactive compounds caffeic acid and chlorogenic acid in coffee peel extracts may be used as alternatives for oral anticoagulation therapy as antiplatelet and antithrombotic agents and may have fewer side effects than other drug treatments. Bioavailability is an important factor in the effectiveness of these phenols in the body (37,38).

4. CONCLUSION

From our results, the three organic coffee husk extracts tested were good sources of macro-antioxidants, exhibiting antioxidant effect, bioaccessibility, and anticoagulant activity. When comparing between extractions, EP proved to be more efficient, but also (H- NEP, and NEP) showed favorable results (in some cases even better than EP). Coffee

husk extracts have the potential to be used as sources of nutraceutical compounds due to their antioxidant and anticoagulant properties. The extractive process, including drying and grinding, and the extract's ability to sequester the DPPH radical and prolong thromboplastin time were studied. The non-extractable polyphenolic fractions of the coffee husks also contributed to the retention and bioaccessibility of the phenolic compounds. These findings suggest that coffee husk extracts can be a valuable resource for the development of new nutraceutical compounds.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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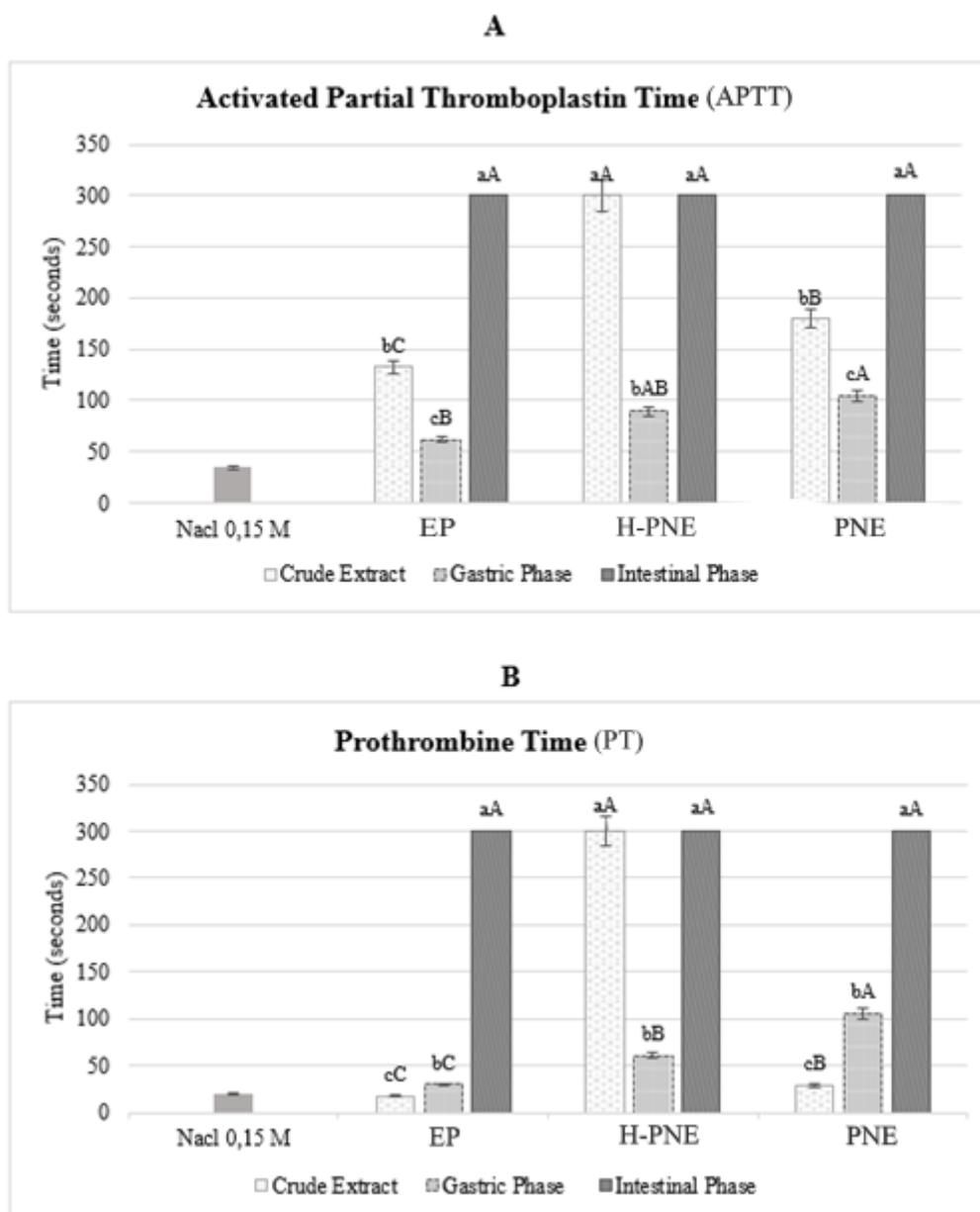
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Figure 1 - Assay of anticoagulant activity. A - Activated Partial Thromboplastin Time (ATTP), B - Prothrombine Time (PT)



EP: Extractable polyphenols; H-NEP: Hydrolyzable non-extractable polyphenols; NEP: Non-extractable polyphenols. Different lower-case letters indicate significant differences ($p < 0.05$) between extracts; different upper case letters indicate significant differences ($p < 0.05$) between digestion stages in the same extract. Source: Prepared by the author, 2022.

Table 1. Determination of physical and chemical parameters of organic coffee husk flour

Parameters	Organic coffee husk meal	Coffee husk*
Yield	20.09%	-
pH	3.97 ± 0.06	4.70 – 6.63
Color L*	45.37 ± 0.73	-
Color a*	5.13 ± 0.52	-
Color b*	9.03 ± 0.29	-
Humidity (g/100g)	13.81 ± 0.49	12.00
Lipids (g/100g)	0.50 ± 0.03	0.50 – 2.00
Proteins (g/100g)	9.41 ± 0.17	9.20
Maltose (g/100g)	3.72 ± 0.95	-
Glucose (g/100g)	3.40 ± 0.35	-
Xylose (g/100g)	3.32 ± 0.11	-
Fructose (g/100g)	1.62 ± 0.10	-
Arabinose (g/100g)	6.44 ± 0.04	-

*Values presented by the literature in research related to coffee husk (8,23,39).

Table 2. Organic acid profile of organic coffee husk flour.

Organic acids (mg/100g)	Organic coffee husk meal
Citric	1.59 ± 0.22
Tartaric	1.10 ± 0.17
Ascorbic	2.09 ± 0.51
Malic	5.05 ± 1.00
Pyruvic	0.14 ± 0.04
Succinic	3.46 ± 0.26
Lactic	0.71 ± 0.15
Formic	0.26 ± 0.05
Propionic	0.68 ± 0.85

Table 3. Bioaccessibility of phenolic compounds and flavonoids from organic coffee husk polyphenol extracts.

Determination	Extracts types	Digestion <i>in vitro</i>			
		Extract	Gastric phase	Intestinal phase Bioaccessible	Bioaccessibility (%)
Total Phenolics mg CAE/100 g	EP	501.00 ± 1.84 ^{aB}	160.46 ± 1.00 ^{bB}	141.04 ± 4.00 ^{bB}	28.51 ^Z
	H-NEP	2.430 ± 3.05 ^{aA}	1039.60 ± 1612 ^{cA}	1355.5 ± 9.70 ^{bcA}	55.78 ^Y
	NEP	60.62 ± 12.45 ^{dB}	101.63 ± 8.11 ^{cB}	180.05 ± 13.42 ^{bB}	297 ^X
Total Flavonoids mg CE/ 100g	EP	9.00 ± 1.05 ^{aA}	1.38 ± 0.30 ^{cA}	1.15 ± 0.19 ^{cA}	12.7 ^Z
	H-NEP	0.99 ± 0.02 ^{bB}	0.42 ± 0.66 ^{bA}	0.68 ± 0.53 ^{bA}	68.6 ^Y
	NEP	0.59 ± 0.41 ^{bB}	0.41 ± 0.68 ^{bA}	1.09 ± 0.06 ^{bA}	184.7 ^X
Gallic acid µg/g ⁻¹	EP	30.37 ± 0.00 ^{aA}	15.05 ± 0.02 ^{bA}	3.33 ± 0.03 ^{cA}	10.96 ^X
	H-NEP	4.18 ± 0.00 ^{aB}	0.74 ± 0.01 ^{bB}	0.27 ± 0.01 ^{cB}	6.45 ^Y
	NEP	3.47 ± 0.00 ^{aC}	2.00 ± 0.01 ^{bB}	0.2 ± 0.00 ^{cC}	5.76 ^Z
Caffeic acid µg/g ⁻¹	EP	33.7 ± 0.36 ^{aA}	9.79 ± 0.01 ^{bA}	3.74 ± 0.00 ^{cA}	11.09 ^X
	H-NEP	5.24 ± 0.00 ^{aB}	1.06 ± 0.01 ^{bB}	0.56 ± 0.01 ^{cB}	10.68 ^Y
	NEP	-	-	-	-
Chlorogenic acid µg/g ⁻¹	EP	7.52 ± 0.02 ^{aA}	2.28 ± 0.01 ^{bA}	0.51 ± 0.01 ^{cA}	6.78 ^Z
	H-NEP	1.05 ± 0.00 ^{aB}	0.30 ± 0.00 ^{bB}	0.47 ± 0.00 ^{cB}	44.76 ^X
	NEP	0.55 ± 0.00 ^{aC}	0.10 ± 0.00 ^{bC}	0.17 ± 0.01 ^{cC}	30.9 ^Y
Syringic acid µg/g ⁻¹	EP	0.27 ± 0.01 ^{aA}	0.03 ± 0.00 ^b	-	-

	H-NEP	0.07 ± 0.01^{aB}	-	0.06 ± 0.00^{bA}	-
	NEP	-	-	-	-
Quercetin $\mu\text{g}/\text{g}^{-1}$	EP	N.m	N.m	N. m	-
	H-NEP	1.17 ± 0.00^{aA}	0.28 ± 0.01^{bA}	-	-
	NEP	1.20 ± 0.00^{aB}	0.21 ± 0.00^{bB}	0.17 ± 0.00^{cC}	14.16

EP: extractable polyphenols; H-NEP: hydrolyzable non-extractable polyphenols; NEP: non-extractable polyphenols. Different lower-case letters indicate significant differences ($p < 0.05$) between gastrointestinal simulation phases (row); different upper case letters indicate significant differences ($p < 0.05$) between extracts (column). X, Y, and Z indicate significant differences ($p < 0.05$) in the bioavailability of the extracts (column).

Table 4. Antioxidant evaluation of organic coffee husk polyphenol extracts and bioaccessibility.

Antioxidant	Extract Types	Digestion <i>in vitro</i>			Bioaccessibility (%)
		Extract	Gastric phase	Intestinal phase Bioaccessible	
DPPH μmol EqT/g	EP	1995,5 ±24,7 ^{aA}	467,6 ±62,7 ^b	205,3± 27,2 ^c	10,28
	H-NEP	215,55±8,32 ^B	N.d	N.d	-
	NEP	N.d	N.d	N.d	-
ABTS μmol EqT/g	EP	2953 ±62,5 ^{aA}	2072,5± 95,7 ^{cA}	2672,5 ± 52,4 ^{bA}	90,50 ^Z
	H-NEP	1332,63± 69,4 ^{bB}	1039,37± 7,6 ^{cC}	1245,9± 47,2 ^{bC}	93,49 ^Y
	NEP	1376,9 ±42,5 ^{bcB}	1210,5 ±31,9 ^{cB}	1526,5 ±7,5 ^{bB}	110,86 ^X
FRAP μmol EqT/g	EP	2043,1±8,21 ^{aA}	1924,18± 2,83 ^{bA}	1812,88± 3,9 ^{dA}	201,08 ^X
	H-NEP	773,82 ± 13,32 ^{bB}	109,33 ±5,53 ^{dB}	489,4± 40,8 ^{cB}	88,72 ^Y
	NEP	607,79±6,41 ^{bcC}	68,16 ±6,19 ^{dC}	374,5 ±23,6 ^{cC}	61,61 ^Z

EP: extractable polyphenols; **H-NEP:** hydrolyzable non-extractable polyphenols; **NEP:** non-extractable polyphenols. Different lower-case letters indicate significant differences ($p < 0.05$) between gastrointestinal simulation phases (row); different upper case letters indicate significant differences ($p < 0.05$) between extracts (column). X, Y, and Z indicate significant differences ($p < 0.05$) in the bioavailability of the extracts (column).

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Artigo 3**ORGANIC ARABICA COFFEE HUSK HAS ANTIOXIDANT AND CELL PROTECTIVE EFFECTS AND INDUCE BENEFICIAL CHANGES IN INTESTINAL MICROBIOTA OF DIABETIC ADULTS DURING *IN VITRO* COLONIC FERMENTATION**

Thaianaly Leite Abreu^a, Antonia Dayane Jenyffer de Farias Marques^a, Juliana Santos de Espindola^b, Bianca Beatriz Torre de Assis^a, Karoliny Brito Sampaio^c, Thatyane Mariano Rodrigues de Albuquerque^c, Maria Teresa Bertoldo Pacheco^b, Fabiana Andrea Barrera^b, Evandro Leite de Souza^c, Marciane Magnani^a, Marta Suely Madruga^a, Taliana Kênia Alencar Bezerra^a

^a Post-Graduate Program in Food Science and Technology, Department of Food Engineering, Technology Centre, Federal University of Paraíba, 58051-900, João Pessoa, PB, Brazil

^b Food Science and Quality Center, Institute of Food Technology (ITAL), Campinas, SP, Brazil

^c Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, PB, Brazil

E-mails: thaianaly.abreu@gmail.com, dayanefaarias@gmail.com, juliana.s.espindola@gmail.com, karolbsampaio@gmail.com, thaty_mra@hotmail.com, bertoldopacheco@gmail.com, fabiana.pe@ital.sp.gov.br, els@academico.ufpb.br, magnani2@gmail.com, msmadruga@uol.com.br, *taliana.kenia@hotmail.com

*Corresponding Author

ABSTRACT

Coffee husks (CH) are the main by-product of the coffee industry and are usually discarded or used as fertilizer. The use of coffee husks can have environmental and social benefits, and when combined with the organic cultivation of a more sustainable crop, these impacts are mitigated. This byproduct is increasingly being studied and used for its antioxidant, anti-inflammatory, and antimicrobial properties, as well as its prebiotic potential. These effects are related to bioactive compounds such as phenols and fiber-bound macroantioxidants that can reduce oxidative stress, regulate gene expression, protect body cells from ROS damage, and modulate the gut microbiome of people suffering from diabetes. The aim of this study was to evaluate organic coffee husks for their antioxidant capacity in vitro (DPPH, ORAC and FRAP), their cytoprotective/cytotoxic effect on Caco-2 cells (MTT and DCF-DA) and their effects on the gut microbiota of diabetic patients in an in vitro gut fermentation system. The results showed a good antioxidant activity of CH, which was maintained under simulated gastric conditions against all tested radicals. The sample also showed cell viability and developed in ROS in all enrollments. As for fecal fermentation, there was a positive change in the release of phenolic compounds in the presence of the bark. A positive prebiotic index was detected for CH during colonic fermentation, indicating an overlap of beneficial bacteria at the expense of the beneficial bacteria that constitute the intestinal microbiota of diabetic patients. These results demonstrate the potential of CH as a source of bioactive compounds associated with important biological and cellular effects in maintaining the microbiota of individuals with diabetes and a disrupted intestinal barrier, and the by-product may open new perspectives for science and food technology as a potential nutraceutical.

Keywords: by-products; organic coffee; phenolics; bioactivities; gut microbiota

1. INTRODUCTION

Coffee is one of the most important commodities around the world. Brazil leads the global coffee production, being responsible for almost one-third of the world production income provided by this culture (ICO, 2021). Sustainability in the coffee productive chain has become an emerging issue to minimize the environmental impacts of the generated by-products, such as husk (CORDOBA et al., 2020).

Coffee is one of the main sources of phenolic compounds and caffeine in the human diet due to its high consumption (DE FARIAS MARQUES et al., 2022). Studies show that coffee bioproducts have phenolics compounds with ability to reduce oxidative stress, which is related to cardiovascular disease and cancer (DAS; RAMANATHAN, 2023; GURUNG et al., 2020; SILVA et al., 2020). Furthermore, polyphenols found in coffee husks are associated with a lower risk of developing type 2 diabetes and improved blood sugar regulation in diabetic individuals (Das & Gnanasambandan, 2023; Mithul Aravind et al., 2021). Different methods must be performed to assess the antioxidant potential of complex matrices since each method indicates the antioxidant activity against specific free radicals Oxygen Radical Absorbance Capacity (ORAC) method can assess the ability of the matrix to capture free radicals close to that found in the human body (CHISTÉ et al., 2011), while Caco-2 cells simulate the uptake of these compounds in the intestine. Caco-2 cells are a human epithelial cell line derived from colon adenocarcinoma with properties like intestinal cells transporting nutrients and bioactive compounds to the blood (BEDOYA-RAMÍREZ et al., 2017a; GRZELCZYK et al., 2022).

Phenolic compounds may also exert prebiotic activity since intestinal bacteria can metabolize the compounds found in coffee husks. The intestinal bacterial metabolism helps to generate simpler and more active compounds with health benefits (Massa et al.,

2022). However, few is known about the effects of coffee husks on bacterial metabolism and their antioxidant potential in the gut (Das & Ramanathan, 2023; Gurung et al., 2020).

This study assessed the bioaccessible phenolic compounds in coffee husk and its antioxidant activity using different *in vitro* models (DPPH, FRAP and ORAC), as well as the effects on selected intestinal bacterial groups through an *in vitro* colonic fermentation using feces from diabetic individuals.

2 MATERIAL AND METHODS

2.1 Material

Organic coffee husks (100% Arabica and typica) were collected in the city of Taquaritinga do Norte, Pernambuco, Brazil (-7.88809 S, 36° 5' 33" W). The organic coffees are produced with the shading technique and were harvested by hand. After coffee wet processing, the husks were separated from the grains and frozen at -18°C until the study was conducted.

2.2 Sample preparation

Coffee husks were dried in a forced-circulation oven at 40 °C for 48 h using previously described conditions (SILVA et al., 2020). The dried shells were ground to 2 mm with a Wiley knife to a fine bran-like mass and stored in anti-hermetically sealed package protected from light, under refrigeration (6 °C).

2.2.1 Preparation of the Organic Coffee Husk Extract

According to the method proposed by Silva et. al (2020), the organic coffee husk flour was weighed directly into a falcon tube and homogenized manually with an

extraction solution of water and ethanol (1:1) for 5 minutes. The ratio of flour to extraction solution was 1:10 (p:v). The resulting mixture was incubated at 60 °C in a water bath for 60 minutes and then centrifuged at $3\,500 \times g$ for 20 minutes at 10 °C. The supernatant was collected and filtered with filter paper. The resulting extract was then rotary evaporated (Fisatom 802, São Paulo, Brazil) at 60°C for 5 hours and then reconstituted with ultrapure water to obtain the original extract volume.

2.3 Cytotoxic/cytoprotective assays

2.3.1 CACO-2 cell culture

Cells were seeded in 75 cm² flasks and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 8.4 mM HEPES, 1% sodium pyruvate, 1% non-essential amino acids, and 1% L-glutamine. The medium was replaced every 2-3 days. Exponentially growing cells were detached from the culture flasks using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded on 96-well plates with 2×10^4 cells per well. After reaching confluence, the growing medium was removed and cells were pre-treated with control or different concentration of coffee husk extract (CH) extracts (1, 10, 100, 500, and 1000 mg/ml) for 3 h. Then, cells were stimulated with control or H₂O₂ at a concentration of 1mM for additional 2 h. The cells in the control group were treated with a medium free of FBS. Cell viability assay and ROS production were evaluated.

2.3.2 Determination of the effects of CH on viability of Caco-2 cells

The method used to evaluate the viability of Caco-2 cells was based on the cellular uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, M5655, Sigma-Aldrich St. Louis, MO, USA), which is endocytosed and mitochondrial

dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals insoluble in aqueous solutions. Cells were incubated with 50 µg/ MTT during the last 30 min of treatment at 37 °C. Afterward, the medium was removed and MTT crystals were dissolved in DMSO. Absorbance values were measured at 560 and 650 nm. The reduction of MTT was calculated as (abs at 560 nm) – (abs at 650 nm) and expressed as a percentage of baseline measurements.

2.4.3 Evaluation of the effects of CH on ROS production in Caco-2 cells

The ROS production in Caco-2 cells was evaluated using DCFDA assay, which is based on the diffusion of DCFDA into the cells, deacetylation by cellular esterases, and oxidation by ROS into 2', 7' –dichlorofluorescein (DCF). DCF-DA is a fluorogenic dye that measures reactive oxygen species (ROS) activity within the cell. In the last 30 min of pre-treatment, cells were incubated with 20 µM of DCF-DA. After the pre-treatment medium was removed, the cells were washed with PBS to remove DCF-DA not internalized and treated with medium (control group), CF concentration, or stimulus plus CF concentration. The fluorescence was measured immediately during 120 min. The AUC was calculated and the difference between sample AUC and control AUC was used as the selective basis of the co-incubation time of DCFH-DA and pure treatment with Caco-2 cells.

2.4 Evaluation of antioxidant activity of CH in vitro and cell model

2.4.1 DPPH and FRAP assays

The ability of CH extracts to scavenge the DPPH radical was determined using a method described by Brand-Williams et al. (1995). The radical scavenging activity and

antioxidant capacity of CH extracts were measured using a UV-VIS spectrophotometer (Quimis, São Paulo, SP, Brazil) at 515 nm.

The antioxidant ability of CH extract to reduce iron (Fe^{3+}) to the ferrous form (Fe^{2+}) was verified at 593 nm in a UV-VIS spectrophotometer (Quimis, São Paulo, SP, Brazil) (BENZIE; STRAIN, 1999). Calibration curves were prepared with different concentrations of Trolox (50-1000 μM) and results were expressed in terms of equivalent Trolox antioxidant activity per gram of sample.

2.4.2 ORAC absorption capacity

The ORAC assay is based on the ability to reduce the peroxy radical ($\text{ROO}\cdot$) generated by the thermal degradation of (2,2'-azobis(2-amidinopropane)dihydrochloride) (AAPH) and protect the fluorescein molecule from the action of the peroxy radical by the antioxidant fluorescein. The fluorescence decay is monitored in kinetic mode with readings per min for 2 h at pH 7.4 (CHISTÉ et al., 2011). Analysis was performed in a 96-well microplate fluorescence reader (Synergy, BioTek®, Gen5 software) with fluorescence filters for excitation at 485 nm and emission at 528 nm at 37 °C. A standard Trolox curve was used to express the ORAC values of the samples at concentrations of 250, 500, and 1000 ppm. A 75 mM potassium phosphate buffer (pH 7.4) was used for curve dilution and sample substrate. In each well of the microtiter plate, 30 μl of sample or standard, 60 μl of 508.25 nM fluorescein solution, and 110 μl of 76 mM AAPH solution were added. Sample and protective standard (AUCnet) were calculated by the difference between the area under the fluorescence decay curve of sample/standard (AUC sample/standard) and the area under the fluorescence decay curve without sample or addition of Trolox (white AUC). Results were expressed in μmol Trolox equivalent/g sample (Chisté et al., 2011).

2.5 *In vitro* digestion and evaluation of the remaining HC antioxidant capacity

The *in vitro* digestion of CH was performed as previously described (BRODKORB et al., 2019). Aliquots of CH were prepared to determine the effects of the simulated gastrointestinal conditions. Stock solutions of electrolytes and enzymes comprising the oral, gastric, and intestinal phases were prepared in accordance with international consensus as reported by INFOGEST. Antioxidant Capacity Remaining (ACR) after digestion was expressed as a percentage and determined according to Equation 1 (Ribeiro, 2018).

$$\text{ACR (\%)} = (\text{CF digested} / \text{CF undigested}) \times 100 \quad (\text{Eq. 1})$$

Where: *CF digested* is the concentration of the analyzed compound from digested CH (intestinal phase), and *CF undigested* is the concentration of analyzed compound from undigested CH (initial extract).

2.6 *Human fecal inoculum and in vitro colonic fermentation of CH*

Fecal samples from four adult diabetic volunteers (two men and two women, aged between 25 and 55 years) were used after approval by a Human Research Ethics Committee (Federal University of Paraíba, João Pessoa, PB, Brazil, CAE number: 2 65756222.4.0000.5188). Inclusion criteria included type 2 diabetics taking medications to control blood glucose levels, who did not suffer from any gastrointestinal or colonic disease, following a regular omnivorous diet without using probiotic foods, concentrated probiotics or prebiotics in the last 48 h and antibiotics during the six months prior to the study. Donors received specific instructions as well as an appropriate collection kit. The stool collection kit included sterilized collection tubes, disposable spatulas, instructions

for sample collection/sending, disposable gloves, and 70% alcohol. In addition, the instructions contained in the kit described the steps for hand hygiene and aseptic handling of the samples. The samples were placed in a jar with an anaerobic generator system (AnaeroGen, Oxoid, Basingstoke, England) and transported to the laboratory. The experiments started immediately after the arrival of the feces to guarantee a fresh fecal microbiota, and the feces were manipulated in an anaerobic environment. The same amount of feces from each donor was pooled and mixed with sterile phosphate-buffered saline (PBS; 0.1 M; pH 7.4; 1:10, w/v) under agitation (200 rpm) for 2 min for use in the experiments.

The colonic fermentation was performed as previously described (Medeiros et al., 2021) using 40% (v/v) of autoclaved (121 °C, 1 atm, 15 min) fermentation medium [4.5 g NaCl, 4.5 g KCl, 1.5 g NaHCO₃, 0.69 g MgSO₄, 0.8 g L-cysteine, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 0.4 g bile salt, 0.08 g CaCl₂, 0.005 g FeSO₄, 1 mL Tween 80, 4 mL resazurin solution (0.25 g/L, as an anaerobic indicator) and 1 L of distilled water], 40% (v/v) of the pooled fecal inoculum, and 20% (w/v) of the digested CH. Fermentation was carried out at 37 °C for 48 h under anaerobic conditions (AnaeroGen). The fermentation medium without the addition of CH corresponded to a negative control. Salts and reagents used to prepare the fermentation medium were purchased from Sigma-Aldrich (de Assis et al., 2021).

2.7 Evaluation of the relative abundance of intestinal bacterial groups and prebiotic index during CH colonic fermentation

The relative abundances of selected intestinal bacterial groups were measured using fluorescent in situ hybridization coupled with flow cytometry using four distinct probes (oligonucleotides) labeled with fluorescent Cy3 and capable of hybridizing the

specific region of the 16S rRNA gene of *Lactobacillus* spp./*Enterococcus* spp (probe Lab 158, facultative anaerobes); *Bifidobacterium* spp. (probe Bif 164, strictly anaerobic), *Bacteroides* spp./*Prevotella* spp (probe Bac 303, strictly anaerobic), and *Clostridium histolyticum* (probe Chis 150, strictly anaerobic) (Sigma-Aldrich) (Rodrigues et al., 2016). The total bacterial population was quantified using SYBR Green (Invitrogen, Carlsbad, CA, USA) (Conterno et al., 2019). These target human intestinal bacterial groups were selected because the modulated of their relative abundances are typically linked to positive and negative metabolic responses and health outcomes in the host (DE ALBUQUERQUE et al., 2020; MASSA et al., 2022). Measurements were performed using a BD Accuri C6 flow cytometer (New Jersey, USA) with 488 nm excitation of a solid-state blue laser. Signals from individual cells through the laser zone were recorded as logarithmic signals. Fluorescence signals were recorded in FL1 (SYBR Green) and FL2 (Lab 158, Bif 164, Bac 303, Chis 150) channels and plotted as cytograms using BD Accuri C6 software.

After calculating the relative abundance of each measured bacterial group, the prebiotic index was calculated using the equation (DE ALBUQUERQUE et al., 2021):

$$\text{Prebiotic index} = \% \text{Lab} + \% \text{Bif} - \% \text{Bac} - \% \text{Chis} \text{ (Eq. 2)}$$

Where: %Lab is the percentage hybridized by Lab 158 at 48 h - percentage hybridized by Lab 158 at time zero; %Bif is the percentage hybridized by Bif 164 at 48 h - the percentage hybridized by Bif 164 at time zero; %Bac is the percentage hybridized by Bac at 48 h - the percentage hybridized by Bac 303 at time zero, and %Chis is the percentage hybridized by Chis 150 at 48 h - the percentage hybridized by Chis 150 at time zero.

A positive prebiotic index indicates a beneficial modulation of the intestinal microbiota during colonic fermentation, resulting in a potential prebiotic effect, while a negative prebiotic index indicates an undesirable modulation of the intestinal microbiota.

2.8 Measurements of pH, sugars, and phenolic compounds during CH colonic fermentation

The pH values (method 981.12) were determined with a digital potentiometer (Quimis, Diadema, SP, Brazil) (AOAC, 2019).

2.8.1 Determination of sugar contents during CH colonic fermentation

The contents of sugars (arabinose, glucose, fructose, maltose, and xylose) profile was determined with high performance liquid chromatography technique as previously described (Zeppa et al., 2001). The data were processed with OpenLAB CDS ChemStation Edition™ software (Agilent Technologies). The peaks of phenolic compounds were identified by comparison of their retention times with those of external standards (Sigma-Aldrich). The quantification of phenolic compounds was done with calibration curves of the external standards (R^2 of ≥ 0.998) (Menezes et al., 2021b).

2.8.2 Determination of phenolic compound contents during

The contents of phenolic compounds were determined in freeze-dried CH before and after exposure to the simulated gastrointestinal digestion (non-dialyzed fraction). Initially, methanol extracts of CH exposed and non-exposed to the simulated gastrointestinal digestion were prepared using previously described procedures (Massa et al., 2020). The separation and quantification of the phenolic compounds (caftaric acid, gallic acid, syringic acid, cyanidin 3-glucoside, delphinidin 3-glucoside, catechin, epigallocatechin gallate, epicatechin, epicatechin gallate, procyanidin A2, procyanidin B1, procyanidin B2, hesperidin, kaempferol 3-glucoside, quercetin 3-glucoside, rutin, and cis-resveratrol) were performed with HPLC using an Agilent chromatograph (model 1260 Infinity LC, Agilent Technologies, St. Clara, CA, USA) and analytical conditions previously described (Massa et al., 2020). A Zorbax C18 (12.6 × 4.6 mm, 5 μm; Agilent

Technologies) precolumn and a Zorbax Eclipse Plus RP-C18 (100 × 4.6 mm, 5 µm; Agilent Technologies) column were used for the determination of phenolic compounds. The data were processed with OpenLAB CDS ChemStation Edition™ software (Agilent Technologies). The peaks of phenolic compounds were identified by comparison of their retention times with those of external standards (Sigma-Aldrich). The quantification of phenolic compounds was done with calibration curves of the external standards (R^2 of ≥ 0.998) (MENEZES et al., 2021b). The results were expressed as g/100g.

2.9 Statistical analysis

The data obtained were analysed using analysis of variance (ANOVA) followed by factor analysis using Minitab ® 19 statistical software based on a 5% significance level. The Pearson's correlation test evaluated the relationship between the values of phenolic compounds. Row for the same bacterial group or prebiotic index denote differences ($p \leq 0.05$), based on Student's t test. GraphPad Prism 6.0 calculation software (GraphPad Software, La Jolla, CA, USA) was used for these analyses.

3 RESULTS AND DISCUSSION

3.1 Antioxidant capacity of CH in Caco-2 cell culture

Caco-2 cells are stimulated with hydrogen peroxide and the ability of CH extracts to attenuate the induced oxidative stress was evaluated. To determine a possible toxic effect of the peroxide concentration and the CH extracts, the viability of the cells was evaluated with MTT assay. A decrease in MTT value compared to the baseline indicate that cells were impaired. Figure 1A shows that no tested concentration of CH extracts

was able to affect cell viability, even when incubated together with hydrogen peroxide, indicating that cellular functions were not disturbed after treatment.

Assessment of (ROS) production in Caco-2 cells was performed using DCF-DA assay and results are shown in Figure 1B. Treatment with Caco-2 cells and CH extract without oxidative stimulation did not change the intracellular ROS level ($p > 0.05$), indicating that CH these by themselves does not cause oxidation. Induction of oxidative stimulation with hydrogen peroxide increased ($p > 0.05$) intracellular ROS level, which was reversed in a dose-dependent manner by increasing the CH extract concentration.

The phenolic compounds present in CH, mainly chlorogenic and gallic acids, are associated with ROS inactivation and contribute to the antioxidant activity of CH extracts (Mcdade et al., 2021). The ability to inactivate ROS is an important function to protect the organism from the penetration of harmful substances into the intestinal lumen, such as pathogenic microorganisms and dietary toxins (Zhou et al., 2022). Impairment of the intestinal barrier can lead to chronic inflammation, including Crohn's disease, inflammatory bowel disease, and intestinal disease (CHEN et al., 2020).

Under oxidative stress, cellular mitochondria produce large amounts of ROS, such as hydrogen peroxide, superoxide, and hydroxyl free radicals. High intracellular ROS levels can change cell membrane permeability, damage DNA, and induce lipid peroxidation and abnormal signal transduction (LIU et al., 2021).

Treatment with Caco-2 cells and CH extract without oxidative stimulation did not alter intracellular ROS levels in control samples ($p > 0.05$), as measured with DCF-DA assay (Figure 1B). The induction of oxidative stimulus caused a difference ($p > 0.05$) in some sample, except for the concentrations of (CH) extracts 100, 500, and 1000 mg/ml. Greater stability of the extracts against the oxidative action of H_2O_2 was observed, possibly due to their antioxidant potential and concentration of polyphenols.

Liu et al (2021) found that low doses of nano-SiO₂ (10, 25, 50, 100, and 200 µg/ml) isolated from five brands of instant coffee did not affect gastrointestinal cell activity and caused no damage to Caco-2 cell membrane, being considered an indicative of safe for the tested nanoparticles. Bedoya-Ramírez et al. (2017b) showed that samples of Colombian coffee after exposure to oxidative stress with H₂O₂ prevented ROS accumulation in Caco-2 cells, indicating a cytoprotective effect and improved cell viability, as found in our study.

Compounds in CH may be directly linked to oxidative protection, such as free chlorogenic acids and their derivatives. Iriando-DeHond et al. (2016) reported that the same compounds could account for the antioxidant capacity of the aqueous extract of silverskin aqueous extract. The results also showed that the tested extract concentrations did not cause cytotoxicity to HaCaT cells, in addition to exert anti-aging properties due to its antioxidant effects.

CH may exert health-promoting effects in Caco-2 cells, including antioxidant capacity. However, it is important to emphasize that further studies are needed to evaluate the effects of CH using *in vivo* models.

3.2 Determination of antioxidant capacity *in vitro*

The results obtained in DPPH, FRAP, and ORAC assays are shown in Table 1. In general, in terms of Antioxidant Capacity Remaining (ACR) after digestion, it can be highlighted that the samples performed well in DPPH, FRAP, and ORAC assays, with more than 50% of ACR, standing out the results with FRAP assay (91.21% ACR). Based on these results, it is believed that the CH may play local antioxidant effect (digestive tract) and after digestion (Ayoub et al., 2016; Shahidi et al., 2019).

CH showed higher antioxidant activity before the digestion process probably due to the oxidation of phenolic compounds during digestive metabolism (MCDADE et al., 2021). This behavior was observed in the samples, but (ACR) was achieved satisfactorily for all observed radicals. The higher antioxidant capacity in the coffee husk samples before simulated digestion can be explained by the composition and with the co-extraction of other alkali-soluble compounds such as carbohydrates and proteins, which may contribute to the total antioxidant capacity (Connolly et al., 2021).

Gallic, caffeic, and chlorogenic acids may have contributed to triggering the action of oxidizing radicals (SILVA et al., 2020). According to Wenzel (2012), such phenolic acids correspond to non-enzymatic antioxidants, i.e., components that help protect foods from chemical deterioration due to autooxidation/peroxidation of lipids, proteins, and genes. In general, phenolic compounds may act by chelating metals against the free radical formation, enzymatic induction, and acting on target and highly specific molecules, with the possibility of cellular involvement (MCDADE et al., 2021).

Considering the values of this ORAC determination for *in vitro* digestion (65.44 - 40.78 and 33.48 $\mu\text{M EqT/g}$), a decrease in the activity of oxygenated radicals at each step can be seen, confirming that the gastrointestinal simulation process leads to a decrease in antioxidant activity.

3.3 Effects of CH on selected intestinal bacterial groups during colonic fermentation

The relative abundance of *Lactobacillus* spp./*Enterococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp./*Prevotella* spp., and *C. histolyticum* medium with CH), as well as in a negative control (NC; without fermentable substrate) during 48 h of *in vitro* colonic fermentation are shown in Figure 2. The CH increased ($p \leq 0.05$) the relative abundance of all measured intestinal bacterial groups during colonic fermentation, with a

predominance of *Bifidobacterium* spp., indicating the availability of components able to reach the colon (e.g., insoluble and soluble fibers and phenolic compounds) to be fermented by gut microbiota (DONG et al., 2020a; MASSA et al., 2022) NC medium had a decrease ($p \leq 0.05$) in the relative abundance of *Lactobacillus* spp./*Enterococcus* spp., and an increase ($p \leq 0.05$) in the relative abundance of *Bifidobacterium* spp., *Bacteroides* spp./*Prevotella* spp., and *C. histolyticum*. However, relative abundance of *Bifidobacterium* spp. was lower in NC medium than in medium with CH ($p \leq 0.05$).

Lactobacillus spp./*Enterococcus* spp. and *Bifidobacterium* spp. are the main microbial markers for evaluating a potential prebiotic ingredient due to their beneficial effects on intestinal health with systemic outcomes, such as in diabetic individuals (ALARCÓN YEMPÉN et al., 2021; GIBSON et al., 2017). The increase of *Lactobacillus* spp./*Enterococcus* spp. found in medium with CH, in contrast to the decrease relative abundance in NC, is important because these bacterial groups could improve the intestinal barrier function (DAS; GNANASAMBANDAN, 2023). Also, the higher relative abundance of *Bifidobacterium* spp. during colonic fermentation of medium with CH shows the potential of this byproduct to stimulate beneficial intestinal bacteria. Increased intestinal *Bifidobacterium* spp. populations are directly linked to improvements in immune system and intestinal barrier, and decreased colonization by pathogens (SANDERS et al., 2019). *Lactobacillus* and *Bifidobacterium* species could help individuals with DM to have lower fasting blood glucose levels, as well as decrease postprandial blood glucose due to the production of metabolites that serve as important molecular bridge between the host and the microbiota, with the ability to modulate different hormones and enzymes in the intestine (Das & Ramanathan, 2023; Wang et al., 2016).

Diabetic individuals have distinct gut microbiota characteristics compared to non-diabetic individuals (ALARCÓN YEMPÉN et al., 2021). The genus *Bacteroides*

corresponds to Gram-negative bacteria predominant in the intestinal microbiota of diabetic individuals (DAS; GNANASAMBANDAN, 2023). The increased in the relative abundance of *Bacteroides* spp./*Prevotella* spp. in medium with CH during colonic fermentation may be related to this specific intestinal microbiota feature in diabetic individuals. Despite reports for these bacterial groups, Gurung et al., (2020) state that some *Bacteroides* and *Prevotella* species could exert positive health effects in patients with DM2.

The increase in the relative abundance of *C. histolyticum* was greater ($p \leq 0.05$) in NC than in medium with CH. It indicates that the presence of CH provided a less favorable environment for the growth of this pathogenic microorganism, which is positive since type 2 diabetic individuals had genes that benefit the growth of opportunistic pathogens (Hameed et al., 2020). These results could still be related to the antimicrobial effects of phenolic compounds present in CH against pathogens (Pérez-Burillo et al., 2019).

Positive prebiotic index was found for medium with CH during colonic fermentation, while negative prebiotic index was found for NC (Table 2), indicating an overlap of beneficial bacteria in detriment of undesirable bacteria during colonic fermentation. The increase of intestinal beneficial bacteria has been associated with the presence of phenolic compounds, especially phenolic acids, which are used as substrates in the human colon promoting prebiotic-like effects (DE SOUZA et al., 2019; MASSA et al., 2022). Prebiotics increase the production of metabolites directly related to the balance of the gut microbiota and lower DM risk (Alarcón Yempén et al., 2021).

3.5 Measurements of sugars and phenolics during colonic fermentation

According to the correlation for the digested fluid, intestinal digestion

significantly reduced the retention of phenolics. The process is capable of reducing the number of phenolic compounds available for absorption due to the degradation of these compounds by digestive enzymes in the stomach and intestine (Castaldo et al., 2020).

The values of pH sugars and individual phenolics were measured before digestion, as well as during fermentation of CH to elucidate the metabolites derived from this process (are shown in Table 3). Media with CH had a decrease ($p \leq 0.05$) in pH values during the colonic fermentation. The main sugars identified in coffee husks were maltose, glucose, xylose, fructose, and arabinose. After colonic fermentation a decrease in all sugar contents was observed. Sugars are consumed by gut microbiota during fermentation with CH to produce metabolites such as acids, which are the metabolism of their end-products decreasing the pH values (MENEZES et al., 2021a)

The pH decrease ($p \leq 0.05$) could cause inhibition of the growth of pathogenic microorganisms, such as observed for *C. histolyticum* (Liu et al., 2020). In addition to sugars, the high content of non-digestible carbohydrates in CH, could induce the production of SCFA and, consequently, health benefits at the intestinal and systemic levels (de Albuquerque et al., 2021; Dong et al., 2020).

CH extract presented higher ($p \leq 0.05$) flavonoid contents before *in vitro* digestion when compared to medium with CH during colonic fermentation. According to Gao et al. (2006), only between 2 and 15% of flavonoids can be completely digested and absorbed by the body during gastrointestinal digestion. Total phenolic and flavonoid content remained similar ($p > 0.05$) throughout colonic fermentation. This behavior may be related to different interactions of various phenolic compounds with pH changes and the action of digestive enzymes and bile acids, as reported in previous studies with vegetable by-products (Quatrin et al., 2019).

Pearson's correlation heat map shown in Figure 3 illustrates the relationship between samples before and after colonic fermentation and phenolics. In this study, the samples were divided into four groups based on the cluster analysis [*e.g.*, extract, digested sample, fermentation with CH, and NC (without CH)].

A total of eighteen phenolic compounds were identified in the samples, including flavan-3-ol, benzoic acids, proanthocyanidins, flavonols, and stilbenes. The presence of CH was positively correlated with phenolic compounds. In contrast, fewer phenolic compounds were present in samples without CH.

Individual compounds present in CH extract, such as chlorogenic acid and gallic acid, correspond to the most expressive concentrations (Gao et al., 2006). Some genera, such as *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, are capable of metabolizing chlorogenic acid in the large intestine. These bacteria have specific enzymes, called chlorogenic enzymes, to degrade chlorogenic acid through colonic fermentation, where bacteria use this compound as a source of carbon and (Gonthier et al., 2006; Mortelé et al., 2022).

Gallic acid is a major compound in coffee husks and has antioxidant properties. Several species of bacteria in the intestinal tract, including *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Ruminococcus*, can metabolize it. These bacteria use specific enzymes to degrade gallic acid into smaller compounds (Mcdade et al., 2021). Studies report that such compounds present in coffee husks can reduce the absorption of glucose, preventing its release by the liver, due to the inhibition of glucose-6-phosphatase activity, as a consequent obstacle to the absorption of glucose in the small intestine, by preventing glucose-6-phosphate translocase 1 (Johnston et al., 2003, 2005; Mcdade et al., 2021).

Isomers of chlorogenic acid, gallic acid and other phenolic compounds found in CH were not detected after 48 h of colonic fermentation. Pérez-Burillo et al. (2019) reported similar effects of an in vitro digestion-fermentation of green and roasted coffee on the intestinal microbiota. Coffee husks contain compounds belonging to the class of hydroxycinnamates, which are metabolized by the intestinal microbiota, and may explain the reduction or not detection of polyphenols in fermented samples (Mcdade et al., 2021).

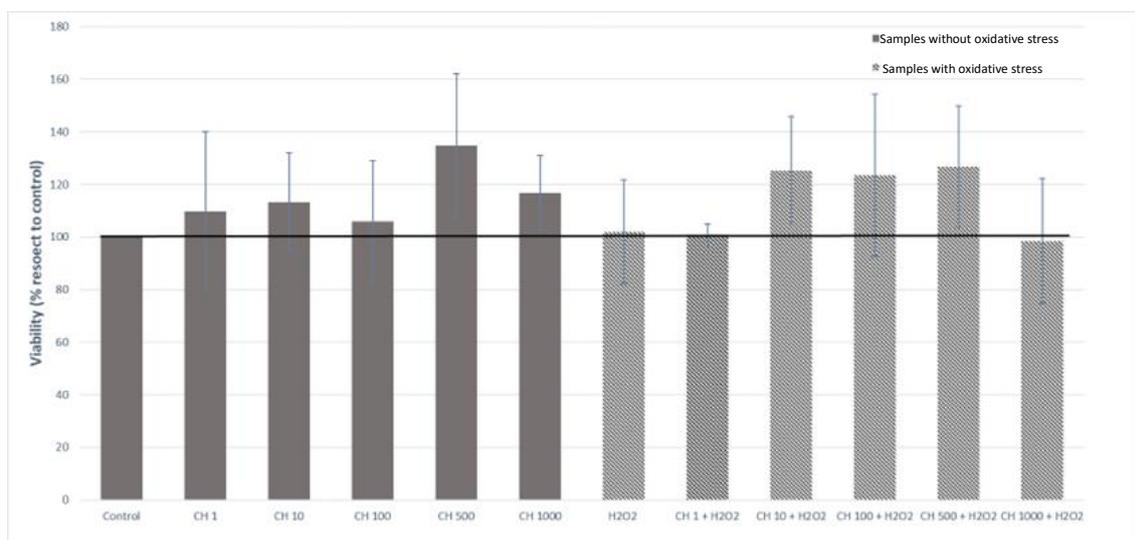
Caffeic acid is an isomer of chlorogenic acid, and caftaric acid is an ester of caffeic acid, meaning it is formed by the union of caffeic acid with tartaric acid (MCDADE et al., 2021; ZHENG et al., 2020) and its presence in the intestinal fluid may be linked to the bioconversion of chlorogenic acid into absorbable metabolites, which together with catechin, denoting the positive Pearson's correlation in its concentrations for the sample of this group. After 48 h of colonic fermentation, it is possible to observe the influence that catechin had on colonic metabolism through the generated metabolites belonging to the class of procyanidins, which are polyphenols found mainly in fruits and nuts, such as grapes, apples, and almonds. They are composed of catechin and epicatechin joined by ester and ester-ester linkages. Some of the procyanidin isomers include epicatechin, catechin, epicatechin-3-catechin, catechin-3-Epicatechin, and epicatechin-3-(2-catechin). These isomers vary in their chemical structure and, consequently, in their biological properties due to their possible positive effects on human health (Mcdade et al., 2021; Smeriglio et al., 2017).

According to a study conducted by van Duynhoven et al. (2011), catechin degradation is carried out by groups of bacteria of the genus *Actinobacteria* and *Clostridium*. In addition, other bacterial species, such as *Bacteroides*, *Eubacterium*, *Enterococcus*, and *Blautia*, are also involved in the process of converting glycosylated

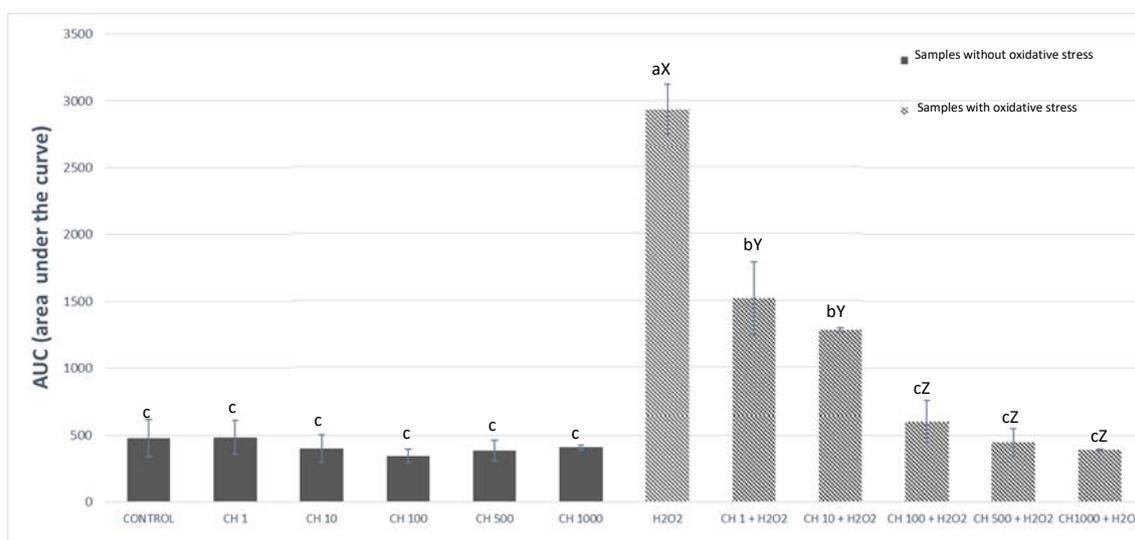
flavonoids into aglycones and their subsequent degradation into smaller phenolic compounds (Mithul Aravind et al., 2021).

4 CONCLUSIONS

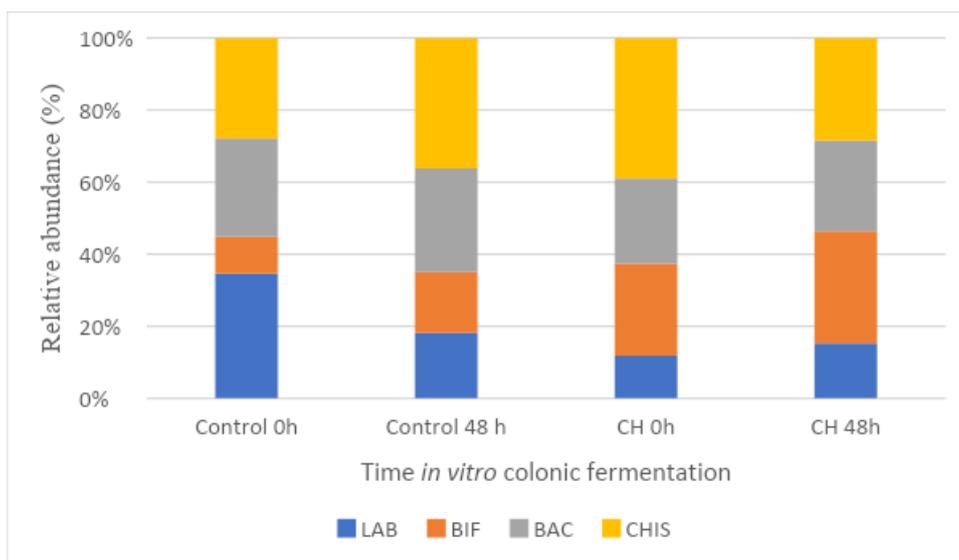
The phenolic compounds contained in coffee husks are administered as antioxidants during digestion, and this protection may help antioxidants reach cells intact and produce the desired effects. Caco-2 cells treated with the CH extract kept viability and were able to delay the induced oxidative damage even at high concentrations. The cytoprotective behavior of the phenolic compounds contained in CH provides support for future research and could lead to effects that promote intestinal function and nutrient absorption. The results of the in vitro colonic fermentation with fecal inoculum from diabetic patients showed a positive prebiotic index for CH, indicating an overlap of bacterial bacteria to the detriment of not beneficial bacteria during colonic fermentation. Moreover, the presence of CH led to an increase in the abundance of *Bifidobacterium* spp. during colonic fermentation. Based on these results, it can be assumed that CH modifies the intestinal microbiota and the metabolism of phenolic compounds, contributing to the improvement of the intestinal microbiota of diabetic patients. Organic CH can be an excellent starting point for the development of new safe and pesticide-free nutraceutical compounds, as they not only fulfill potential health-related claims, but also allow better exploration of bioactive compounds present in commonly discarded by-products.

Figure 1- Cytoprotection and ROS Accumulation Assay**A - Cell viability test**

Coffee husk extracts (CH) and H₂O₂ did not affect cell viability. Caco-2 cells were treated at different concentrations of coffee extracts (1, 10, 100, 500 and 1000 mg/ml) with or without oxidative stimulus (H₂O₂ 1 mM) for 3h. Values are expressed as mean \pm standard deviation (n = 3). The values did not indicate statistically significant differences (p < 0.05).

B – Evaluation of (ROS) production in Caco-2 cells

Coffee extract prevent oxidative stress induced by H₂O₂. Caco-2 cells were treated at different concentrations of coffee extracts (1, 10, 100, 500 and 1000 mg/ml) with or without oxidative stimulus (H₂O₂ 1 mM) for 3h. Values are expressed as mean \pm standard deviation (n = 3). Different letters indicate statistically significant differences (p < 0.05). (a, b, c) indicate statistical difference between all samples. The averages referring to the samples without the oxidative stimulus did not differ statistically (p < 0.05). (x, y, z) correspond to samples exposed to H₂O₂.

Figure 2 - Relative abundance of different bacterial groups

Relative abundance of different bacterial groups (% average \pm standard deviation, $n = 3$) in media with digested organic coffee husk bran (CH) and negative control (NC; without fermentable substrate) at zero (baseline), and 48 h of colonic fermentation. Lab 158 specific to *Lactobacillus* spp./*Enterococcus* spp.; Bif 164 specific to *Bifidobacterium* spp.; Bac 303 specific to *Bacteroides* spp./*Prevotella* spp. and Chis 150 specific to *C. histolyticum*.

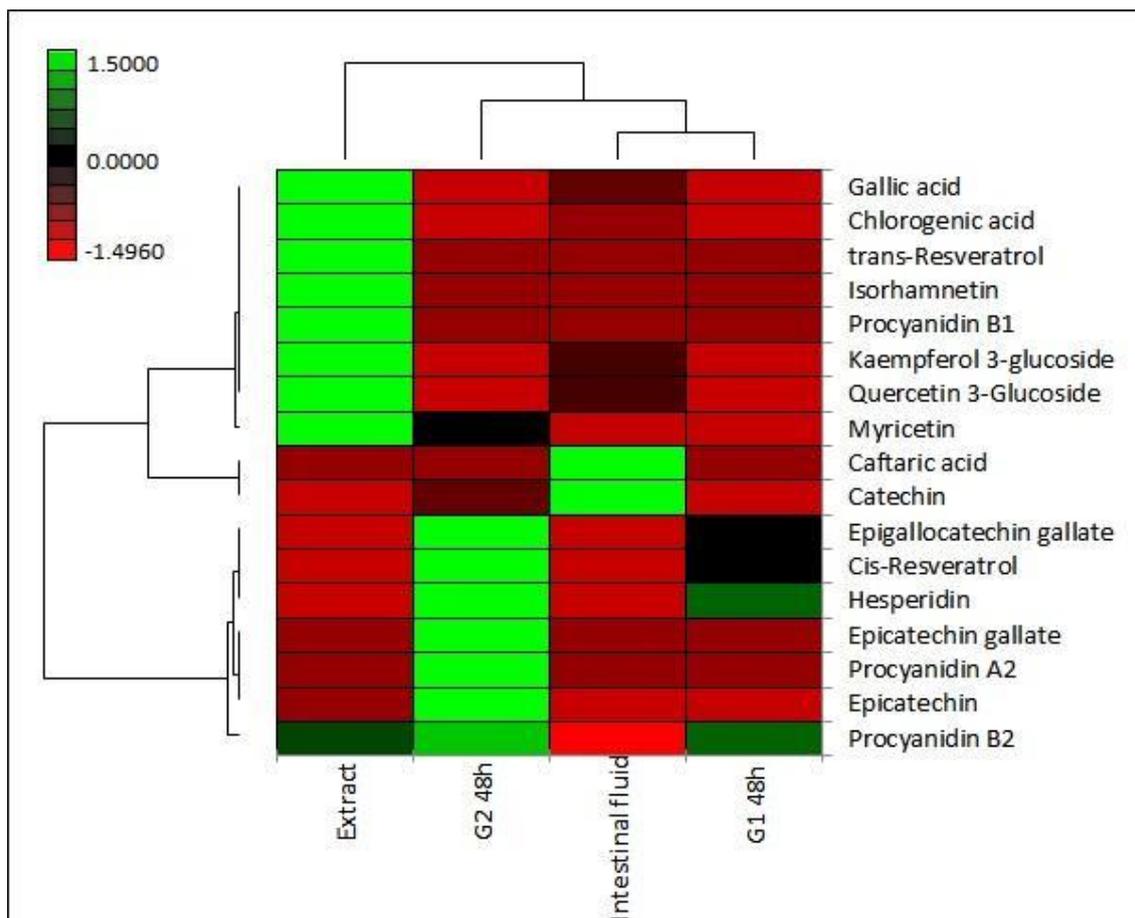


Figure 3 - Map of the Pearson's correlation for phenolic compound profile variable data measured in digested organic coffee husk medium during 48 h of in vitro colonic fermentation. Intestinal fluid corresponds to zero fermentation time. G1 represents the negative control (fermentation medium without husk), G2 the intestinal fluid digested with coffee husk and extract of organic coffee husk.

Table 1. Antioxidant activity of organic coffee husk before and during digestion ($\mu\text{mol EqT/g}$), and antioxidant capacity remaining (%) (average \pm standard deviation; $n = 3$).

Method	Antioxidant activity			
	Coffee Husk	Gastric phase	Intestinal phase Bioaccessible	ACR (%)
DPPH $\mu\text{mol EqT/g}$	1995,5 \pm 1,84 ^A	1171,2 \pm 4,1 ^B	1139,0 \pm 4,00 ^B	57,07*
FRAP $\mu\text{mol EqT/g}$	2043,1 \pm 8,21 ^A	1931,66 \pm 3,27 ^B	1883,98 \pm 2,75 ^C	92,21**
ORAC $\mu\text{M EqT/g}$	65,44 \pm 3,12 ^A	40,78 \pm 0,65 ^B	33,48 \pm 0,19 ^C	51,16***

A – C: Different superscript capital letters in the same row for the same method denote differences ($p \leq 0.05$), based on Tukey's test; */**/** in the antioxidant capacity remaining (ACR) of the fluids (column).

Table 2. Prebiotic index (mean \pm standard deviation; n = 3) calculated for media with digested organic coffee husk bran (CH) and negative control (NC; without fermentable substrate) at 48 h of colonic fermentation.

Bacterial groups	Relative difference	
	NC	CH
<i>Lactobacillus</i> spp./ <i>Enterococcus</i> spp.	-0.83 \pm 0.12 ^{Bc}	1.03 \pm 0.06 ^{Ac}
<i>Bifidobacterium</i> spp	0.94 \pm 0.15 ^{Bb}	2.02 \pm 0.18 ^{Aa}
<i>Bacteroides</i> spp./ <i>Prevotella</i> spp.	0.83 \pm 0.07 ^{Bb}	1.43 \pm 0.22 ^{Ab}
<i>Clostridium histolyticum</i>	1.55 \pm 0.21 ^{Aa}	0.74 \pm 0.13 ^{Bd}
Prebiotic index	-2.27 \pm 0.19 ^B	0.88 \pm 0.05 ^A

A – B: Different superscript capital letters in the same row for the same bacterial group or prebiotic index denote differences ($p \leq 0.05$), based on Student's t test; a-d: different superscript small letters in the same column for the same fermentation media denote difference ($p \leq 0.05$), based on Tukey's test.

Table 3 – pH values, contents of total phenolics (mg CAE/g), flavonoids (mg CE/g) and sugars (g/100g) (average \pm standard deviation; n = 3) in medium with digested organic coffee husk bran (CH) before digestion and after colonic fermentation.

Parameters	Fermentation Time		
	Before digestion	0 h	48 h
pH	4.97 \pm 0.06 ^C	8.77 \pm 0.01 ^A	7.79 \pm 0.02 ^B
Flavonoids mg CE/g	9.00 \pm 1.05 ^A	3.24 \pm 0.12 ^B	3.81 \pm 0.35 ^B
Maltose (g/100g)	3.72 \pm 0.95	<LOD	<LOD
Glucose (g/100g)	3.40 \pm 0.35	<LOD	<LOD
Xylose (g/100g)	3.32 \pm 0.11	<LOD	<LOD
Fructose (g/100g)	1.62 \pm 0.10 ^A	0.25 \pm 0.01 ^B	<LOD
Arabinose (g/100g)	6.44 \pm 0.04	<LOD	<LOD

<LOD: below the limit of detection. A – C: Different superscript capital letters in the same row denote differences ($p \leq 0.05$), based on Tukey's test.

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5 CONCLUSÃO

A partir dos resultados obtidos na referente pesquisa executada, pode-se relatar que os três extratos de casca de café orgânico foram eficazes como fonte de macroantioxidantes e cumpriram os efeitos antioxidantes, bioacessibilidade e atividade anticoagulante.

Sobre a comparação entre as formas de extração, PE destacou-se como mais eficiente na maioria das estimativas, no entanto (PNE - H e PNE) também apresentaram resultados favoráveis e até superiores ao primeiro extrato (PE), mostrando que seus compostos foram capazes de resistir as condições empregadas, dando ênfase a atividade antioxidante do radical ABTS e atuando na extensão do tempo da tromboplastina e protrombina, o que caracteriza possível potencial determinante em benefício da homeostase. As frações de polifenóis não extraíveis foram capazes de ocasionar aproveitamento total da matriz, assim como propriedades adicionais, pois favoreceram a retenção/bioacessibilidade de compostos fenólicos mesmo em pequenas concentrações.

Compostos fenólicos presentes na casca do café contribuíram para o bom potencial antioxidante durante a digestão e essa proteção pode ajudar a garantir que os antioxidantes cheguem intactos até as células exercendo efeitos benéficos. As células Caco-2 tratadas com extrato de CH alcançaram viabilidade e mesmo em baixas concentrações foi capaz de retardar o dano oxidativo induzido. O comportamento citoprotetor de compostos fenólicos presentes em CH dão aporte para futuras pesquisas e podem direcionar a efeitos que auxiliam a função intestinal e absorção de nutrientes.

Os resultados deste estudo demonstraram que a fermentação colônica *in vitro*, com inóculo fecal de diabéticos apresentou índice prebiótico positivo para CH, enquanto índice prebiótico negativo foi encontrado para NC, indicando uma sobreposição de bactérias benéficas em detrimento de bactérias indesejáveis durante a fermentação. Além disso, a presença de CH resultou no aumento da abundância do gênero *Bifidobacterium*. Com base nesses achados é possível considerar que CH modifica a microbiota intestinal e metabolização de compostos fenólicos podendo contribuir para melhorar a microbiota intestinal de indivíduos diabéticos, onde essas evidências podem influenciar na integridade da barreira intestinal, assim como a melhora de absorção e controle da glicemia nesses indivíduos.

Tais resultados sugerem que os extratos de polifenólicos da casca de café orgânico e a própria casca de café podem servir como um excelente ponto de partida para desenvolvimento de

novos agentes nutracêuticos seguros e livres de defensivos agrícolas, uma vez que além de cumprir alegações e potencial de saudabilidade, também incentiva a melhor exploração de compostos bioativos presentes nos subprodutos comumente descartados.

ANEXOS

ANEXO A – CERTIDÃO DO COMITÊ DE ÉTICA EM PESQUISA

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: CASCA DE CAFÉ ORGÂNICO: UM ESTUDO DA BIOACESSIBILIDADE DE COMPOSTOS FENÓLICOS E SUAS POTENCIALIDADES BIOLÓGICAS

Pesquisador: Thaianaly Leite Abreu

Área Temática:

Versão: 2

CAAE: 65756222.4.0000.5188

Instituição Proponente: Centro De Ciências da Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.907.145

Apresentação do Projeto:

O projeto intitulado: CASCA DE CAFÉ ORGÂNICO: UM ESTUDO DA BIOACESSIBILIDADE DE COMPOSTOS FENÓLICOS E SUAS POTENCIALIDADES, pertence ao PPGCTA/UFPB/CT.

Introdução:

O Brasil destaca-se como o principal produtor e exportador mundial de café, apresentando forte impacto na economia do país. De acordo com a International Coffee Organization (ICO, 2021a), na safra 2020/2021, que compreende o período de abril de 2020 até março de 2021, o país foi responsável por 39,4% da produção internacional e 78,2% da produção do continente sul-americano (ICO, 2021b). Segundo Baqueta et al. (2017), o processamento do grão de café, ocasiona elevada quantidade de subprodutos, com destaque para as cascas, que após beneficiamento dos grãos, constitui aproximadamente o percentual de 50% da produção total, ou seja, o peso de café beneficiado é aproximadamente igual ao peso de subproduto gerado casca (pele, polpa, mucilagem e pergaminho). Os consumidores de café mudaram seus hábitos e adotaram uma nova percepção da bebida, somada a produção, com redução de impactos ambientais e particularidades de cultivos que implicam na segurança alimentar. Desta forma o processamento do café orgânico, juntamente ao aproveitamento de seus subprodutos é

Endereço: Prédio da Reitoria da UFPB ç 1º Andar

Bairro: Cidade Universitária

CEP: 58.051-900

UF: PB

Município: JOAO PESSOA

Telefone: (83)3216-7791

Fax: (83)3216-7791

E-mail: comitedeetica@ccs.ufpb.br

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incentivado visando agregar valor econômico e reduzir os desperdícios, consequente mantendo as propriedades nutricionais e os compostos bioativos (MATOSO et al., 2020; MEYBECK; GITZ, 2017). Nos últimos anos estudos avaliam a composição química da casca de café, com ênfase nos compostos bioativos, atividade antioxidante, assim como seu metabolismo, bioacessibilidade, propriedades biológicas resultantes do elevado teor de compostos fenólicos e macroantioxidantes vinculados às fibras dietéticas (JANISSEN; HUYNH, 2018a; SILVA et al., 2020). A fibra alimentar ou dietética (FD), componente não digerível presente em materiais vegetais, pode ser dividida em fibra dietética solúvel (FDS) e insolúvel (FDI) de acordo com a solubilidade em água, podem agregar funcionalidade e aspectos tecnológicos a matriz alimentar (DONG et al., 2020). Os macroantioxidantes vinculados as fibras são divididos em duas diferentes frações: os polifenóis hidrolisáveis (PFH), fenólicos de baixo peso molecular fortemente associados a proteínas e polissacarídeos; e os polifenóis não extraíveis (PNE) e as proantocianidinas não extraíveis (PANE) que são estruturas mais complexas de alto peso molecular. (PÉREZ -JIMÉNEZ; SAURA-CALIXTO, 2018). Esses compostos representam uma fração emergente e bastante promissora, porém ainda são pouco estimados, visto que correspondem a uma quantidade significativa que permanece nos resíduos das extrações convencionais de fenólicos e não são recuperados, contudo, são capazes de agregar efeitos nas propriedades biológicas da matriz alimentar além causar influência direta na resposta fisiológica digestiva e cardiovascular (AYOUB; DE CAMARGO; SHAHIDI, 2016; MACAGNAN et al., 2015a) Avaliar a bioacessibilidade dos compostos fenólicos das cascas de café é o fator primordial a ser levado em consideração. A bioacessibilidade condiz ao efeito da digestão gastrointestinal (enzimas, pH e microbiota) nos biocompostos que se tornam acessíveis para potencial absorção na circulação sistêmica e tecidos alvo (PEÑA-VÁZQUEZ et al., 2022). Choi e Kim (2017) e Nam, Park e Nam (2020) alegam que componentes majoritários associados aos subprodutos de café (ácido clorogênico e ácido cafeico) desempenham importante papel no efeito da atividade antitrombótica in vitro, atividade de coagulação, efeito na função plaquetária e viabilidade celular, havendo assim a possibilidade de aprofundamento em novas pesquisas que incentivem tais efeitos e propriedades biológicas advindas da matriz (IRIONDO-DEHOND et al., 2019a). Nesse aspecto, a prospecção de compostos bioativos dos subprodutos oriundos do beneficiamento do café orgânico, faz-se necessário para incentivar a utilização e aproveitamento do ponto de vista nutricional e tecnológico desses subprodutos, visando a agregação de valor e redução da geração de resíduos com potencialidades inexploradas.

Hipótese:

Endereço: Prédio da Reitoria da UFPB ç 1º Andar
Bairro: Cidade Universitária **CEP:** 58.051-900
UF: PB **Município:** JOAO PESSOA
Telefone: (83)3216-7791 **Fax:** (83)3216-7791 **E-mail:** comitedeetica@ccs.ufpb.br

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Os macroantioxidantes presentes em subprodutos do processamento de café orgânico tropicais são bioacessíveis e possuem atividade biológica.

Metodologia Proposta:

ENSAIO DA DIGESTÃO GASTROINTESTINAL E FERMENTAÇÃO COLÔNICA in vitro O estudo de liberação de compostos bioativos presentes nas partículas utilizando fluidos gástrico e intestinal simulados, será realizado de acordo com adaptações da metodologia utilizada por Minekus et al. (2014). Uma alíquota de cada extrato: PE- etanol, PNE-H e PNE, será preparada para determinação do efeito das condições do trato gastrointestinal simulado. De acordo com o consenso internacional relatado pela INFOGEST, serão preparadas soluções estoques de eletrólitos e enzimas que compreendem as fases bucal, gástrica e intestinal. Todos os ensaios serão realizados em tubos Falcon estéreis de 50 mL, mantidos em um Shaker termostático agitado a 120 rpm a 37°C, para simulação das fases da digestão. Na fase oral, 5 g de amostra serão misturadas a 4 mL de fluido salivar (Tabela 5), 25 µL de CaCl₂ 0,3 M, 0,5 mL de solução aquosa de amilase 75 U.mL⁻¹ e 0,475 mL de água grau Milli-Q, mantendo-se a mistura incubada por 2 min. Após este período, inicia-se a etapa gástrica da digestão. Para tal, serão adicionados 8 mL de solução gástrica, 1 mL de solução aquosa de pepsina 2000 U.mL⁻¹ e 0,5 µL de CaCl₂ 0,3 M. Após a mistura das soluções, o pH será corrigido para 3,0 com auxílio de HCl 1M e o volume final (20 mL) será alcançado pela adição de água grau Milli-Q. As amostras serão, então, incubadas por 2 h. Ao fim da fase gástrica, a ação enzimática será interrompida em banho de gelo por 10 minutos. As amostras serão centrifugadas a 4°C e 5000 g por 30 minutos, sendo os sobrenadantes separados e armazenados sob congelamento até a realização das análises. A bioacessibilidade será expressa em porcentagem e determinada de acordo com a Equação 4 (DE ASSIS et al., 2021). Bioacessibilidade (%) = (CF digerido/CF não digerido) x 100 (Eq. 4) Onde: CF digerido corresponde à concentração do composto analisado das cascas de café digerida (fase intestinal) e CF não digerido a concentração do composto analisado das cascas de café não digeridas (extrato inicial).

3.5.1 Inóculo fecal humano e Sistema de Fermentação Colônica O procedimento será realizado conforme metodologia adaptada descrita por Medeiros et al. (2021). Amostras fecais de 4 voluntários adultos diabéticos (dois homens e duas mulheres, com idade entre 25 e 55 anos) serão utilizadas após aprovação do Comitê de Ética em Pesquisa com Seres Humanos (Universidade Federal da Paraíba, João Pessoa, Paraíba, Brasil). Os critérios de inclusão serão pessoas que não

Endereço: Prédio da Reitoria da UFPB ç 1º Andar
Bairro: Cidade Universitária **CEP:** 58.051-900
UF: PB **Município:** JOAO PESSOA
Telefone: (83)3216-7791 **Fax:** (83)3216-7791 **E-mail:** comitedeetica@ccs.ufpb.br

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sofressem de nenhuma doença gastrointestinal ou colônica, seguindo uma dieta onívora regular sem usar alimentos probióticos, probióticos concentrados ou prebióticos nas últimas 48h e sem antibióticos durante os seis meses anteriores ao estudo. Os doadores receberão instruções específicas além de um kit de coleta adequado. O kit de coleta de fezes incluiu tubos coletores esterilizados, espátulas descartáveis, instruções para coleta/envio da amostra, luvas descartáveis e álcool 70%. Além disso, as instruções contidas no kit descreverão as etapas de higienização das mãos e manuseio asséptico das amostras. As fezes serão coletadas em tubos estéreis. As amostras serão dispostas em uma jarra com um sistema gerador de anaerobiose (AnaeroGen, Oxoid, Basingstoke, Inglaterra) e transportados para o laboratório. Os experimentos serão iniciados imediatamente após a chegada das fezes para garantir uma microbiota fecal fresca, e as fezes serão manipuladas em ambiente sob anaerobiose. Para os experimentos, a mesma quantidade de fezes de cada doador será reunida e misturada com solução salina tamponada com fosfato estéril (PBS; 0,1 M; pH 7,4; 1:10, p/v) sob agitação (200 rpm) por 2 min. Cada lote será composto por 40% do meio de fermentação (v/v), 40% de inóculo fecal humano (v/v) e 20% da farinha de casca de café digerida (p/v). A fermentação será realizada a 37°C por 24, 48 e 72h em condições anaeróbicas (AnaeroGen).

Critério de Inclusão:

Sujeitos de ambos os sexos diabéticos com idade entre 25-55

Critério de Exclusão:

Sujeitos que façam o uso de qualquer antibiótico nos 6 meses anteriores a pesquisa, tabagistas, etilistas ou portadores de doenças intestinais crônicas ou agudas (diarreia) e portadores de doenças infecto-contagiosas.

Objetivo da Pesquisa:

Objetivo Primário: Investigar o conteúdo de macroantioxidantes, a bioacessibilidade e potenciais biológicos de polifenóis presentes em casca de café orgânico.

Objetivo Secundário: · Avaliar o farelo obtido da casca de café orgânico quanto ao conteúdo e composição, · Determinar o perfil de polifenóis extraíveis, polifenóis não extraíveis hidrolisáveis e proantocianidinas não hidrolisáveis no farelo de casca de café orgânico, · Determinar a atividade antioxidante das frações de polifenóis e das fibras alimentares no farelo de casca de café orgânico,

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Bairro: Cidade Universitária **CEP:** 58.051-900
UF: PB **Município:** JOAO PESSOA
Telefone: (83)3216-7791 **Fax:** (83)3216-7791 **E-mail:** comitedeetica@ccs.ufpb.br

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· Avaliar a bioacessibilidade e fermentação colônica dos polifenóis presentes no farelo de casca de café orgânico; · Determinar o potencial prebiótico da casca de café orgânico; · Avaliar o potencial hipoglicemiante do farelo de casca de café orgânico

Avaliação dos Riscos e Benefícios:

A pesquisa não oferece nenhum risco aos participantes voluntários.

Comentários e Considerações sobre a Pesquisa:

Para o desenvolvimento da pesquisa será necessária a adesão de 4 voluntários diabéticos adultos, doadores de amostra fecal (dois homens e duas mulheres, com idade entre 25 e 55 anos). As amostras serão utilizadas para execução da pesquisa após aprovação do Comitê de Ética em Pesquisa com Seres Humanos (Universidade Federal da Paraíba, João Pessoa, Paraíba, Brasil). Os critérios de inclusão serão pessoas que não sofressem de nenhuma doença gastrointestinal ou colônica, seguindo uma dieta onívora regular sem usar alimentos probióticos, probióticos concentrados ou prebióticos nas últimas 48h e sem antibióticos o durante os seis meses anteriores ao estudo. Os doadores receberão instruções específicas além de um kit de coleta adequado. O kit de coleta de fezes incluiu tubos coletores esterilizados, espátulas descartáveis, instruções para coleta/envio da amostra, luvas descartáveis e álcool 70%. Além disso, as instruções contidas no kit descreverão as etapas de higienização das mãos e manuseio asséptico das amostras. As fezes serão coletadas em tubos estéreis sem custo algum.

Considerações sobre os Termos de apresentação obrigatória:

Considerando que os termos do projeto encontram-se de acordo com a Resolução 466/12, e a Normativas Operacionais 001/2013.

Recomendações:

Recomenda-se aprovar com uma ressalva!

A pesquisadora tem que colocar no projeto que existe risco na pesquisa, mesmo que esse risco seja um simples constrangimento do avaliador(a) na hora da participação das análises!

Conclusões ou Pendências e Lista de Inadequações:

Aprovado

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UF: PB	Município: JOAO PESSOA
Telefone: (83)3216-7791	Fax: (83)3216-7791
	E-mail: comitedeetica@ccs.ufpb.br

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Considerações Finais a critério do CEP:

Certifico que o Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal da Paraíba – CEP/CCS aprovou a execução do referido projeto de pesquisa. Outrossim, informo que a autorização para posterior publicação fica condicionada à submissão do Relatório Final na Plataforma Brasil, via Notificação, para fins de apreciação e aprovação por este egrégio Comitê.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_P ROJETO_1999449.pdf	19/12/2022 10:04:46		Aceito
Cronograma	CRONOGRAMA.docx	02/12/2022 12:21:24	Thaianaly Leite Abreu	Aceito
Folha de Rosto	FolhaDeRostoThaianalyOk.pdf	02/12/2022 12:10:23	Thaianaly Leite Abreu	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoCEP.pdf	14/09/2022 00:18:48	Thaianaly Leite Abreu	Aceito
Parecer Anterior	CertidaoParaComite.pdf	14/09/2022 00:18:14	Thaianaly Leite Abreu	Aceito
Outros	questionario2.pdf	14/09/2022 00:15:23	Thaianaly Leite Abreu	Aceito
Declaração de Instituição e Infraestrutura	Anuencia.pdf	14/09/2022 00:06:17	Thaianaly Leite Abreu	Aceito
Orçamento	orcamento.docx	12/09/2022 01:13:07	Thaianaly Leite Abreu	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Pesquisa.pdf	12/09/2022 01:08:36	Thaianaly Leite Abreu	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Telefone: (83)3216-7791 **Fax:** (83)3216-7791 **E-mail:** comitedeetica@ccs.ufpb.br

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JOAO PESSOA, 23 de Fevereiro de 2023

Assinado por:
Eliane Marques Duarte de Sousa
(Coordenador(a))

Endereço: Prédio da Reitoria da UFPB 6 1º Andar
Bairro: Cidade Universitária **CEP:** 58.051-900
UF: PB **Município:** JOAO PESSOA
Telefone: (83)3216-7791 **Fax:** (83)3216-7791 **E-mail:** comitedeetica@ccs.ufpb.br

ANEXO B – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**UNIVERSIDADE FEDERAL DA PARAÍBA****CENTRO DE TECNOLOGIA****PROGRAMA DE PÓS – GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE
ALIMENTOS**

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Título do Projeto de Pesquisa: Casca de Café Orgânico: Um Estudo Da Bioacessibilidade de Compostos Fenólicos e suas Potencialidades Biológicas

Pesquisador Responsável: Mst^a. Thaianaly Leite Abreu

Instituição/Departamento: Universidade Federal da Paraíba – CT/PPGCTA

Telefone para contato: (83)99852-9642 E-mail: thaianaly.abreu@academico.ufpb.br

Pesquisadores participantes: Dr^a Taliana Kênia Alencar Bezerra e Dr^a Marta Suely Madruga

Prezado (a) Senhor (a)!

Esta pesquisa é sobre avaliação do efeito prebiótico de casca de café orgânico utilizando inóculo fecal humano e está sendo desenvolvida pela pesquisadora Mst^a Thaianaly Leite Abreu, aluna regular do Programa de Pós-Graduação em Ciências e Tecnologia de Alimentos, da Universidade Federal da Paraíba (UFPB), sob a orientação do Prof^ª. Dr^a. Taliana Kênia Alencar Bezerra, professora do mesmo programa de pós-graduação e do departamento de Engenharia de Alimentos.

Você está sendo convidado(a) para participar, como voluntário, em uma pesquisa. Você precisa decidir se quer participar ou não. Por favor, não se apresse em tomar a decisão. Leia cuidadosamente o que se segue e pergunte ao responsável pelo estudo qualquer dúvida que você tiver. Após ser esclarecido(a) sobre as informações a seguir, no caso de aceitar fazer parte do

estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa você não será penalizado(a) de forma alguma.

Solicitamos a sua colaboração para realizar a doação de material fecal para execução de um projeto de pesquisa intitulado “Casca de Café Orgânico: Um Estudo Da Bioacessibilidade de Compostos Fenólicos e suas Potencialidades Biológicas” que tem como objetivo avaliar se a casca café orgânico é uma fonte de prebióticos para fermentação colônica *in vitro* da microbiota de indivíduos diabéticos. Os voluntários serão convocados em duas ocasiões. Na primeira ocasião, após o recrutamento, você será convidado a responder o questionário, aplicado pelo pesquisador responsável, sobre hábitos alimentares e saúde. Na segunda ocasião, previamente agendada para a entrega das fezes frescas de consistência sólida.

RISCOS E DESCONFORTOS PARA OS PARTICIPANTES

O voluntário que se sentir constrangido ao responder ao questionário ou alguma pergunta específica do questionário poderá neste momento abandonar este estudo sem prejuízo algum a ambas as partes.

Além disso, você poderá se sentir constrangido durante a coleta das fezes e no transporte das mesmas podendo desistir de participar do estudo neste momento. Não há benefício direto para o participante.

BENEFÍCIOS ESPERADOS

A realização desta pesquisa fornecerá informações científicas relevantes e atualmente escassas no campo de estudo.

GARANTIA DE SIGILO

O pesquisador tomará todas as medidas para manter suas informações pessoais (como nome, endereço e outras) em sigilo. Durante todo o estudo e mesmo depois de sua conclusão, quando os resultados deste estudo forem publicados em revistas científicas ou apresentados em congressos ou reuniões, a sua identidade será guardada em segredo, não sendo revelada qualquer informação a seu respeito que possa identificar você publicamente. Contudo, durante o estudo, as pessoas envolvidas diretamente na pesquisa e o Comitê de Ética poderão ter acesso aos seus dados. Mesmo assim, os seus dados serão preservados e não serão divulgados publicamente.

PERÍODO DE PARTICIPAÇÃO

Sua participação na pesquisa terá duração de 15 dias. Você poderá retirar-se da pesquisa em qualquer momento, antes ou durante a mesma, sem penalidades ou prejuízo.

GARANTIA DE ACESSO

Em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas.

Consentimento da participação da pessoa como sujeito

Eu, _____,

portador do RG _____ abaixo assinado, concordo em participar do presente estudo, como voluntário. Fui suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo “UM ESTUDO DA BIOACESSIBILIDADE DE COMPOSTOS

FENÓLICOS E SUAS POTENCIALIDADES BIOLÓGICAS”. Eu discuti com os pesquisadores sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo.

Local e data

Nome e Assinatura do Voluntário

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste voluntário de pesquisa.

João Pessoa _____, de _____ de 20____

Pesquisador responsável

Atenciosamente,

Endereço (Setor de Pesquisa): Laboratório de Processos Microbianos em Alimentos,
Departamento de Engenharia de Alimentos do Centro de Tecnologia da Universidade Federal
da Paraíba (UFPB), Campus I - Cidade Universitária – CEP 58051-900 – João Pessoa – PB,
Telefone: (83) 3216-7576

Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal da
Paraíba Campus I - Cidade Universitária - 1º Andar – CEP 58051-900 – João Pessoa/PB

(83) 3216-7791 – E-mail: comitedeetica@ccs.ufpb.br