LAISE TRINDADE PAES

FUNCTIONAL POTENTIAL OF PHENOLIC-RICH TOASTED SORGHUM FLOURS IN CANCER PREVENTION AND GUT MICROBIOTA MODULATION

Thesis submitted to the Graduate Program in Food Science and Technology of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Advisor: Frederico Augusto Ribeiro de Barros

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VIÇOSA – MINAS GERAIS
2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
P126f
2024

Paes, Laise Trindade, 1994-
Functional potential of phenolic-rich sorghum toasted flours in cancer prevention and gut microbiota modulation / Laise Trindade Paes. – Viçosa, MG, 2024.
1 tese eletrônica (137 f.): il. (algumas color.).

Texto em inglês.
Inclui anexos.
Orientador: Frederico Augusto Ribeiro de Barros.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, 2024.
Referências bibliográficas: f.33-41.
DOI: https://doi.org/10.47328/ufvbbt.2024.555
Modo de acesso: World Wide Web.

1. Farinha de sorgo - Uso terapêutuco. 2. Antioxidantes. 3. Colón (Anatomia) - Câncer - Prevenção. 4. *Plasmodium falciparum*. 5. Fenóis. 6. Microbioma gastrointestinal. I. Barros, Frederico Augusto Ribeiro de, 1983-. II. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

CDD 22. ed. 664.7207

Bibliotecário(a) responsável: Alice Regina Pinto Pires CRB-6/2523

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APPROVED ON 6TH OF JUNE 2024:

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VIÇOSA – MINAS GERAIS
2024

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to the people who have supported me throughout this journey. First and foremost, to my mother, Domingas, for her unwavering love and encouragement. To my sisters, Nágila and Jamille, whose support has been invaluable.

I am deeply grateful to my friends Raphael, Tiago, Warryson, Bianca, Jeisa, Laura, Gustavo, Karina, and Tatiana. Your emotional support helped me endure the difficult days, and for that, I am profoundly thankful. To Laura Cabral, thank you for assuring me that everything would work out and for your admiration, which made me believe I could achieve more even when I felt like a failure.

To the team at the Biocarb laboratory for being with me, especially Leonara, Fabiana, and Júlia, thank you for the insightful conversations and assistance with analyses. Your help was essential. Rodrigo, your words prevented me from giving up on this doctorate. Thank you for your encouragement.

A special thank you to my advisor, Frederico, for motivating me to persevere and for guiding me through each step with your academic advice. I have grown tremendously over these four years, and it is largely thanks to you.

I am also grateful to my co-advisors, Luciana Azevedo, Hércia Martino, and Bruce Hamaker, for receiving me in their laboratories to perform this work and for their valuable contributions to my research.

I extend my gratitude to Capes for the scholarship (finance code 001), to the program Capes PrInt for the scholarship to do my research at Purdue University, Fapemig for the financial support, and the Department of Food Technology and the Federal University of Viçosa, the most beautiful university in Brazil. I am proud to have written my story with this team and in this place.

From where I came, in my childhood, I never dreamed of becoming a doctor. I will be the first in my family to achieve a doctorate, but I will not be the last.

Thank you all for being a part of this journey!

RESUMO

PAES, Laise Trindade, M.Sc./D.Sc., Universidade Federal de Viçosa, junho de 2024. **Potencial Funcional das Farinhas Torradas de Sorgo Ricas em Fenólicos na Prevenção do Câncer e Modulação da Microbiota Intestinal**. Orientador: Frederico Augusto Ribeiro de Barros. Co-orientadores: Bruce R. Hamaker, Hércia Stampini Duarte Martini e Luciana Azevedo.

O sorgo, rico em fibras alimentares e compostos fenólicos, oferece promissores benefícios à saúde. O objetivo desta tese foi investigar: 1) a bioatividade de extratos ricos em fenólicos de sorgo contra células cancerosas e o plasmódio causador da malária; 2) os potenciais efeitos protetores das farinhas torradas de sorgo branco e com taninos contra os estágios iniciais do câncer de cólon induzido por dimetilhidrazina (DMH) em estágio inicial em ratos; e 3) a influência das farinhas torradas de sorgo e dos seus extratos fenólicos na microbiota intestinal *in vitro*. No estudo *in vitro*, utilizando água aquecida e 70% etanol/água para gerar extratos fenólicos das farinhas torradas de sorgo, foram identificados 145 compostos fenólicos. O extrato etanólico de sorgo branco (WSE) mostrou o menor IC_{50} nas células cancerosas EA.hy926 e A549, enquanto o extrato etanólico de sorgo com taninos (TSE) apresentou o menor IC_{50} nas células HCT-8. Todos os extratos reduziram a adesão e invasão celular de HCT-8, indicando potencial antimetastático. O WSE também demonstrou toxicidade para as cepas de *Plasmodium falciparum* resistentes e sensíveis à cloroquina. No estudo *in vivo*, 40 ratos foram divididos em 4 grupos e submetidos a várias dietas, incluindo controle (G1), dieta com farinha torrada de sorgo com taninos e com farinha torrada de sorgo branco com indução por DMH (G2, G3, respectivamente) e apenas DMH (G4). As avaliações de estresse oxidativo indicaram uma atividade aumentada de superóxido dismutase (SOD) e glutationa (GSH) no fígado de ratos alimentados com farinha torradas de sorgo com taninos, sugerindo uma defesa contra o estresse oxidativo induzido por DMH. Além disso, os ensaios de focos de criptas aberrantes (ACF) demonstraram uma menor incidência de ACF em ratos suplementados com farinhas de sorgo (G2: 51,75 ± 26,84, G3: 64,38 ± 18 ACF ≤ 3 criptas), sugerindo um efeito preventivo contra a carcinogênese. Adicionalmente, a determinação de ácidos graxos de cadeia curta (SCFA) revelou níveis mais altos de acetato no grupo suplementado com farinha de sorgo branco (G3: 36,12 ± 7,01 mM) em comparação com outros grupos, e maior concentração de propionato observados no mesmo grupo (G3: 10,51 ± 1,43 mM) em comparação com o controle negativo (G1). A fermentação fecal *in vitro* das farinhas de sorgo branco (WSD) e com taninos (TSD) digeridas, bem como dos extratos de sorgo branco (WSE) e com taninos (TSE) com frutooligossacarídeos (FOS) mostrou a maior concentração de acetato em amostras de extratos fenólicos de sorgo branco (WSE) e extratos fenólicos de sorgo com taninos (TSE) com FOS.

Os níveis de propionato foram mais altos em WSE + FOS, seguidos por TSE + FOS, em comparação com FOS sozinho. O WSD suplementado com seu extrato fenólico, WSE, mostrou níveis aumentados de acetato e propionato, ao contrário de TSD + TSE, enfatizando a influência dos compostos fenólicos na modulação de SCFA. Esses achados destacam o papel promissor das farinhas torradas de sorgo no combate ao estresse oxidativo e na proliferação de células cancerosas *in vitro*, na mitigação da carcinogênese de cólon em estágio inicial e na modulação da microbiota intestinal e dos SCFA, justificando uma exploração adicional de seus potenciais preventivos e terapêuticos.

Palavras-chave: Atividade antioxidante; Carcinogênese do cólon; *Plasmodium falciparum*; Composição fenólica; Modulação da microbiota intestinal.

ABSTRACT

PAES, Laise Trindade, M.Sc./D.Sc., Universidade Federal de Viçosa, June, 2024. **Functional Potential of Phenolic-Rich Sorghum Toasted Flours in Cancer Prevention and Gut Microbiota Modulation**. Advisor: Frederico Augusto Ribeiro de Barros. Co-advisors: Bruce R. Hamaker, Hércia Stampini Duarte Martini, and Luciana Azevedo.

Sorghum, rich in dietary fibers and phenolic compounds, offers promising health benefits. The aim of this thesis was to investigate: 1) the bioactivity of sorghum phenolic-rich extracts against cancer cells and malaria-causing Plasmodium; 2) the potential protective effects of white and tannin toasted sorghum flours against early-stage dimethylhydrazine (DMH)-induced colon cancer in rats, and 3) the influence of these flours and their phenolic extracts on gut microbiota. In the *in vitro* study, using warm water and 70% ethanol/water as solvents to generate phenolic-rich extracts from sorghum toasted flours, 145 phenolic compounds were identified. White sorghum ethanolic extract (WSE) showed the lowest IC_{50} on EA.hy926 and A549 cancer cells, while tannin sorghum ethanolic extract (TSE) showed the lowest IC_{50} on HCT-8 cells. All extracts reduced the adhesion and invasion of HCT-8 cells, indicating antimetastatic potential. WSE also demonstrated toxicity to both chloroquine-resistant and sensitive strains of *Plasmodium falciparum*. In the *in vivo* study, 40 male rats were divided into 4 groups and subjected to various diets, including control (G1), tannin and white toasted sorghum flour diets with DMH colon induction (G2, G3, respectively), and DMH only (G4). Oxidative stress assessments indicated an enhanced activity of superoxide dismutase (SOD) and glutathione (GSH) in the liver of rats fed with tannin-rich sorghum flour, suggesting improved defense against oxidative stress induced by DMH. Moreover, aberrant crypt foci (ACF) assays demonstrated a lower incidence of ACF in rats supplemented with sorghum flours (G2: 51.75 ± 26.84, G3: 64.38 ±18 ACF ≤ 3 crypts), suggesting a preventive effect against carcinogenesis. Additionally, determination of short-chain fatty acids (SCFAs) revealed higher acetate levels observed in the group supplemented with white sorghum flour (G3: 36.12 ± 7.01 mM) compared to other groups, and higher propionate levels observed in the same group (G3: 10.51 ± 1.43 mM) compared to the negative control (G1). *In vitro* fecal fermentation of digested white (WSD) and tannin sorghum flours (TSD), as well as white (WSE) and tannin sorghum extract (TSE) with fructooligosaccharides (FOS) showed the highest acetate concentration in samples of white sorghum phenolic extracts (WSE) and tannin sorghum phenolic extracts (TSE) with FOS. Propionate levels were highest in WSE + FOS, followed by TSE + FOS, compared to FOS alone. WSD supplemented with its phenolic extract, WSE, showed increased acetate and propionate levels, unlike TSD + TSE, emphasizing the influence of phenolic compounds on modulation of SCFA. These findings highlight the promising role of toasted

sorghum flours in fighting oxidative stress and cancer cells proliferation *in vitro*, in mitigating early-stage colon carcinogenesis, and modulating gut microbiota and SCFA, warranting further exploration of their preventive and therapeutic potentials.

Keywords: Antioxidant activity; Colon carcinogenesis; *Plasmodium falciparum*; Phenolic composition; Gut microbiota modulation.

SUMMARY

1. INTRODUCTION

In the landscape of modern dietary habits, there is a concerning rise in health issues associated with poor nutrition. Non-communicable chronic diseases such as cardiovascular diseases, type 2 diabetes, and intestinal-related conditions are becoming more common, being associated to dietary factors and posing significant challenges for affected individuals worldwide (Shan et al., 2020; Magliano et al., 2019; Timmis et al., 2022; Narula et al., 2021). These conditions often stem from diets rich in processed foods, high in sugars, unhealthy fats, and low in essential nutrients like fiber, vitamins, and minerals (English et al., 2021; Shan et al., 2020). In fact, dietary pattern poses as a risk factor even for various types of cancer, including colon cancer (Sawicki et al., 2021; World Cancer Research Fund International, 2018). Therefore, this dietary imbalance not only compromises overall health but also increases the risk of chronic diseases, thereby diminishing individuals' quality of life.

In response to these alarming trends, there is a growing interest in identifying healthier dietary options. In this context, whole grain flours offer a multitude of advantages for human health (Seal et al., 2021). They are abundant sources of dietary fiber, which plays a crucial role in promoting intestinal health (Gong et al., 2018), regulating blood sugar and cholesterol levels, and aiding in weight management (Reynolds et al., 2020). Additionally, these flours contain a diverse array of bioactive compounds, such as phenolic compounds, which possess antioxidant, anticancer, and anti-inflammatory properties (Xu et al., 2021; Ma et al., 2021). These compounds act synergistically to protect against cellular damage, reduce inflammation, and mitigate the risk of chronic diseases (Whent et al., 2012; van Hung, 2015).

Sorghum (*Sorghum bicolor* (L.) Moench), an ancient cereal grain, has emerged as a promising candidate in this quest for improved nutrition. With its rich nutritional profile, including high levels of dietary fiber and bioactive compounds, sorghum offers a compelling alternative. In particular, whole grain flours derived from sorghum have garnered attention for their potential health benefits (Xu et al., 2021; Silva et al., 2020; Martínez et al., 2021). The phenolic compounds from various sorghum genotypes exhibited cytotoxicity against cancer cells *in vitro* (Yang et al., 2009; Awika et al., 2009; Smolensky et al., 2018), with this bioactivity varying depending on the extraction method, as different types of solvents produce distinct phenolic profiles (Cox et al., 2019; Paes et al., 2024). Moreover, sorghum phenolics have recently demonstrated toxicity against the malaria-causing Plasmodium (Paes et al., 2024).

Sorghum has also demonstrated various health effects *in vivo* results, ranging from an antidiabetic activity (Chung et al., 2011), modulation of adiposity and lipogenesis (Martinez et al., 2021a; Arbex et al., 2018), reduction of inflammation (Silva et al., 2020; de Sousa et al., 2019), and to combating oxidative stress (Martinez et al., 2021b; de Sousa et al., 2019). Sorghum has also contributed to promoting gut health by attenuating colitis-induced inflammation through upregulating repair mechanisms and short-chain fatty acid (SCFA) transporter expression (Ritchie et al., 2017). In another study, phenolic extracts from the bran of this cereal showed potential for colon cancer prevention by inhibiting proliferation, inducing apoptosis, and suppressing tumor formation in mice (Lee et al., 2021).

Gut microbiota and their SCFA metabolites are also important for intestinal health, where their composition exerts effects beyond the gastrointestinal system, influencing glucose metabolism, obesity (Portincasa et al., 2022), and preventing cardiovascular diseases (Overby & Fergunson, 2021) and colon cancer (Hou et al., 2022). Furthermore, dietary fibers are essential in modulating gut microbiota. In this sense, sorghum, rich in dietary fibers, has also proven effective in altering and improving microbiome composition *in vivo* (de Sousa et al., 2019; Tuncil et al., 2018). Similarly, sorghum phenolic compounds also show the capacity to modulate gut microbiota and its production of SCFA (Ashley et al., 2019).

Among the various forms of processing sorghum flours, toasting uses dry heat being effective to preserve the health-promoting components of sorghum, dietary fibers and phenolic compounds. A previous study (Silva et al., 2020) had already shown the potential of toasted sorghum flours from both white and tannin genotypes. It was demonstrated that these flours lowered the ALT levels and improved lipid metabolism in rats induced to oxidative stress with paracetamol, highlighting the anti-inflammatory and anti-hyperlipidic properties of toasted sorghum flours. Therefore, in addition to the possible health benefits, these flours also offer both nutritional value and culinary appeal, as they can be integrated into a traditional Brazilian dish called “farofas”. This work aimed to explore the functionalities of toasted sorghum flours phenolic-rich extracts *in vitro* in cancer cells, malaria Plasmodium, and a fecal fermentation study; and *in vivo* investigating the potential attenuating effects of these flours against early stages of colon cancer.

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2. JUSTIFICATION

There is growing evidence suggesting that the consumption of sorghum flours can confer health benefits. These flours are rich in phenolic compounds and dietary fiber, which have been associated with anti-inflammatory and antioxidant properties, as well as intestinal health benefits. Research has shown that sorghum phenolic compounds, including phenolic acids, flavonoids, and condensed tannins, have demonstrated bioactivity against oxidative stress, which is implicated in various chronic diseases, including cancer.

However, there is still a need for more consistent data and further investigation into the promotion of intestinal health by sorghum flours. Additionally, the idea of toasted sorghum flours is to create a product with minimal processing to preserve the characteristics of high resistant starch content and phenolic compounds, besides analyze its impact *in vitro* and *in vivo*. By addressing this gap in knowledge, this research endeavors to provide valuable insights into the health-promoting effects of toasted sorghum flour consumption and its potential applications.

The incorporation of toasted sorghum flours into everyday dishes like "farofas" holds promise for enhancing the nutritional quality of traditional Brazilian cuisine and promoting public health. Given the rising incidence of chronic diseases globally, including colon cancer, there is a growing need for dietary interventions that can mitigate disease risk and improve overall health outcomes. By leveraging the functional properties of toasted sorghum flours, particularly their antioxidant and anticancer activities, this study shows an opportunity to position sorghum as an important staple in its culinary landscape. This not only supports the preservation of traditional food culture but also offers a sustainable and health-promoting alternative in an era of increasing demand for nutritious and functional foods.

3. OBJECTIVES

3.1 GENERAL OBJECTIVES

Evaluate the health impacts of toasted white and tannin sorghum flours *in vitro* and *in vivo*.

3.2 SPECIFIC OBJECTIVES

- Characterize toasted sorghum flours (proximate composition, resistant starch, phenolic composition, and antioxidant capacity).

- Evaluate the *in vitro* antiproliferative, antimalarial and antimetastatic properties of sorghum flour phenolic extracts.
- Analyze the effect of toasted sorghum flours intake in an *in vivo* model on biomarkers, oxidative stress, and colon cancer in early stages.
- Examine the impacts of toasted sorghum flours intake on the morphology, short-chain fatty acids production, and intestinal microbiota both *in vitro* and *in vivo*.

4. HYPOTHESIS

The first hypothesis is that toasted sorghum flours may exert cytotoxic activity *in vitro* in cancer cells, as well as present antioxidant, anti-metastatic, and anti-malarial activities, through phenolic compound-rich extracts from these flours, and that even eco-friendly extracts made with heated water are capable of exerting such activities.

Another hypothesis is that whole toasted flours of white sorghum (BRS 501) and tannin sorghum (BRS 305) ingested in an *in vivo* experiment could attenuate the effects of early-stage colon cancer in rats induced by DMH, characterized by the presence of aberrant crypts foci. This would be due to the composition of these flours, rich in dietary fiber and phenolic compounds, both of which have ample evidence of promoting intestinal health. The effects on intestinal health may include a reduction in number of aberrant crypts foci and oxidative stress in liver, where the DMH is metabolized, as well as an increase in short-chain fatty acids and beneficial bacteria in gut microbiota.

Lastly, it was hypothesized that in an *in vitro* fermentation experiment with fecal samples from human volunteers, the toasted flours and phenolic extracts from sorghum could alter the production of short-chain fatty acids and modulate the intestinal microbiota, providing insights into the influence of sorghum components on the human gut.

5. CHAPTER 1: LITERATURE REVIEW

5.1 DIETARY PATTERNS AND NONCOMMUNICABLE DISEASES

Industrialization during the 19th and 20th centuries introduced significant changes to our diet, including the incorporation of dairy products, cereals, processed foods with additives, refined sugars and vegetable oils, fatty meats, and salt (Adolf & Tilg, 2024). This Westernized diet – which is characterized by low intake of fruits and vegetables, while being high in fat, sodium, large portions, high calories, and excess sugar – has health consequences for the population, leading to weight gain and obesity and acting as a risk factor for various other non-communicable diseases, such as cardiovascular, metabolic, inflammatory, and malignant diseases, such as cancers (Rakhra et al., 2020; Kwan et al., 2016; NCD-RisC, 2019; WCR International, 2018). Despite the name, even Eastern and isolated populations have been affected by this new dietary pattern, as has been happening in China (Ye & Leeming, 2023), further increasing concerns about health and nutrition.

This change in dietary patterns has led to a considerable rise in obesity rates, compounded by sedentary lifestyles, resulting in an imbalance between calorie intake and expenditure (WHO, 2020). This trend was not observed in earlier times when humans experienced periods of starvation (Blüher, 2019), indicating multiple factors at play. Researchers have delved into understanding how food cravings are disrupted in obese individuals, how hormones regulate feelings of satiety and appetite in the hypothalamus, and how dysfunction in adipose tissue can give rise to secondary health issues (Blüher, 2019; Heymsfield & Wadden, 2017; Murray et al., 2014).

Obesity and overweight, according to the World Health Organization (WHO), are defined as the accumulation of abnormal or excessive fat that can adversely affect an individual's health. WHO data from 2016 revealed that over 1.9 billion adults aged 18 and above were overweight, with more than 650 million categorized as obese. This accounts for 39% and 13% of overweight and obese adults, respectively. This issue has also become prevalent among children, with an estimated 38.2 million children under the age of 5 being overweight or obese in 2019 (WHO, 2020).

Health issues related to obesity are many. Obesity results in an increase in the size of lipid droplets, known as steatosis, in hepatocytes, leading to conditions such as non-alcoholic fatty liver disease, fatty liver, and cirrhosis (Heymsfield & Wadden, 2017; McCullough, 2004). Additionally, research by Gross et al. (2004) highlighted the correlation between refined

carbohydrate consumption and the prevalence of type 2 diabetes, indicating that the increase in consumption of refined carbohydrate-rich foods and the decrease in fiber-rich foods in diets were linked to the rise in type 2 diabetes cases during the 20th century. Also, changes in the global diet towards higher consumption of high glycemic index carbohydrates can lead to increased food intake due to postprandial hypoglycemia following blood glucose spikes (Ludwig, 2002).

The link between obesity and cardiovascular diseases may be attributed to several factors: increased dyslipidemia due to elevated free fatty acid release, heightened activity of the renin-angiotensin-aldosterone system, mechanical stress leading to systemic and pulmonary hypertension, as well as associations with type 2 diabetes and liver-related diseases (Heymsfield & Wadden, 2017). Cardiovascular diseases, including coronary heart disease, strokes, and hypertension, rank as the leading cause of death worldwide, with 17.9 million deaths recorded in 2016 (WHO, 2020).

Additionally, obesity, characterized by a low-grade chronic inflammation, contribute to cancer incidence as inflammation plays a pivotal role in tumor development and progression. Adipocyte hypertrophy leads to increased secretion of leptin, a pro-inflammatory adipokine that stimulates the production of various cytokines and tumor necrosis factor α (TNF-α), along with reactive oxygen species (ROS), favoring the tumor microenvironment. This explains the strong association between obesity and various types of cancer, particularly those developing adjacent to adipocytes. Insulin resistance, hyperglycemia, and dyslipidemia – often associated with obesity – are also risk factors for cancer development (Heymsfield & Wadden, 2017; Deng et al., 2016).

Despite the existence of many other factors, diet is a risk factor for the development of various types of cancers (WCRF International, 2018). Steck & Murphy (2019) demonstrate in their study associations between dietary patterns and cancer risk, where pro-inflammatory diets, characterized by a high score on the Dietary Inflammatory Index, were linked to an increased risk of developing lung, pancreatic, colorectal, breast, and prostate cancers. The same authors point out that the associations between different dietary patterns and cancer risk are likely influenced by the combined biological effects of various dietary components, involving mechanisms such as gut microbiota and their metabolites, epigenetics, inflammation, immune function, metabolic or hormonal disruption, and oxidative stress (Steck & Murphy, 2019).

On the other hand, the study by Kliemann et al. (2023) showed that replacing 10% of processed foods in the diet could reduce the risk of colon and liver cancers (Ma et al., 2019; Adolf & Tilg, 2024). Additionally, healthy diets and regular physical activity could also lower

the risk of carcinogenesis (Kerr et al., 2017). Addressing the complexity of the obesity issue and westernized diets requires multifaceted approaches, driven by public policies advocating for lifestyle changes and exploration of epigenetic factors (Blüher, 2019). Therefore, WHO recommendations for obesity prevention emphasize physical activity and diets rich in whole grains, fruits, and vegetables due to their dietary fiber and antioxidant content.

5.2 IMPACTS OF WHOLE GRAIN FOODS ON HEALTH

5.2.1 The Role of Whole Grains in Health Promotion

According to AACC (2000), whole grains are defined as intact, ground, cracked, or flaked caryopses containing the main anatomical components in the same form as they occur in nature, namely, an intact caryopsis. These components include the starchy endosperm, germ, and bran (Okarter & Liu, 2010). The most popular and commonly consumed cereal grains include corn, wheat, and rice, while oats, barley, rye, millet, and sorghum are considered minor grains (FAO, 2022; Pedersen et al., 1989).

Whole grains are renowned for their richness in dietary fiber, low fat content, and balanced proportions of starch and proteins, as well as vitamins, minerals, and phenolic compounds (Slavin et al., 1997; Thompson, 1992). Due to their high dietary fiber and phenolic compound content, whole grains have demonstrated beneficial effects in studies targeting several non-communicable diseases, prompting recommendations by the WHO for their consumption, along with other fiber-rich foods (Wu et al., 2015; Kazemzadeh et al., 2014; Schatzkin et al., 2007; Montonen et al., 2003; Steffen et al., 2003).

The cardioprotective benefits of consuming whole grains have been observed in various studies. A meta-analysis conducted by Aune et al. (2016), pooling data from numerous studies, demonstrated an inverse relationship between whole grain consumption and the risk of coronary heart disease and cardiovascular disease. Moreover, a long-term follow-up study of both men and women in the United States associated high whole grain intake with a decreased risk of cardiovascular disease-related mortality, irrespective of lifestyle factors and dietary habits (Wu et al., 2015). Among the grains consumed by participants, whole wheat, oats, cornmeal, rye, barley, and brown rice were reported.

Whole grain consumption has also been linked to improvements in metabolic markers, promoting cardiovascular health. Lairon et al. (2005) found that a high intake of dietary fiber from cereals was associated with lower blood pressure and homocysteine concentration, a risk

factor for cardiovascular disease. Similarly, Kazemzadeh et al. (2014) reported that brown rice consumption decreased diastolic blood pressure and hs-CRP inflammatory marker levels in overweight or obese women compared to white rice consumption.

In terms of preventing type 2 diabetes and obesity, whole grains have shown promising results. Whole grain intake has been associated with lower BMI and reduced weight gain (Lairon et al., 2005; Bazzano et al., 2005). The study by Bazzano et al. (2005) indicated that consuming whole breakfast cereals was inversely related to BMI values > 25 and limited weight gain to a maximum of 10 kg over an 8-year period. To better understand why whole grain exert protective effects and promote health, it is essential to delve into the impact of dietary fiber and phenolic compounds – two components present in grains – which will be discussed further in subsequent sections.

Regarding their protective effects against cancer, longitudinal studies have established a correlation between whole grain consumption and a reduced risk of cancer (Zong et al., 2016; Kyro et al., 2013). Research examining the association between dietary fiber from various sources and colorectal cancer risk revealed that grain fibers and whole grains were linked to a lower risk of developing this disease (Schatzkin et al., 2007). Similar findings were reported by Larsson et al. (2005) for colon cancer risk and by Chan et al. (2007) for pancreatic cancer. The cancer prevention mechanism involved in the consumption of whole grains may include increased fecal bulk and reduced gastrointestinal transit time, leading to lower absorption of carcinogens, as well as the greater production of short-chain fatty acids (SCFAs) (Gaesser, 2020), which can have various health effects as reviewed below.

5.2.2 Effects of Dietary Fibers On Health and Gut Microbiota

One of the recommendations from the World Health Organization for healthier dietary habits is the consumption of dietary fiber. According to the Codex Alimentarius (2009), dietary fibers are "carbohydrate polymers with ten or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans". For a carbohydrate to be considered a dietary fiber, it must meet one of the following criteria: a) it must be an edible carbohydrate polymer naturally present in food; b) it must be a carbohydrate polymer obtained from raw foods through chemical, physical, or enzymatic methods, with a proven physiological effect on the body to benefit health; or c) it must be a synthetic carbohydrate polymer with a proven physiological effect on the body to benefit health. This definition of dietary fiber includes non-starch polysaccharides such as cellulose, hemicellulose, and β-glucans;

oligosaccharides; lignins; certain plant-associated substances such as phytates; and resistant starch (RS) (DeVries, 2003).

Upon ingestion, dietary fiber reaches the large intestine intact, where it undergoes fermentation by the intestinal microbiota, conferring numerous benefits to the host. Fermentation products include methane, hydrogen, and carbon dioxide gases; SCFAs such as butyrate and propionate (Figure 1); organic acids like lactate and succinate; as well as methanol and ethanol alcohols, albeit in smaller quantities (Lockyer & Nugent, 2017). Butyrate is primarily utilized by colonocytes as an energy source, while propionate is predominantly metabolized by the liver (Schoeler & Caesar, 2019). Consequently, the pH of the intestinal lumen decreases, promoting the proliferation of colon epithelial cells (Carabin & Flamm, 1999; Saad et al., 2011).

SCFAs act as signaling molecules on G-protein coupled receptors, such as GPR43, inhibiting lipolysis (Fig. 1). Additionally, GPR43 activation in L-cells stimulates increased secretion of glucagon-like peptide-1 (GLP-1), enhancing glucose and lipid metabolism (Schoeler & Caesar, 2019). Activation of proliferator-activated receptor γ (PPARγ) by butyrate and propionate helps regulate lipid metabolism, reduce body weight, and decrease triglyceride accumulation in the liver (Schoeler & Caesar, 2019; den Besten et al., 2015; Alex et al., 2013; Gao et al., 2009).

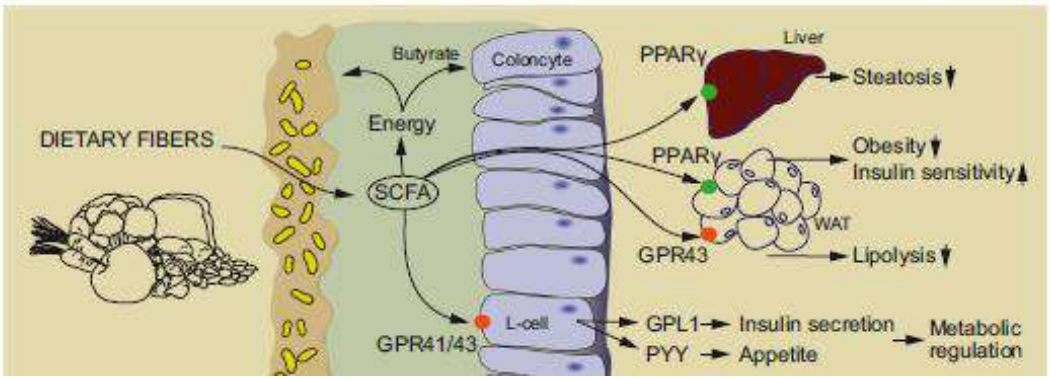

Figure 1. Mechanisms of direct and indirect action of dietary fibers. Source: Schoeler & Caesar, 2019.

This mechanism of action elucidates the physiological and preventive effects against chronic diseases in the body, yielding effects that extend beyond the gastrointestinal system. Dietary fibers aid in obesity management through multiple factors, including chemical satiation signals, which improve appetite control and the feeling of fullness, consequently reducing food intake (Papathanasopoulos & Camilleri, 2010; Tucker & Thomas, 2009; Woods, 2005). Satiety is attributed to the release of two hormones: glucagon-like peptide (GLP-1) and peptide YY

(PYY) (Lattimer & Haub, 2010). Research by Tucker & Thomas (2009) correlated dietary fiber consumption with reduced body weight in middle-aged women, primarily attributed to fat loss, irrespective of factors such as age and physical activity level.

Given the interconnectedness previously observed between obesity, cardiovascular disease, type 2 diabetes, and inflammation, it is expected that dietary fiber would counteract these diseases along with obesity. The association between high dietary fiber consumption and reduced risks of developing cardiovascular diseases (CVDs) (Reynolds et al., 2022) may be explained by improved lipid metabolism (Schoeler & Caesar, 2019; Gao et al., 2009; Anderson et al., 2009). Some dietary fibers and phenolic compounds can bind to bile acids, impairing micelle formation and consequently increasing the excretion of cholesterol and bile acids in feces (Naumann et al., 2020). Moreover, due to their structural characteristics and resistance to digestion, dietary fiber consumption reduces postprandial glucose levels and improves insulin sensitivity (Weickert & Pfeiffer et al., 2018).

Regarding potential effects on inflammation, dietary fiber intake was inversely associated with the inflammatory markers C-reactive protein (CRP) and interleukin-6 (Ma et al., 2021; Wannamethee et al., 2009). CRP is a chronic inflammation biomarker, and its reduction is associated with an improvement in patients' metabolic profile. One mechanism for this anti-inflammatory action, and consequently an anticancer effect, is through increased production of butyrate in high-fiber diets (Chen & Vitetta, 2018). The study by Bishehsari et al. (2018) demonstrated that butyrate exerts an effect on anti-inflammatory T-regulatory cells (Treg), reducing colon tumorigenesis. Additionally, Zhang et al. (2016) found that besides aiding Treg cell maturation, butyrate promoted the inhibition of interleukin-17 through T helper 17 cells, thereby reducing colitis in rats.

Several studies aim to investigate the correlation between dietary fiber consumption and the incidence of cancers, particularly colon cancer. Arayici et al. (2022) found that the consumption of both soluble and insoluble fibers appears to be correlated with a lower incidence of colorectal cancer (CRC), while hyperinsulinemic and pro-inflammatory diets seem to favor the development of this same type of cancer (Steck & Murphy, 2019). Moreover, the type of dietary fiber also seems to influence the protective effect against cancer. Hullings et al. (2020), in their cohort study among 478,994 US adults aged 50–71 years, found that dietary fiber from cereals was associated with a lower incidence of CRC, but fibers from other sources did not show the same results.

As dietary fibers are fermented by intestinal microorganisms, they play a crucial role in modulating this microbiota, generating health benefits for the host, and reducing the risk of

developing diseases, such as cardiovascular and inflammatory diseases (Ma et al., 2021; Bishehsari et al., 2018; Lairon et al., 2005). Therefore, as eubiosis (the balance of the intestinal microbiota) contributes to homeostatic regulation, its opposite, dysbiosis, can lead to the development of various diseases (Hand et al., 2016). According to Makki et al. (2018), the intestinal microbiota affects the modulation of juvenile growth, the immune system, and the modulation of lipid and glycosidic metabolism.

Depending on the long-term dietary pattern, specific enterotypes are associated, such as Bacteroides with animal protein and fat, and *Prevotella* with carbohydrates and dietary fiber (Makki et al., 2018; Wu et al., 2011). *Prevotella* was positively related to higher SCFA production as it is a fiber degrader (Ma et al., 2021; Filippo et al., 2010). Populations with fiber-rich diets exhibit a microbiota adapted for fermenting these plant-derived carbohydrates, enriched with *Treponema* and *Succinivibrio*, in addition to *Prevotella* (Makki et al., 2018; Filippo et al., 2010). Although they have relatively low amounts of enzymes responsible for metabolizing fibers, species belonging to the phyla Actinobacteria and Firmicutes benefit from dietary fiber consumption (Makki et al., 2018; Deehan et al., 2017).

The enzymatic machinery and substrate acceptance of a microorganism in the intestine are crucial for its enrichment, along with its ability to tolerate the low pH generated by fermentation products, which can either benefit or inhibit some taxa (Deehan et al., 2017). Competitive exclusion increases resistance to pathogens, preventing potentially pathogenic microorganisms from colonizing the intestinal mucosa through competition for adhesion sites and nutrients (Coyte et al., 2015).

Alterations in the gut microbiota of unhealthy individuals have been observed, associated with inflammation. Ma et al. (2021) analyzed the gut microbiota of 307 healthy men and found that the gut microbiota characteristic of inflammatory conditions was associated with a higher level of CRP. Furthermore, shifts in microbiota composition were observed in individuals with inflammatory bowel diseases, with a decrease in beneficial microorganisms, such as *Faecalibacterium prausnitzii* (Hand et al., 2016; Sokol et al., 2009). Increased consumption of dietary fiber favors the growth of bacteria, such as *Clostridiales*, which utilize these types of carbohydrates as an energy source (Ma et al., 2021).

Interestingly, Sonnenburg et al. (2016) studied the consequences of a lack of dietary fiber intake in mice containing a human microbiota and found that within just three generations, microbiota diversity was progressively reduced to a level beyond recovery when mice reverted to a fiber-rich diet. These results are concerning, as Westernized diets, which are more refined,

rich in simple sugars, and low in fiber, exhibit less microbial diversity compared to non-Westernized diets (Makki et al., 2018; Martínez et al., 2015).

5.2.3 Effects of Phenolic Compounds on Health and Gut Microbiota

In fruits and vegetables, dietary fiber is commonly associated with phenolic compounds, combining their health effects (Quirós-Sauceda et al., 2014). Phenolic compounds are substances with a broad spectrum of structures and functions, but they share the presence of at least one aromatic ring and one hydroxyl group (Haminiuk et al., 2012; Okarter & Liu, 2010). There are several classes of phenolic compounds: phenolic acids, referred to as simple phenolics due to their low molecular weight compared to other phenolics; stilbenes; flavonoids, which can bind to sugar molecules; lignans; and tannins, which exhibit a high degree of polymerization (Haminiuk et al., 2012).

Phenolic compounds are secondary metabolites widely distributed in plants, where they serve a protective role, possessing bioactive properties such as the sequestration of free radicals (Dykes et al., 2005). This antioxidant activity demonstrated by phenolic compounds holds significance both from a technological standpoint for the food industry and from a health perspective in combating oxidative stress (Shahidi & Ambigaipalan, 2015).

Oxidative stress stands as one of the main contributors to the development of chronic diseases (Figure 2), prompting numerous studies to investigate compounds with antioxidant activities (Dykes et al., 2013; Yang et al., 2009; Awika et al., 2009; Cilla et al., 2008; Awika et al., 2004). The mechanism of action of phenolic compounds as antioxidant agents involves the donation of a hydrogen atom to bind to free radicals, thereby reducing their reactivity (Shahidi & Ambigaipalan, 2015). Consequently, the antioxidant capacity of a particular compound is contingent upon the number of hydroxyl groups in its structure (Cao et al., 1997).

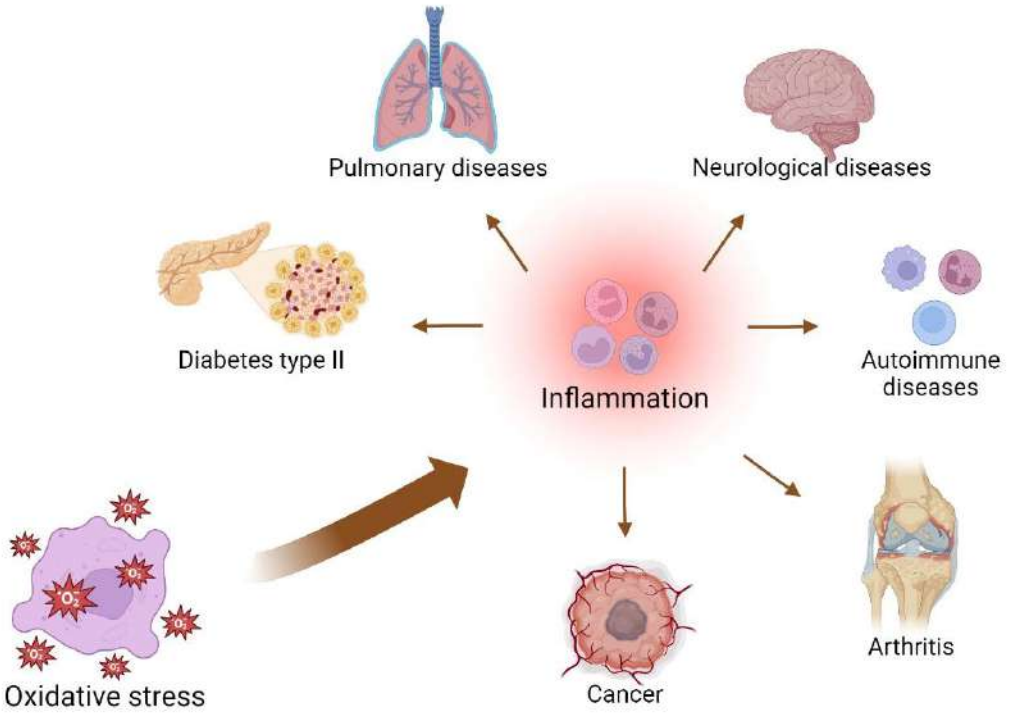

Figure 2. Relationship between oxidative stress, inflammation, and chronic diseases. Source: Awika, 2017, Simpson & Oliver, 2020, Ansari et al., 2020, Charlton et al., 2021, Hayes et al., 2020, Ibáñez-Cabellos et al., 2023.

Chronic inflammation, stemming from oxidative stress, has been the focus of research concerning antioxidants. Phenolic compounds exhibit bioactivity that can be advantageous to health, including anti-inflammatory and anti-cancer properties (García-Lafuente et al., 2014; Agah et al., 2017), as well as benefits against obesity, dyslipidemia, cardiovascular diseases, and hypertension (De Morais Cardoso et al., 2017). However, the efficacy of phenolics in providing health benefits relies on several factors. Following the ingestion of phenolic compounds, various events may occur contingent upon the nature of the polyphenol, its molecular weight, formation of complexes with other molecules, and the food matrix in which it is situated. The bioaccessibility and bioavailability of phenolic compounds found in fruits and cereals are determined by a) the quantity of these compounds released from their food matrix during digestion for absorption, and b) the extent to which this compound, after absorption into the bloodstream, can exert its antioxidant effect (Haminiuk et al., 2012; Parada & Aguilera, 2007; Manach et al., 2005).

Phenolic compounds from various sorghum varieties have demonstrated antioxidant potential both *in vitro* (Dykes et al., 2005) and *in vivo* (Silva et al., 2020; Moraes et al., 2012). In the study by Awika et al. (2009) phenolic compounds extracted from white sorghum

exhibited less antiproliferative capacity compared to extracts of tannin-rich sorghum, which showed the highest value. Tests on Wistar rats fed a high-lipid diet evaluated the potential of phenolics from sorghum varieties to reduce inflammation and oxidative stress (Moraes et al., 2012). Contrary to *in vitro* findings, greater antioxidant activity did not necessarily translate to greater anti-inflammatory potential. Interestingly, the sorghum variety with lower antioxidant capacity *in vitro* produced less TNF-α in rats, correlating with reduced inflammation.

In several studies, 3-deoxianthocyanin (3-DXA), a flavonoid present in sorghum, has outperformed its analogous anthocyanins. Shih et al. (2007) demonstrated that luteolinidin exhibited greater cytotoxicity against cancer cells than cyanidin. Yang et al. (2009) investigated the potential of sorghum 3-DXA to induce the activity of phase II enzymes and inhibit the growth of cancer cells. Extracts from various sorghum types demonstrated antiproliferative activity, particularly non-methoxylated forms of 3-DXA against cancer cells. Additionally, O-methylation reduced the antioxidant potential of the polyphenols (Yang et al., 2009), indicating a potential effect *in vivo*.

Due to the antioxidative action of phenolic compounds, effects in combating non-communicable diseases are also evident. Enzymatic inhibition of α-amylase and α-glucosidase has been researched as a means to control hyperglycemia, leading to more controlled glucose absorption and prevention of insulin spikes. Phenolic compounds exhibit this ability to bind to other molecules, such as tannins binding to these digestive enzymes to delay glucose release (Espitia-Hernandez et al., 2020; Links et al., 2015).

Sorghum phenolic extracts have demonstrated interesting hypoglycemic activity in both healthy and diabetes-induced rats, decreasing serum glucose in both groups and increasing serum insulin in diabetic rats only (Chung et al., 2011). Arbex et al. (2018) found that rats induced with obesity due to a high-fat diet and given extruded sorghum meal exhibited lower adiposity percentages and blood glucose levels. Luteolinidin and 5-methoxy-luteolinidin were the deoxyanthocyanidins found in greater quantities in this extruded sorghum flour, potentially impacting metabolic markers.

Phenolic compounds have been associated with various cardiovascular health benefits, including a lower risk of coronary artery disease (Duffy et al., 2001), improved lipid metabolism (Silva et al., 2020), reduced levels of total cholesterol and triglycerides (Chung et al., 2011), and prevention of LDL oxidation (Duthie et al., 2000) owing to flavonoids' ability to chelate iron and copper and scavenge free radicals (Okarter & Liu, 2010; Yao et al., 2004).

Phenolic acids are typically linked to dietary fibers, such as ferulic, p-coumaric, and caffeic acids in cereals (Quirós-Sauceda et al., 2014; Vitaglione et al., 2008). This association

results in the metabolism of bound phenolic acids differently from free phenolic acids in the body. While unbound phenolic compounds can be absorbed in the upper intestine, bound phenolic acids traverse the stomach and small intestine intact before being fermented and released by the colon microbiota (Girard & Awika, 2018; Neacsu et al., 2017; Vitaglione et al., 2015). These bound compounds, including polymeric phenolic compounds like tannins, are not absorbed in the upper gastrointestinal tract (Awika et al., 2018), underscoring their importance in influencing the intestinal microbiota.

Bound or high molecular weight phenolic compounds are liberated by specific bacteria capable of hydrolyzing their bonds (Duncan et al., 2016). However, the products of this hydrolysis are not always monomers but rather microbiota metabolites that have undergone various reactions (Cardona et al., 2013). The maintenance of phenolic compounds' antioxidant properties post-release by the microbiota depends on their form and potential reactions (Awika et al., 2018). Consequently, the combined effects of phenolic compounds and their microbial metabolites, along with fiber fermentation metabolites, both possessing anti-inflammatory properties, may synergistically benefit the host (Vitaglione et al., 2015).

While *in vitro* antioxidant activity does not guarantee a reduction in oxidative stress *in vivo*, data on antioxidant activity are valuable for identifying the potential of phenolic compounds in promoting health. Thus, *in vitro* studies serve as an initial step towards animal and human testing.

5.3 SORGHUM

Sorghum (*Sorghum bicolor* (L.) Moench) is an ancient cultivated cereal renowned for its drought resistance, playing a vital role in feeding populations in semi-arid regions of Africa (Awika, 2017). Despite its significance, global sorghum production remains lower than that of other major cereals such as rice, corn, and wheat. FAO data from 2018 indicates that sorghum production totaled 59.3 million tons worldwide, considerably less than the production of rice, corn, and wheat, which reached 782 million, 1.141 billion, and 734 million tons, respectively.

In Brazil, sorghum production exceeded 2.27 million tons in 2018, establishing the country as one of the leading producers globally, ranking 7th in sorghum production worldwide. The primary sorghum-producing states in Brazil include Goiás, Mato Grosso, and Minas Gerais, with production exceeding 100 thousand tons in each state (Conab, 2019). Notably, only about 4% of sorghum planted in Brazil contains tannins, with the majority grown in the Bagé region of Rio Grande do Sul (Embrapa, 2012).

Sorghum production in Brazil has been steadily increasing, particularly during the off-season, and it has become an essential crop in rotation systems (Embrapa, 2012). While historically sorghum in Brazil has been primarily used for animal consumption, there is a growing emphasis on human consumption, with sorghum-based products already available in the market. According to Dicko et al. (2006), sorghum finds application in various food preparations worldwide, including tortillas in Latin America, thin porridge in Africa and Asia, beers in Africa, and baked products in the USA, Africa, and Japan. Moreover, significant research efforts have been dedicated to developing new sorghum-based products such as cereal bars (Queiroz et al., 2012), gluten-free cookies (Soares et al., 2019), and bread (Mtelisi et al., 2020).

Sorghum is a tall plant, capable of reaching heights of up to 6 meters, with panicles containing its grains. The sorghum grain is typically rounded, measuring approximately 4 mm in length, with an average weight of 28 mg (Whistler et al., 2012). A mature sorghum caryopsis comprises three main parts: the pericarp, endosperm, and germ (Rooney et al., 1979). The pericarp consists of three layers: the epicarp, mesocarp, and endocarp (Earp & Rooney, 1982; Earp et al., 2004), with the grain testa situated between the pericarp and the aleurone (Earp et al., 2004).

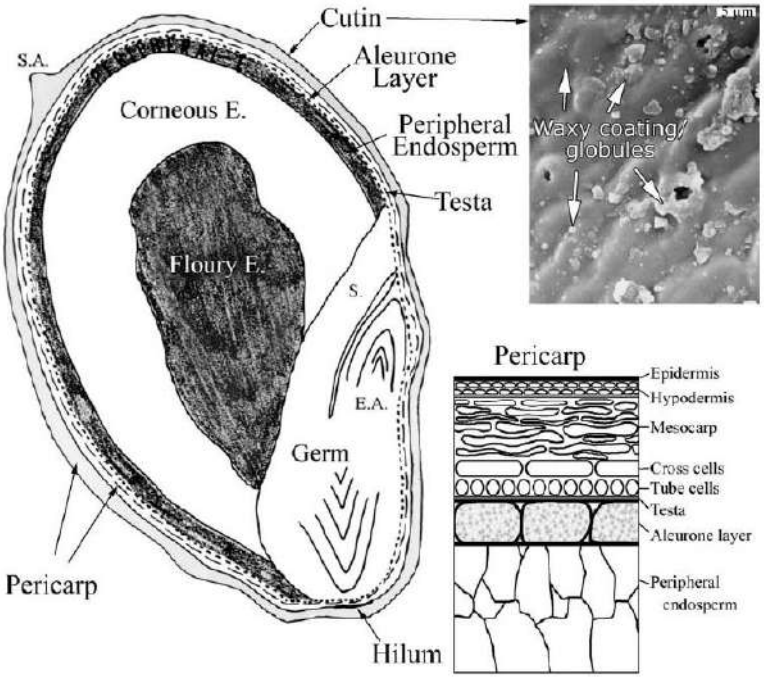

Figure 3. Sorghum grain scheme. The upper right is the cuticular layer that covers the grain. S means scutellum, E. A. means embryonic axis, and S. A. means stylar area. Source: Earp et al., 2004.

Sorghum grain exhibits a diverse range of colors, including pale orange, brown, red, and

dark reddish-brown hues (Whistler et al., 2012), as depicted in Figure 4. The coloration of sorghum kernel pericarp is governed by the interaction of specific genes, namely R and Y genes (Rooney, 2000), while genes B1 and B2 determine the presence or absence of a pigmented testa layer (Dykes et al., 2005; Earp et al., 2004). Additionally, genes *P* and *Q* contribute to secondary coloration, influencing the production of red, purple, and tan pigmented sorghum plants (Dykes et al., 2005; Rooney, 2000).

Figure 4. Black (left), white (center) and red (right) varieties of sorghum in the field. Source: Awika, 2017.

Phenolic compounds, carotenoids, and non-starch polysaccharides are primarily located in the pericarp and testa layers of the sorghum grain, while starch, proteins, B vitamins, and minerals are predominantly found in the endosperm. The germ contains lipids, fat-soluble vitamins, and minerals (De Morais Cardoso et al., 2017). Sorghum exhibits variations in composition depending on its phenotype, with commercially available types including black, white, and red sorghum (Awika, 2017). Structurally and compositionally, sorghum grain shares similarities with corn (Table 1), with minor differences such as slightly lower moisture content, 1–2% higher starch and protein content, and lower total fat content (Whistler et al., 2012). Starch constitutes the major component of sorghum, comprising about 70% of the whole grain (Sang et al., 2008; Hoseney et al., 1981) and approximately 86% of the endosperm on a dry basis (Awika, 2017). The composition of sorghum starch can vary depending on the type, ranging from 0 to 23.1% amylose content in normal, waxy, or heterowaxy sorghum varieties (Sang et al., 2008).

Table 1. Typical sorghum whole grain composition.

	Range (%)
Moisture	8- 20*
Carbohydrates	60 – 75
Protein	8.6 – 15.6
Lipids	2.3 – 4.9
Ash	1.1 – 2.5
Dietary fiber	8.7 – 13.0

All values on a dry basis. *Only result in wet basis. Source: Virupaksha & Sastry, 1968; Awika et al., 2005; Da Silva & Ciocca, 2005; Sang et al., 2008; Whistler et al., 2012; Awika, 2017.

Sorghum proteins can be categorized based on their solubility into albumins, globulins, kafirins, cross-linked kafirins, and glutelins (Dicko et al., 2006; Jambunatan et al., 1975). Kafirins, which are aqueous alcohol-soluble prolamins, constitute the majority of sorghum proteins and are further divided into three classes: α-kafirins, β-kafirins, and γ-kafirins, accounting for approximately 79% of total proteins (De Morais Cardoso et al., 2017; Taylor & Schüssler, 1986). Sorghum proteins generally exhibit lower digestibility compared to proteins from other cereals, primarily due to the resistance of kafirins to peptidases (Belton et al., 2006).

Apart from prolamines (kafirins), sorghum also contains non-prolamin proteins such as albumin, globulin, and glutelin, which have higher nutritional value owing to their elevated lysine content in comparison to kafirins (Taylor & Schüssler, 1986). Taylor & Schüssler (1986) observed higher levels of albumin and globulin in the germ, while globulins were more abundant in the endosperm compared to prolamines. Although sorghum proteins are relatively low in lysine, they are rich in other essential amino acids like glutamic acid, proline, leucine, and alanine (Moraes et al., 2012). Not only do sorghum proteins alter the digestibility of the present starch, but also other bioactive compounds present in the grains called phenolic compounds, which exhibit a wide variety and functional properties upon ingestion.

5.3.1 Sorghum Bioactive Compounds

Phenolic compounds found in sorghum are abundant and possess an intriguing and distinct composition compared to other cereals, with numerous studies underscoring their potential in promoting health (Girard & Awika, 2018; Awika et al., 2018; De Morais Cardoso et al., 2017; Yang et al., 2009; Awika & Rooney, 2004). Sorghum exhibits a complex phenolic profile, predominantly comprising phenolic acids, flavonoids (such as flavones, flavanones, and anthocyanins), and condensed tannins or proanthocyanidins (Dicko et al., 2006; Awika, 2017; De Morais Cardoso et al., 2017).

Among the phenolic compounds found in cereals, phenolic acids have garnered the most attention (Girard & Awika, 2018). Most sorghum phenolic acids are derived from benzoic and cinnamic acids (Figure 5), primarily concentrated in the outer layers of the grain and can be present in free form or bound to the plant cell structure (Awika & Rooney, 2004). Phenolic acids are prevalent in the plant cell wall structure of cereals, often esterified with hemicellulose, and the bound forms are primarily derived from ferulic acid (Girard & Awika, 2018; Chiremba et al., 2012; Dykes & Rooney, 2006).

Hahn et al. (1983) conducted a study to identify phenolic acids in free and bound forms in seven sorghum varieties using high-performance liquid chromatography. The total phenolic content was found in higher quantities in varieties of white, lemon yellow, and red sorghum in the bound form, with only one red variety exhibiting a higher content of phenolics in the free form. Eight phenolic acids were detected, including protocatechuic, p-hydroxybenzoic, caffeic, p-coumaric, and ferulic acids, which were found in both free and bound forms. Gallic acid was exclusively found in the bound form, while vanillic and cinnamic acids were detected in free form and/or bound, depending on the sorghum variety.

The content of phenolic acids can vary depending on the sorghum variety, with values ranging from 135.5 to 479.4 µg/g (De Morais Cardoso et al., 2017; Chiremba et al., 2012; Afify et al., 2012). Hahn et al. (1983) reported amounts of phenolic compounds ranging from 558 to 2892 µg/g in the free form and 820 to 1280 µg/g in the bound form, depending on the sorghum cultivar. In sorghum grains, derivatives of ferulic acid constitute around 90% of the bound phenolic acids, contributing to the hardness of the grain (Chiremba et al., 2012). The content of protocatechuic and ferulic acids in sorghum ranges from 150.3 to 178.2 µg/g and 120.5 to 173.5 µg/g, respectively, with higher quantities found compared to other subclasses (De Morais Cardoso et al., 2017; Afify et al., 2012; Svensson et al., 2010).

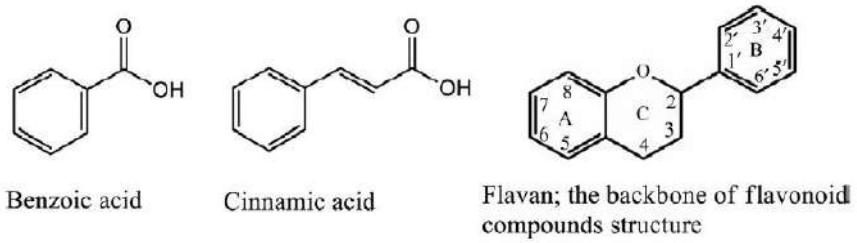

Figure 5. Basic structure of most phenolic compounds found in sorghum. Source: Awika, 2017.

Flavonoids constitute the most abundant and diverse group of phenolic compounds in plants, sharing the flavan structure (Figure 6) and varying based on the substituent groups around the heterocyclic ring C (Awika, 2017). There are seven classes of flavonoids: flavonols, flavononols, flavones, flavanols or catechins, flavanones, anthocyanins, and isoflavonoids (Haminiuk et al., 2012; Shahidi & Ambigaipalan, 2015). Sorghum grains contain flavones, flavanones, and anthocyanins in higher quantities, along with some presence of flavonols and flavanols (De Morais Cardoso et al., 2017; Dykes & Rooney, 2006). As these compounds are primarily located in the outer layers of the sorghum grain, they contribute to the grain color, pericarp thickness, and the presence of pigmented testa (De Morais Cardoso et al., 2017; Awika et al., 2005).

Flavones, a pale-yellow group, are typically found in small amounts in cereals, but certain sorghum varieties exhibit high levels of these compounds, particularly the aglycone forms luteolin and apigenin (Awika, 2017; Dykes et al., 2009; Dykes et al., 2011). For instance, specific sorghum varieties have flavone concentrations ranging from 1400 to over 2000 µg/g (Ravisankar et al., 2018), while other cereals contain less than 400 µg/g (Awika et al., 2018). Sorghum varieties displaying red and lemon-yellow colors with a tan secondary hue tend to have the highest levels of this class of compounds (Dykes et al., 2009; Dykes et al., 2011). The flavones present in sorghum are associated with disease prevention due to their potent bioactivity even at low concentrations (Yang et al., 2012; Awika, 2017).

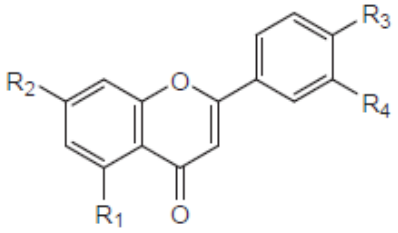

Figure 6. Structure of sorghum flavone. In apigenin derivatives, R3 and R4 correspond to OH and H, respectively; in luteolin derivatives, both R3 and R4 are a OH group. Source: Girard & Awika, 2018.

The main flavanones identified in sorghum are the aglycone forms of eriodictyol and naringenin (De Morais Cardoso et al., 2017), with lower levels found in varieties with white pericarp and higher levels in those with yellow pericarp (Dykes & Rooney, 2006). Interestingly, the presence of flavanones in sorghum often coincides with the presence of flavones, and the main structural difference between these two classes of flavonoids is the absence of a double bond between C2 and C3 in flavanones (Figure 7) (Awika, 2017). The content of flavanones in sorghum varieties such as white, red, and lemon-yellow can vary depending on the cultivar, ranging from 134 to 1780 µg/g in lemon-yellow sorghum, which is higher compared to other varieties and is associated with the yellow color of the pericarp (Dykes et al., 2011). Flavanones have also demonstrated potential health benefits, particularly in the prevention of colon cancer (Yang et al., 2015).

Flavanols represent another class of flavonoids, consisting of compounds known as catechins, epicatechins, and galocatechins (Shahidi & Ambigaipalan, 2015; Haminiuk et al., 2012). While flavanols are typically found in nature as monomers or oligomers, in sorghum, they are often present in the form of condensed polymers with a degree of polymerization ranging from 15 to 20 (Awika et al., 2003; Girard & Awika, 2018). One study revealed that among the flavonoids consumed in the United States, flavan-3-ols constitute the majority of flavonoids in the diet, accounting for 83.5% (Chun et al., 2007).

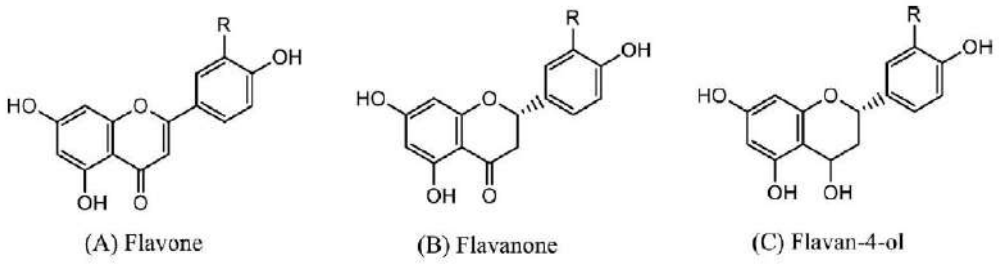

Figure 7. Backbone structures of the main classes of non-pigmental monomeric flavonoid aglycone found in sorghum. Source: Awika, 2017.

In sorghum, the predominant anthocyanins are 3-deoxyanthocyanins (3-DXA), which differ from typical anthocyanins in their substituted glycosyl groups at C3 (Figure 8) (Awika & Rooney, 2004). Unlike other pigmented grains where the anthocyanins responsible for grain color have substitutions at the C3 position, 3-DXA in sorghum lack this substitution but maintain stability. Interestingly, the absence of glycosylation at C3 in 3-deoxyanthocyanins enhances their hydrophobicity, rendering them less susceptible to nucleophilic attacks and

consequently more stable across a wide pH range (Awika, 2017; Yang et al., 2014; Awika, 2008; Awika et al., 2004).

The 3-DOA identified in sorghum are derived from apigeninidin and luteolinidin, which are non-methoxylated forms, as depicted in Figure 6 (Dykes et al., 2009; Awika et al., 2004). Studies by Awika et al. (2004) revealed a higher anthocyanin content in black sorghum (average 10.1 mg/g bran) compared to brown and red varieties (2.8 - 4.3 mg/g bran), with only 3-DXA being identified in their analyses. Apart from exhibiting greater stability over a wide pH and temperature range (Yang et al., 2014; Awika et al., 2004), which makes them potential natural colorants in the food industry, 3-DXA have also demonstrated antioxidant activity in numerous studies (Dykes et al., 2013; Yang et al., 2009; Awika et al., 2009; Awika et al., 2004).

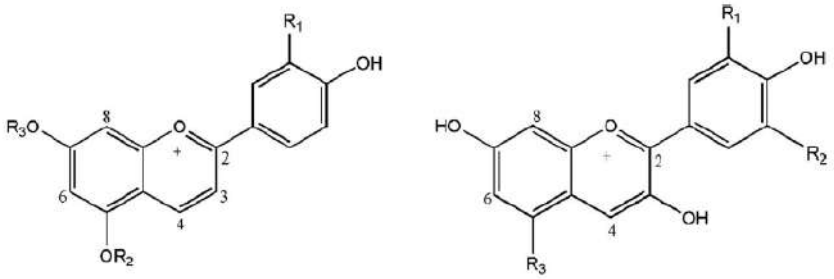

Figure 8. Structure of 3-deoxyanthocyanins (left), where apigeninidin has in the position R_1, R_2 and R_3 = H, and luteolinidin has R_1 = OH and R_2 and R_3 = H. Structure of anthocyanins (right) commonly found in plants, cereals, and fruits. Source: Awika & Rooney, 2004.

Condensed tannins, also known as proanthocyanidins (PA), are secondary metabolites found in plants, serving as a defense mechanism against predators. According to Awika and Rooney (2004), the tannins present in sorghum are predominantly condensed tannins, formed from polymers of flavan-3-ols and/or flavan-3,4-diols. These flavanol units are linked together in the C4→C8 position, which is characteristic of type B proanthocyanidins. In contrast, type A proanthocyanidins have an additional bond between C2 and C7 (Dykes & Rooney, 2006).

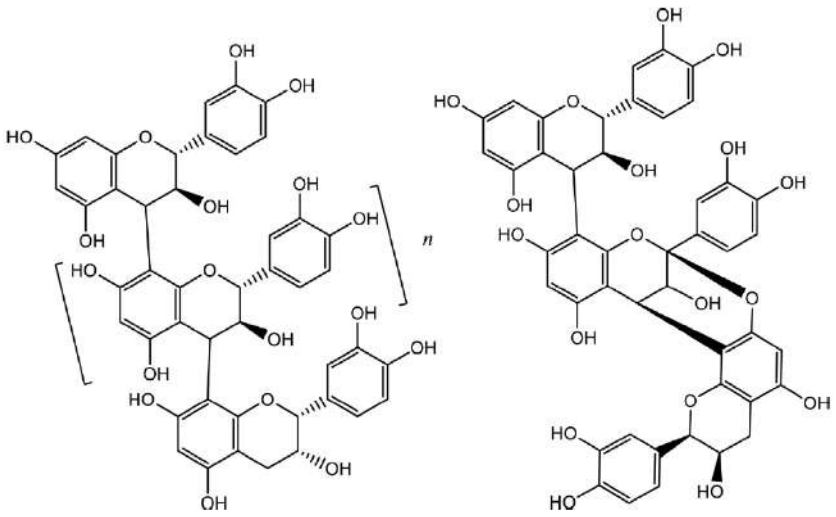

Figure 9. Structure of sorghum proanthocyanidins with type B interflavan linkage (left); and type A linkage (right), both found in sorghum. Source: Awika, 2017.

Sorghum can be categorized based on its proanthocyanidins (PA) into three types: type I (lacking significant levels of tannins), type II (containing tannins extractable only in acidified methanol), and type III (having tannins extractable by both methanol and acidified methanol) (Hahn & Rooney, 1986; Barros et al., 2014). Despite the constant association of tannins with sorghum, most sorghum varieties cultivated are tannin-free (Awika & Rooney, 2004). The presence or absence of a pigmented testa is linked to the B1 and B2 genes (Hahn & Rooney, 1984). The degree of polymerization (DP) of tannins found in sorghum is generally greater than 10 (Awika et al., 2003), and this high molecular weight is associated with a stronger affinity for other molecules, such as proteins and carbohydrates (Amoako & Awika, 2019; Girard et al., 2018; Barros et al., 2014).

Proanthocyanidins (PA) are the phenolic compounds in sorghum that have garnered the most attention due to their abundance and anti-nutritional characteristics, as they strongly bind with proteins, thus reducing their digestibility (Girard et al., 2018). PA can also bind to minerals, decreasing their absorption, and to starch, which in this case can offer advantages in decreasing digestibility (Barros et al., 2012; Barros et al., 2014; Mkandawire et al., 2013; Amoako & Awika, 2019), thereby reducing blood insulin levels. Hence, reduced digestibility can be considered beneficial for certain individuals, such as those suffering from chronic diseases related to obesity.

5.3.2 Sorghum Health Benefits

Sorghum grain consumption has been associated with various health benefits due to its nutritional composition and the presence of phytochemicals, which have the potential to prevent non-communicable diseases (Stefoska-Needham et al., 2015). Numerous studies have highlighted these advantages, ranging from its antioxidant and anti-inflammatory properties to its metabolic effects on obesity and glycemic indexes (Martinez et al., 2021a; Silva et al., 2020; Arbex et al., 2018; Martinez et al., 2021b; Arbex et al., 2018; Moraes et al., 2015).

In vitro studies investigating the health potential of sorghum have shown promising results. For example, Awika et al. (2009) demonstrated that extracts from various sorghum varieties, including those with high tannin content, exhibited strong antiproliferative activity against colon (HT-29) and esophageal (OE33) cancer cells. Sorghum extracts containing tannins were particularly effective, requiring lower concentrations to inhibit cell proliferation compared to white sorghum extracts. Similarly, Cox et al. (2019) found that extracts from a high-phenolic sorghum variety and sumac sorghum (containing tannins) inhibited the growth of human colorectal adenocarcinoma (HCT-15) and human hepatocellular carcinoma (HEPG2) cell lines.

Sorghum has not only shown action against the growth of cancer cells but also in the process of cell apoptosis and in the generation of reactive oxygen species (ROS). Smolensky et al. (2018) showed that high-polyphenol extract of sorghum bran decreased the cell viability of HepG2 and Caco2 cells. The results of cell apoptosis rate and ROS help to explain the mechanisms that lead to the reduction of this cell viability, where there was an increase in the level of apoptosis from 9.9% of the control to 35.6% after 18h of exposure to 2.5 mg/mL high-polyphenol sorghum extract. There was also an increase in ROS by 37.9% after 18h of exposure to the extracts (2.5 mg/mL), which means that the treatment had a prooxidant action. It is not uncommon for polyphenols with antioxidant activities in vitro to show prooxidant activities in cells, since this anticancer effect may be due to favoring the formation of ROS (Léon-González et al., 2015).

Although most studies of sorghum as a health promoter show effects in combating oxidative stress in *in vitro* analyses, recent studies have also explored whether this potential is also observed in animal models. Arbex et al. (2018) studied the effect of extruded sorghum flour on adiposity and inflammation modulation in obese rats. Results showed that rats fed on extruded sorghum flour diets presented lower levels of TNF-α, glucose, evidencing the anti-inflammatory potential and hypoglycemic effect of sorghum. Also, extruded sorghum in the diet was able to reduce adiposity, total fat, and epididymal adipose tissue in obese rats.

Regarding liver function and health, in many studies (Martinez et al., 2021b; Silva et

al., 2020; Arbex et al., 2018), it was observed a reduction of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), enzymes involved in liver disease, in rats treated with sorghum. Martinez et al. (2021b) sought to evaluate the effect of whole sorghum flour on parameters such as insulin resistance, glucose tolerance, adiposity, and hepatic stasis in rats fed a high fat high fructose (HFHF) diet. Sorghum flour showed efficacy in improving glucose metabolism, increasing insulin sensitivity and glucose tolerance, lipid metabolism, decreasing triglyceride concentration, in addition to decreasing ALT, reducing hepatic steatosis. These results highlight the role of sorghum not only in maintaining health but also in restoring the health of animals with biochemical markers and altered conditions.

The phenolic compounds present in sorghum may be associated with the health benefits seen in animal studies. Deoxyanthocyanidins and condensed tannins were found in extruded sorghum flour and associated with improvements in biometric parameters, inflammation, and adiposity (Arbex et al., 2018), while sorghum total phenolics and their antioxidant activity were inversely correlated with the glycemic index of rats (Moraes et al., 2015). In addition, sorghum flour with high tannin content was able to reduce inflammation and oxidative stress in rats fed on a HFHF diet (Martinez et al., 2021a).

In the study by Silva et al. (2020), spontaneously hypertensive rats were induced to oxidative stress with paracetamol and received toasted sorghum flour with and without tannins (white sorghum). There was an improvement in lipid metabolism and reduction of oxidative stress with similar results in both groups treated with toasted white sorghum flour and with tannins, even if white sorghum had presented lower total phenolic compounds and antioxidant capacity. This suggests that the anti-inflammatory and antioxidant effect may be given by small phenolic compounds rather than tannins, which have a high degree of polymerization.

Despite all the satisfactory results of how sorghum can bring health benefits, there is still a lot to be clarified regarding the changes that its consumption can cause in both healthy living organisms and metabolically altered ones.

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6. CHAPTER 2: *IN VITRO* STUDY WITH SORGHUM PHENOLIC EXTRACTS

Food Research International 176 (2024) 113739

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Phenolic-rich extracts from toasted white and tannin sorghum flours have distinct profiles influencing their antioxidant, antiproliferative, anti-adhesive, anti-invasive, and antimalarial activities

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ARTICLE INFO

Keywords:
Antioxidant supplement
Cytotoxicity
Antiplasmodial effect
ROS
Bioactive compounds
Phenolic profile

ABSTRACT

Sorghum is a gluten-free cereal commonly used in foods, and its consumption has been associated with the prevention of human chronic conditions such as obesity and cancer, due to the presence of dietary fiber and phenolic compounds. This study aimed to evaluate, for the first time, the antiproliferative, antioxidant, anti-adhesion, anti-invasion, and antimalarial activities of phenolic extracts from toasted white and tannin sorghum flours to understand how different phenolic profiles contribute to sorghum biological activities. Water and 70 % ethanol/water (v/v), eco-friendly solvents, were used to obtain the phenolic extracts of toasted sorghum flours, and their phenolic profile was analyzed by UPLC-MS^E. One hundred forty-five (145) phenolic compounds were identified, with 23 compounds common to all extracts. The solvent type affected the phenolic composition, with aqueous extract of both white sorghum (WSA) and tannin sorghum (TSA) containing mainly phenolic acids. White sorghum (WSE) and tannin sorghum (TSE) ethanolic extracts exhibited a higher abundance of flavonoids. WSE demonstrated the lowest IC_{50} on EA.hy926 (IC_{50} = 46.6 μg/mL) and A549 cancer cells (IC_{50} = 33.1 μg/mL), while TSE showed the lowest IC_{50} (IC_{50} = 70.8 μg/mL) on HCT-8 cells (human colon carcinoma). Aqueous extracts also demonstrated interesting results, similar to TSE, showing selectivity for cancer cells at higher IC_{50} concentrations. All sorghum extracts also reduced the adhesion and invasion of HCT-8 cells, suggesting anti-metastatic potential. WSE, rich in phenolic acids and flavonoids, exhibited greater toxicity to both the W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) strains of *Plasmodium falciparum* (IC_{50} = 8 μg GAE/mL and 22.9 μg GAE/mL, respectively). These findings underscore the potential health benefits of toasted sorghum flours, suggesting diverse applications in the food industry as a functional ingredient or even as an antioxidant supplement. Moreover, it is suggested that, besides the phenolic concentration, the phenolic profile is important to understand the health benefits of sorghum flours.

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench), an ancient cultivated cereal resistant to drought, is a food with potential to be studied by combining a high content of dietary fiber and an interesting profile of phenolic compounds (Martinez et al., 2021; Girard & Awika, 2018). Studies have

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https://doi.org/10.1016/j.foodres.2023.113739
Received 29 June 2023; Received in revised form 3 November 2023; Accepted 22 November 2023
Available online 23 November 2023

reported the presence mainly of phenolic acids, flavonoids, such as 3-deoxyanthocyanins, and condensed tannins or proanthocyanidins in sorghum genotypes (Cardoso et al., 2014; Dykes & Rooney, 2006). Phenolic compounds have bioactivity against oxidative stress, which occurs due to the accumulation of free radicals produced by cellular aerobic metabolism and the non-elimination of these reactive oxygen species (ROS) (Lee & Paull, 2020). Sorghum phenolic compounds with different pigmentations (black, red, white, brown) have shown antioxidant properties *in vitro*, with a higher antioxidant activity observed in the bran of varieties containing condensed tannins (Barros et al., 2013; Dykes et al., 2013; Moraes et al., 2015).

Excessive ROS generation can stimulate oncogenesis by interfering with pathways that influence cell proliferation, differentiation, and apoptosis (Smolensky et al., 2018). In vitro studies have shown the health potential of sorghum phenolic compounds through antiproliferative activity against cancer cell lines (Cox et al., 2019; Yang et al., 2009). Yang et al. (2009) tested the potential to induce the activity of phase II enzymes and the growth- inhibitory properties of cancer cells of extracts from black, red, and white sorghum genotypes. They found higher antiproliferative activity in black sorghum extracts containing high concentrations of methoxylated 3-deoxyanthocyanins.

In addition to diseases related to oxidative stress, phenolic compounds may also act against parasites, such as the malaria-causing *Plasmodium falciparum*. Although there are no available studies on the antimalarial potential specifically of the sorghum phenolic compounds in the literature, there is evidence of the action of phenolics from other sources against *Plasmodium* species (Noronha et al., 2022; Carmo et al., 2020). Bioactive compounds present in both camu-camu extracts and black tea kombucha, such as epicatechin, catechin, quercetin, and gallic acid, were negatively correlated with *P. falciparum* strains (Noronha et al., 2022; Carmo et al., 2020).

Sorghum extracts have been prepared using different solvents, like methanol, ethanol, acetone, and even water, to analyze the phenolic profile of sorghum and its properties (Cox et al., 2019; Barros et al., 2013). Extraction parameters are important because they can influence the biological response of *in vitro* tests, as in the study by Cox et al. (2019). The authors found that the antiproliferative effects of sorghum extracts made with 70 % ethanol (v/v) and 5 % citric acid (w/v) were more effective than with 50 % ethanol. This is because different extraction methods can generate extracts with different phenolic compositions.

Moreover, previous *in vivo* studies have demonstrated that rats fed toasted white (BRS501) or tannin sorghum (BRS305) flour exhibited a reduction in oxidative stress and an improvement in lipid profiles (Silva et al., 2020); these are the same genotypes evaluated in the current study. These findings suggest that the phenolic profile, rather than the phenolic concentration, is a crucial parameter for investigating the beneficial effects of sorghum flour, as both flours exhibited similar *in vivo* effects despite the significantly higher phenolic concentration in BRS305. Identifying further health benefits associated with toasted sorghum flour can drive its application in the food industry, including its use in the formulation of *farofas*, a traditional Brazilian food, as an ingredient in other sorghum-based products, or antioxidant supplements. Therefore, this study aimed to evaluate the antiproliferative, antioxidant, anti-adhesive, anti-invasive, and antimalarial activities of phenolic-rich extracts derived from toasted white and tannin sorghum flours using environmentally friendly solvents.

2. Materials and methods

2.1. Samples, reagents, and cell lines

Two sorghum [*Sorghum bicolor* (L.) Moench] genotypes harvested in the mature stage were used in this study: one sorghum genotype with high tannin content (BRS 305) and a white sorghum genotype (BRS 501), which is without tannins, selected from the germplasm bank of Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais, Brazil.

Folin-Ciocalteu's phenol reagent, ethanolamine, gallic acid, (+)-catechin, vanillin, potassium persulfate, ABTS [2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)], Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], DMSO (dimethyl sulfoxide), RPMI-1640 medium, penicillin, Trition X-100, EDTA (disodium ethylenediaminetetraacetate dihydrate), Sybr Safe, DCFH-DA (dichlorodihydro-fluorescein diacetate), MTT (2, 5-diphenyl tetrazolium bromide), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM), and Matrigel® were purchased from Sigma-Aldrich (São Paulo, Brazil). Fetal bovine serum and Albumax II were purchased from Gibco (Grand Island, NY, USA). Ethyl alcohol was acquired from Neon (São Paulo, Brazil). All chemical reagents used were of analytical grade. Also, A549 (lung adenocarcinoma epithelial cells), EAhy926 (noncancerous cell line derived from human endothelium), and HCT-8 (human colon carcinoma) cell lines were obtained from the Rio de Janeiro cell bank (Brazil).

2.2. Toasted sorghum flour preparation

Toasted sorghum flours were prepared similarly to the method of Silva et al. (2020), with some changes. Sorghum grains were ground in a knife mill (C.W. Brabender, Duisburg, Germany) with a 1 mm opening to obtain the white raw sorghum flour (WRSF) and tannin raw sorghum flour (TRSF). Both flours were dried in an oven with air circulation at 35 °C for 15 h and then stored in a freezer (-20 °C) until use. The flours were toasted in direct heat with an average temperature of 200 °C for 6 min. The tannin toasted sorghum flour (TTSF), from BRS 305, and white toasted sorghum flour (WTSF), from BRS 501, were stored at −20 °C until use.

2.3. Extraction of phenolic compounds from the toasted sorghum flours

Phenolic compounds were extracted using GRAS (generally recognized as safe) solvents. Extractable phenolic compounds from toasted sorghum flours were obtained by using two different solvents: a) flour and distilled water (1:10 w/v), kept under stirring at a temperature of 50 °C for 2 h; b) flour and a solution of 70 % ethanol in water (v/v), kept at room temperature (25 °C) for 2 h. The suspension was filtered and then centrifuged at 3100 x *g* for 10 min to reduce the water content and remove the ethanol in the samples. The supernatant of each extract was concentrated using a rotary evaporator (IKA RV 10, Staufen, Germany) at 45 °C, 200 rpm for approximately 90 min, and then freeze-dried (Liotop-L101, Liobras, Brazil). The samples were named as follows: WSA (white sorghum flour aqueous extract), WSE (white sorghum flour ethanolic extract), TSA (tannin sorghum flour aqueous extract), and TSE (tannin sorghum flour ethanolic extract).

2.4. Characterization of toasted sorghum flours

2.4.1. Proximate composition of the toasted sorghum flours

The proximate composition of toasted sorghum flours was investigated to identify the proportions of nutrients. Moisture, proteins, lipids, dietary fiber, ash, and carbohydrates were determined according to the AOAC (1998). Resistant starch was determined using a Megazyme kit (AACC, 2000, method 32–40).

2.4.2. Determination of total extractable phenolics and tannins

For the analysis of free/extractable phenolic compounds, first, the samples were extracted as mentioned previously, using water at 50 °C and ethanol in water (70 % v/v) to obtain free phenolic compounds present in the supernatant. Total extractable/free phenolic compounds concentration, present in toasted sorghum flours, was determined according to the Folin Ciocalteu method, described by Singleton & Rossi (1965). The phenolic concentration is expressed in milligrams of gallic acid equivalent per gram of sample (mg GAE/g). Additionally, the

condensed tannins were quantified by the vanillin method (Price et al., 1978), and the results were expressed in mg of (+)-catechin/g of sample.

2.4.3. Determination of the antioxidant capacity

Antioxidant capacity was determined according to the methodology by Awika et al. (2003). Briefly, the ABTS stock solution was prepared by mixing an equal volume of 8 mM ABTS with 3 mM potassium persulfate, let for reaction in the dark for at least 12 h. The ABTS working solution should have an original absorbance of around 1.5 at 734 nm. The different samples of sorghum extracts (0.1 mL) were added with 2.9 mL ABTS working solution followed by 30 min in the dark at room temperature for reaction. The absorbance was read at 734 nm, and the antioxidant capacity was calculated and expressed as μmol Trolox equivalent /g sample.

2.4.4. Metabolomic analysis of sorghum phenolic profile by UPLC-MS^E

To determine the profile of phenolic compounds in sorghum extracts, they were subjected to evaporation using a Savant SpeedVac centrifuge (Thermofisher) and subsequently reconstituted in 1 mL of a solution containing 2 % methanol (LC-MS grade), 5 % acetonitrile (LC-MS grade), and 93 % Milli-Q water. The reconstituted extracts were then filtered through a hydrophilic PTFE filter (0.22 μm, Analytical) and stored in vials.

Thirty-three analytical standards (vanillic acid, p-coumaric acid, catechin, caffeic acid, ellagic acid, *trans*-ferulic acid, kaempferol, myricetin, pyrogallol, flavanone, quercetin, syringic acid, gallic acid, epicatechin, 4-hydroxy benzyl alcohol, 4-hydroxy benzaldehyde, 4-hydroxybenzoic acid, 4-hydroxy phenylacetic acid, synapinic acid, benzoic acid, quercetin 3-O-glucoside, 3,4-dihydroxy phenylacetic acid, epigallocatechin, epigallocatechin gallate, chlorogenic acid, 2,5-dihydroxy benzoic acid, p-anisic acid, 2-hydroxycinnamic acid, vanillin, *trans*-cinnamic acid, 3-methoxy cinnamic acid, 4-methoxy cinnamic acid, and L-(−)-3-phenylacetic acid) of phenolic compounds (10 ppm) were prepared in a mix and injected in triplicate prior to the injection of the samples in order to ensure the reproducibility and to identify phenolic compounds.

A total of 5 μL of each sample was filtered at a 0.22 μm hydrophilic PTFE syringe filter (Analitica, Diadema, SP/Brazil) and injected into Ultra-Performance Liquid Chromatography (UPLC) Acquity system (Waters Co., Milford, MA), coupled with XEVO G2S Q-Tof (Waters Co., Manchester, UK) and equipped with ionization source electrospray, as described by Cardoso et al. (2020). A UPLC HSS T3 C18 column was used (100 x 2.1 mm, 1.8 μm particle diameter; Waters) with the following parameters: temperature of 30 °C, flow rate of 0.5 mL/min of the mobile phases (A: ultra-pure water containing 0.3 % formic acid and 5 mM ammonium formate, and B: acetonitrile containing 0.3 % formic acid), applying the gradient: 0 min, 97 % A; 11.80 min, 50 % A; 12.38 min, 15 % A; 14.23 min, 15 % A; 14.70 min, 97 % A. Data were acquired using the MS^E negative and centroid mode between *m/z* 50 and 1200; collision energy ramp from 30 to 55 V; cone voltage of 30 V; capillary voltage of 3.0 kV; 1,200 L/h desolvation gas (N_2) at 600 °C; 50 L/h cone gas; source at 150 °C. Leucine enkephalin was used for calibration (Leu-Enk, *m/z* 554.2615, [M−H]-).

Data were processed using Progenesis QI (Waters), and the identification and annotation were performed based on standard run parameters, such as: isotope distribution of neutral mass, exact mass, retention time, and MS/MS fragments spectra. PubChem was used to aid the non-targeted annotation by applying MetaScope, an integrated search tool. Other parameters were applied in descending order of importance in the software, namely exact mass error (<10 ppm), isotopic similarity (>80 %), score (>30), and score of fragmentation. Annotation was also done for unknown compounds using the phenol explorer database, published data from the literature, and chemical features of the molecule. The only compounds considered as tentatively identified were the ones present in the three technical replicates (3/3), where each vial contains a pool of three true replicates and CV < 30 %.

2.5. In vitro biological activities

2.5.1. In vitro cell-based cytotoxicity

Freeze-dried phenolic extracts were suspended in ultrapure water, Tween 80, and DMSO before their use in the biological assays. The following cell lines were used to evaluate the cytotoxic effect of the sorghum phenolic extracts: human lung cancer cells (A549), human colon carcinoma cells (HCT-8), and noncancerous cell line derived from human endothelium (EA.hy926). All cells were cultured in DMEM/Ham-F12 medium, supplemented with 10 % fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Briefly, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well and with 100 μL/well. Incubation was conducted in a humid atmosphere containing 5 % CO_2, 5 % O_2, and 95 % N_2 at 37 °C for 24 h. Based on the quantification of free phenolic compounds, the tested concentrations were established, wherein lower concentrations of treatments were adopted for the WSA and WSE samples for the cells to have sufficient culture medium to grow. Cells were treated with the phenolic extracts of toasted sorghum flours at different concentrations (WSA and WSE: 5, 10, 25, 40, and 50 μg GAE /mL; TSA and TSE: 5, 25, 50, 75, and 100 μg GAE /mL). After 48 h of incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to each well and incubated for 4 h at 37 °C. MTT is reduced to blue formazan crystals by metabolically active cells (Geinaert et al., 2017). The supernatant was removed, and 100 μL of DMSO was added to dissolve these crystals. Absorbance was read at 570 nm wavelength. The methodology used by Escher et al. (2018) was applied to calculate the parameters IC_{50}, GI_{50}, and LC_{50}. IC_{50} is the concentration of the agent that inhibits growth by 50 %, $(T/C) \times 100 = 50$, where T is the number of cells at time t treatment, and C is the control cells at time t treatment. GI_{50} is the concentration of the agent that inhibits growth by 50 %, relative to untreated cells, the concentration at which $([T - T0] / [C - T0]) \times 100 = 50$, where T and C are the number of treated and control cells, respectively, at treatment time t and $T > T0$, and T0 is the number of cells at time zero. LC_{50} is the concentration of the agent that results in a net loss of 50 % of cells, relative to the number at the start of treatment, the concentration at which $([T - T0] / T0) \times 100 = -50$; $[T] < [T0]$.

2.5.2. Clonogenic assay

This assay evaluated the ability of different sorghum extracts to inhibit the formation of colonies of HCT-8 ileocecal adenocarcinoma cells (Franken et al., 2006). Briefly, HCT-8 cells (3000 cells per well) were seeded onto a 6-well plate and incubated overnight at 37 °C under 5 % CO_2 atmosphere. Subsequently, the exhausted medium was changed with fresh medium (2 % FBS) containing 2, 5, or 10 μg/mL of WSA, WSE, TSA, or TSE. Culture treatment was carried out for 24 h, and the cells were cultured for 14 days with growth medium (2 % FBS) replaced at every two days. On the 15th day, colonies were then fixed with methanol for 30 min and stained with crystal violet (0.5 %) for 30 min. Colonies were counted using ImageJ, and the results were expressed as a percentage of the untreated control cultures (Mao et al., 2016; Lima et al., 2018).

2.5.3. Reactive oxygen species (ROS) generation

The effects of sorghum extracts on the scavenging of intracellular ROS were assessed using the DCFH-DA (2′,7′-dichlorofluorescin diacetate) assay. Noncancerous (EA.hy926) and cancerous (A549 and HCT-8) cells (6×10^4 per well) were treated for 1 h with different sorghum extracts concentrations to treat cells below their IC_{50} values and avoid cell death, (WSA: 5, 10, and 25 μg GAE /mL for all cells; WSE: 0.25, 0.50, 1, 2 μg GAE /mL for EA.hy926 cells, and 1, 2 and 5 μg GAE /mL for cancer cells; TSA and TSE: 5, 25, 50, 75 and 100 μg GAE/mL for all cells) or 15 μmol/L H_2O_2 (positive control) or culture medium (negative control). The variable concentrations are due to free phenolic compounds results to maintain the culture medium and avoid reaching the half inhibitory concentration (IC_{50}). After treatment, H_2O_2 was added at

15 μmol/L in the wells, and the fluorescence intensity ($\lambda_{emission}$ = 538 nm and $\lambda_{excitation}$ = 485 nm) was measured (Escher et al., 2018). The data is expressed as percentage of fluorescence intensity relative to the untreated group (negative control).

2.5.4. Cell adhesion and invasion assays

TSA, TSE, WSA, and WSE sorghum extracts at 2, 5, and 10 μg/mL were subjected to a cell adhesion assay without Matrigel®, a screening assay. This assay aimed to select the most active concentration, which would be used in subsequent assays. In the cell adhesion assay without Matrigel®, HCT-8 cells were treated with sorghum extracts for 1 h. Following this treatment, cells were seeded in a 96-well plate, with 8 × 10^4 cells/well, and incubated overnight at 37 °C under a 5 % CO_2 atmosphere. A gentle wash with PBS was performed to prevent detachment of adhered cells, eliminating non-adherent cells. Subsequently, adherent cells were stained with crystal violet (0.5 % v/v) for 30 min, solubilized with sodium dodecyl sulfate (SDS) (1 % m/v) at 37 °C for 30 min, and quantified at 570 nm. The results were normalized considering the control culture (Moreira et al., 2018; Marvi et al., 2022).

After the screening step using the cell adhesion method without Matrigel®, sorghum extracts at the most active concentration were subjected to the cell adhesion assay using Matrigel®. Briefly, HCT-8 cells were treated with 10 μg/mL of sorghum extracts for 1 h and plated in a 96-well plate (8 × 10^4 cells/well) precoated with 60 μL of Matrigel®. The plate was then incubated for 12 h at 37 °C to facilitate adhesion. Subsequently, the cells were washed with PBS, and the adherent cells were stained with crystal violet and analyzed as previously described.

Finally, the cell invasion assay was performed using transwell inserts (Corning, Corning, New York, USA) with a pore diameter of 8 μm, following a previously established methodology (Lima et al., 2018). For gel formation, 60 μL of Geltrex (Thermo Fisher Scientific) diluted (1:4) in serum-free medium was added to the upper chambers and incubated for 8 h at 37 °C. HCT-8 cells at 8 × 10^4 cells/well were then diluted in serum-free medium and treated with sorghum extracts at a concentration of 10 μg/mL for 1 h. The cells were seeded onto the upper surface, while the lower surface contained a medium with 20 % FBS. After 24 h of incubation, the inserts were removed from the wells. The cells on the upper surface of the inserts were wiped with a cotton swab, whereas the cells that had invaded the lower surface were fixed with methanol for 30 min and stained with crystal violet (0.5 % v/v) for 30 min. Ten fields were randomly chosen and captured using an inverted microscope (BEL Photonics) to acquire images. The cells were counted using the Image J software, and the results were expressed as a percentage of cell invasion (Moreira et al., 2018; Marvi et al., 2022).

2.5.5. Antimalarial properties

The anti-plasmodial effect of sorghum phenolic compounds was performed according to Carmo et al. (2020), using strains W2 and 3D7, resistant and sensitive to chloroquine, respectively. Plasmodium strains were cultivated in RPMI culture medium, with 10 % albumax II and 4 % hematocrit, incubated at 37 °C using the candle jar method. The culture medium was changed daily, and parasitemia was checked on Giemsa-stained smears. Sorbitol solution was used to synchronize the parasites and obtain ring shapes. Subsequently, the parasites were diluted and incubated on 96-well plates with sorghum extracts (concentrations 5, 25, 50, 100 μg/mL) or culture medium as a positive control. After 48 h, the supernatant was removed, and subsequently, 100 μL of lysis buffer solution (20 mM, pH 7.5), EDTA (5 mM), saponin (0.008 %; wt/vol), and Trition X-100 (0.08 %; v/v), and 0.2 μL/mL Sybr Safe were added. The microplates were incubated in the dark for 30 min, and the samples were read in a microplate reader with excitation at 485 nm and emission at 535 nm.

2.5.6. Target fishing analysis

To understand the potential mechanisms of action of phenolic-rich extracts from toasted sorghum flour on cell lines used in biological tests and against plasmodia that cause malaria, the phenolic compounds identified in the samples were subjected to target fishing analysis using Similarity Ensemble Approach (SEA) webserver (Keiser et al., 2007; Perez-Castillo et al., 2021). This analysis was carried out using the chemical structure of compounds presented in Table 2, which were the most relatively abundant. Isomers and not completely identified compounds were discarded from the computational analysis. The input was provided in SMILES format for each compound, obtained from PubChem. Predicted targets unrelated to used cell lines' metabolic pathway or associated with organisms other than *Plasmodium falciparum* were excluded from further analysis and discussion. More details of the employed strategy can be found in Azevedo et al. (2022).

2.6. Statistical analysis

All analyses were performed in triplicates, and the results were represented by mean and standard deviation. The raw UPLC-MS^E data was first submitted to a normalization process using Progenesis QI to enable quantitative comparisons among the different samples. The abundance values obtained from the ion mass spectra were utilized for relative quantification and statistical evaluation of the data. Statistical analyses were conducted, including one-way ANOVA and post-hoc Tukey test, with a significance level of $p < 0.05$, was conducted. Data generated were exported to perform a Principal Component Analysis (PCA) by XLSTAT (Addinsoft, France) and a heatmap by Metaboanalyst 3.0 web server. The differential abundance of compounds in the samples was performed by pairwise comparison, and the volcano plots were applied considering the compounds present in two compared conditions after application of two filters: log2 fold change ± 1.0 and ANOVA $p < 0.05$. Dose-response cytotoxicity analysis was determined by non-linear regression (curve fitting), and a *t*-test was performed for all *in vitro* analyses. The experiments were analyzed using the GraphPad Prism® (GraphPad Software, Inc., San Diego, CA, USA). All tests were considered statistically significant at $P \leq 0.05$.

3. Results and discussion

3.1. Proximate composition

The results of the proximate analysis are shown in Table 1. As expected, toasted flours had lower moisture content due to the heat treatment. Overall, there were no significant changes in the proximate composition of the toasted and raw flours.

Regarding the toasted flours, focus of our work, protein content slightly varied for TTSF compared to the findings by Silva et al. (2020)

Table 1
Proximate composition of toasted sorghum flours.

(%)	WRSF	WTSF	TRSF	TTSF
Moisture	11.15 ± 0.56^a	6.29 ± 0.07^b	11.82 ± 0.18^x	5.84 ± 0.08^y
Protein	11.32 ± 0.41^a	12.92 ± 0.40^a	11.64 ± 0.18^x	10.33 ± 0.09^x
Lipid	4.04 ± 0.03^a	3.59 ± 0.03^b	3.75 ± 0.04^x	3.48 ± 0.11^x
Ash	1.60 ± 0.01^a	1.66 ± 0.01^a	1.60 ± 0.01^x	2.21 ± 0.13^y
Carbohydrate	71.90	75.54	71.19	75.33
Resistant starch	7.4 ± 0.92^a	7.6 ± 1.21^a	35.3 ± 1.88^x	35.9 ± 2.53^x
Insoluble fiber	9.74 ± 0.13^a	10.82 ± 0.33^a	19.97 ± 0.25^x	21.88 ± 0.28^y
Soluble fiber	0.36 ± 0.00^a	0.66 ± 0.28^a	0.49 ± 0.06^x	0.97 ± 0.12^y
Total dietary fiber	10.09 ± 0.14^a	11.48 ± 0.04^b	20.45 ± 0.18^x	22.86 ± 0.40^y

All results are on a dry-basis, except for the moisture content. WRSF = White Raw Sorghum Flour; WTSF = White Toasted Sorghum Flour; TRSF = Tannin Raw Sorghum Flour; TTSF = Tannin Toasted Sorghum Flour. Means followed by the same letter within a row, between raw and toasted treatments (WRSF *vs* WTSF, and TRSF *vs* TTSF), are not significantly different (P > 0.05).

Table 2
Most abundant phenolic compounds and standard reference compounds found in sorghum extracts.

Name of compound	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Error (ppm)	IS (%)	Class	Extracts*
Daidzin/Puerarin	$C_{21}H_{20}O_9$	415.1025	10.02	57.0	88.7	−2.35	99.30	F	WSE[1], TSE[1], TSA[5], WSA[2]
Dihydroquercetin	$C_{15}H_{12}O_7$	303.0495	8.41	51.0	61.5	−4.98	99.44	F	TSA[1], TSE[3]
Naringenin 7-O-glucoside	$C_{21}H_{22}O_{10}$	433.1136	8.16	56.3	83.9	−1.07	99.12	F	TSE[2], TSA[2]
Trihydroxyflavanone isomer VII	$C_{15}H_{12}O_5$	271.0601	10.92	57.9	94.7	−4.20	99.83	F	TSA[3], TSE[7], WSE[5], WSA
Tetrahydroxyisoflavone isomer	$C_{15}H_{10}O_6$	285.0394	10.26	57.9	94.5	−3.85	99.56	F	TSE[4], WSE[3], TSA, WSA
Methylgalangin	$C_{16}H_{12}O_5$	283.0597	8.51	50.6	60.2	−5.20	98.73	F	TSE[5], TSA
Trihydroxyflavone isomer I	$C_{15}H_{10}O_5$	269.0444	7.93	52.6	70.0	−4.09	97.83	F	TSE[6], TSA
Hydroxybenzoic acid isomer/ protocatechuic aldehyde/sesamol	$C_7H_6O_3$	137.0232	4.91	55.2	86.2	−8.69	99.47	NI	WSE[2], TSE[9], TSA[7], WSA[1]
Kaempferol	$C_{15}H_{10}O_6$	285.0391	8.41	47.2	42.2	−4.66	99.36	F	TSA[4], TSE[8]
Caffeic acid	$C_9H_8O_4$	179.0337	6.42	57.5	96.3	−7.23	99.54	PA	TSA[6], TSE, WSE[7], WSA[6]
Eriodictyol	$C_{15}H_{12}O_6$	287.0551	10.10	53.1	70.1	−3.42	99.27	F	WSE, TSE, TSA[8], WSA
4-Hydroxybenzoic acid	$C_7H_6O_3$	137.0232	5.23	56.9	95.2	−8.49	98.81	PA	TSA, TSE, WSE[6], WSA[3]
5-Caffeoylquinic acid	$C_{16}H_{18}O_9$	353.0864	6.02	54.7	80.7	−4.11	97.67	PA	TSA, TSE, WSE, WSA
m-Coumaric acid	$C_9H_8O_3$	163.0387	7.64	37.9	0.0	−8.29	98.71	PA	TSA, WSE[8], WSA[5], TSE
Dicaffeoylquinic acid isomer	$C_{25}H_{24}O_{12}$	515.1233	0.73	35.6	0.0	7.45	86.38	PA	WSA[4], WSE
Hydroxycoumarin isomer I	$C_9H_6O_3$	161.0232	6.73	52.6	77.0	−7.47	94.52	NI	WSE, WSA[7], TSA, TSE

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids; L = lignans; OP = other polyphenols. Italic means reference compounds not tentatively identified. Numbers mean the order of the most abundant compounds in the extracts. * Means the extract samples follow the order of higher concentration first and lower concentration later.

work (13.17 ± 1.19 g/100 g). Similarly, the lipid content for the TTSF was higher (6.29 %) than the values found previously for toasted sorghum flours of 3.83 ± 0.13 g/100 g (Silva et al., 2020). When comparing the proximate composition of toasted sorghum flours with raw flours of the same genotypes in previous works (Martino et al., 2012; Bernardo et al., 2019; Correia et al., 2023), there was no great difference in the protein (9.91–12.23 % and 9.68–12.16 % for BRS 501 and BRS 305, respectively), carbohydrate (55.14–69.33 % and 57.88–71.23 % for BRS 501 and BRS 305, respectively) and ash content (1.51–1.80 % and 1.24–1.35 % for BRS 501 and BRS 305, respectively). This evidences how direct dry heat processing does not cause major changes in sorghum flour.

Resistant starch (RS) values found for the BR 501 white sorghum genotype (7.6 ± 1.21 %) were higher than those found for white sorghum by Khan et al. (2013) (2.21 ± 0.06 g/100 g). Although sorghum grains, as well as other cereals, already have a certain RS amount, the sorghum genotype with tannin BR 305 stands out for having high levels of this type of dietary fiber, presenting 35.9 % ± 2.53 of RS; this is the national sorghum genotype with the highest RS concentration (De Carvalho Teixeira et al. 2016). Additionally, compared to other cereals, sorghum also has a higher RS amount, as shown by Giuberti et al. (2012). The authors found lower RS content in maize, barley, rice, and oats (191 ± 6.1, 143 ± 9.7, 142 ± 9.8, 99 ± 28 g/kg, respectively) than in sorghum (275 ± 12.3 g/kg).

The total fiber content was higher for TTSF than for WTSF, which can be mainly explained by the high RS content found in sorghum with tannins. Many factors may influence starch digestion rate and contribute to RS formation, including strong interactions between starch and protein as noted by Benmoussa et al. (2006), as well as the presence of tannins, non-starch polysaccharides (NSP), or alpha-amylase inhibitors (Taylor & Duodu, 2022; Weselake et al., 1985). Dry heat processing applied to sorghum flours contributes to maintaining high RS content and, thus, dietary fiber levels compared to wet heat processing.

3.2. Phenolic characterization and antioxidant capacity

The free total phenolic content (FPC), tannin content, and antioxidant capacity, determined in the extracts of toasted sorghum flours were 1.31 ± 0.04, not detected, and 4.2 ± 0.34 for the white toasted sorghum flour, and 45.34 ± 1.44, 75.72 ± 6.54 and 194.1 ± 0.88 for the tannin toasted sorghum flour, respectively.

As expected, the white sorghum genotype showed lower phenolic than tannin-containing sorghum. Similarly, Silva et al. (2020) found higher values of phenolic compounds for the toasted tannin sorghum flour (12.44 ± 0.31 mg GAE/g) than for toasted white sorghum flour (0.69 ± 0.03 mg GAE/g). It is also worth noting that the quantification of phenolic compounds can vary depending not only on the genotype, but also on the solvent used for extraction (Dia et al., 2016; Barros et al., 2013).

Regarding the TTSF sample, the tannin content (TC) was in accordance with our previous study for the BRS 305 genotype (Silva et al., 2020), and higher than that found in studies for other tannin sorghum genotypes (Punia et al., 2021; Dlamini et al., 2007). Tannins are well-known for their strong binding affinity with proteins (Girard et al., 2018). However, this property may also benefit human health by reducing starch digestibility (Barros et al., 2012), lowering blood glycemic index, promoting gut health, and aiding in the prevention of obesity (Lockyer & Nugent, 2017).

The antioxidant capacity was also evaluated by ABTS assay, where WTSF and TTSF showed values of 4.2 ± 0.34 and 194.1 ± 0.88 μmol TE/g sample, respectively. These results follow the trend of free phenolic values. Thus, higher phenolic content also meant higher antioxidant capacity. Silva et al. (2020) reported lower values than our findings on the antioxidant capacity by ABTS for toasted sorghum flours BRS 501 (1.51 ± 0.05 μmol TE/g sample) and BRS 305 (81.10 ± 2.74 μmol TE/g sample). In addition to increasing knowledge about the characterization of toasted sorghum flours, these results also guide the concentration standardization for biological activity analyses.

3.3. Metabolomic analysis of sorghum phenolic compounds by UPLC-MS^E

Globally, 145 phenolic compounds were tentatively identified (Table S1) in the aqueous and ethanolic extracts of sorghum flour, belonging to the classes of flavonoids (95), phenolic acids (26), other polyphenols (12), as well as lignans (1), stilbenes (2), and other compounds that did not have an attributed class (9). Among them, 23 were common to the four samples, shown in the Venn diagram (Fig. 1A). Fig. 1B depicts the abundance of these compounds represented in general for better visualization of proportions, with flavonoids comprising 85 %, followed by phenolic acids and unidentified compounds (8 % each), and other phenolics representing the smallest portion (1 %).

Total relative ion abundance by sample is demonstrated in Fig. 1C, where it is possible to note the variations in each class of phenolics for each extract. In all extracts, flavonoids and phenolic acids were the most abundant groups, standing out mainly in TSA and TSE extracts, as occurred in other studies on sorghum with tannins (D'Almeida et al., 2021). The ethanolic extracts were expected to have a higher abundance of phenolics than aqueous extracts due to the greater affinity that the

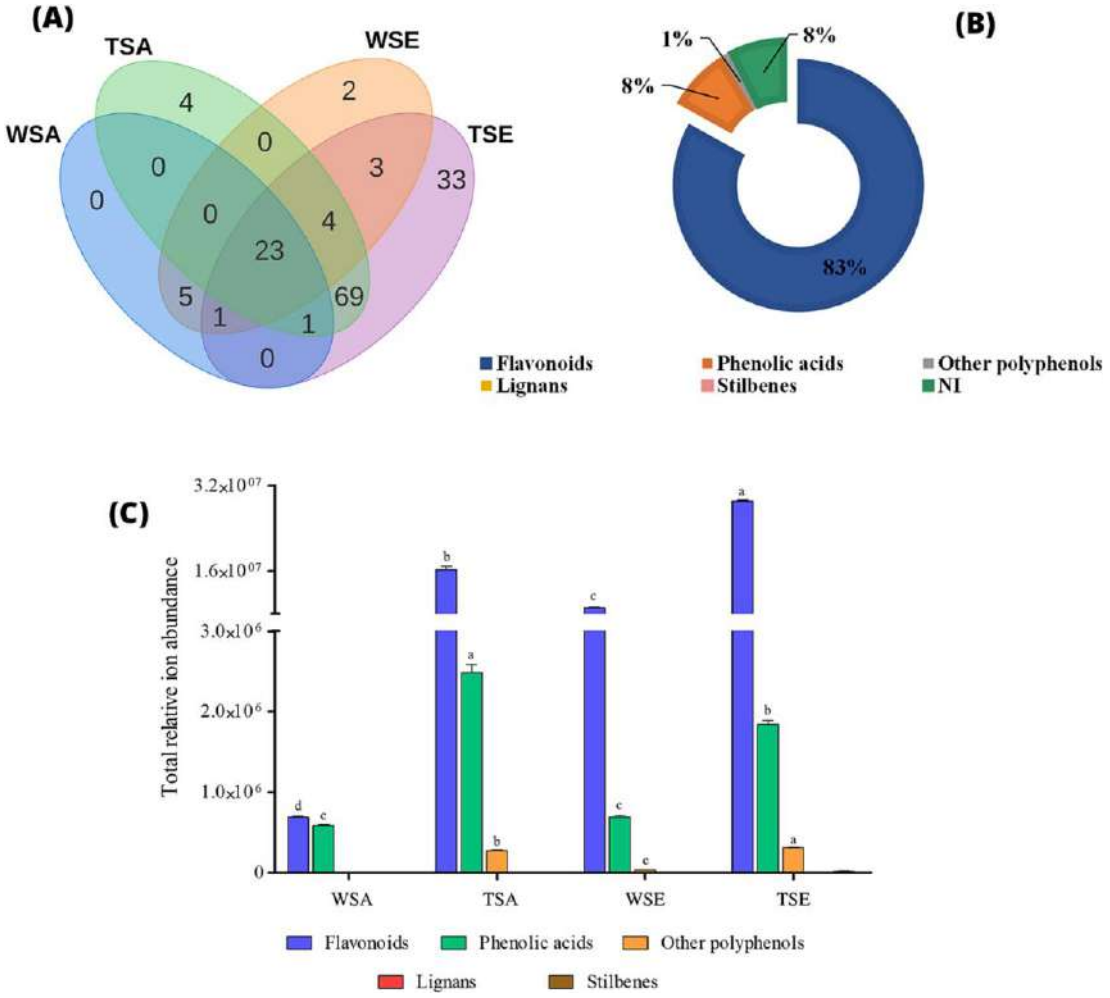


Fig. 1. Metabolomic analysis: (A) Venn Diagram of phenolic compound identifications in all samples; (B) overall distribution of the samples' phenolic compounds; (C) total relative ion abundance of phenolic compounds. WSA: white sorghum aqueous extract, WSE: white sorghum ethanolic extract, TSA: tannin sorghum aqueous extract, TSE: tannin sorghum ethanolic extract.

ethanol/water solvent has to extract phenolic compounds compared to pure water alone.

It was expected that extraction with warm water would be able to remove mainly phenolic acids and be more effective than water at 25 °C, while ethanolic extracts would have more flavonoids, as happened in the study by Fidelis et al. (2020). These authors found a higher content of flavonoids in aqueous and ethanolic extracts from camu camu. This can be observed when comparing white sorghum extracts WSA and WSE, which have similar relative abundances of phenolic acids. However, more flavonoids were obtained with ethanol in the WSE sample than in the WSA sample. TSA had more phenolic acids than TSE, as phenolic acids, the smallest phenolic compounds and therefore more polar, are more easily extracted by the solvent with greater polarity, in this case, water.

It should be noted that these compounds were identified tentatively through a non-targeted comparison against a curated database available on PubChem. Identification was performed using strict identification parameters, such as isotopic similarity and exact mass error. However, it is important to mention that these compounds were not fully confirmed with analytical standards.

The 23 compounds common to all samples can be considered as characteristic of sorghum, present regardless of genotype or extraction method. These include phenolic acids such as caffeic and m-coumaric acid and flavonoids like isorhamnetin 3-O-rutinoside and eriodictyol (Table S2). Many of the listed compounds are in line with the findings by D'Almeida et al. (2021) and Xiong et al. (2020), even with different extraction methods. These authors identified phenolics from tannin-rich with an 80 % ethanol (v/v) extraction and white sorghum with an 80 % methanol (v/v) extraction, respectively. Trans-ferulic acid, the most common phenolic acid found in cereals (Angelino et al., 2017), was also identified in all extracts. In the study by D'Almeida et al. (2021), daidzin/puerarin isoflavones, that were attributed only to soybeans, were also found in sorghum samples.

To investigate the degree of similarity or dissimilarity of the phenolic compounds identified in each extract, a PCA was performed according to their relative abundance (Figure S1). The sum of PC1 and PC2 explained 93.5 % of the variability between samples, indicating a clear distinction between mainly sorghum genotypes. PC1 represents the compounds responsible for differentiation and separation of genotypes, whereas PC2 considers the compounds responsible for separating in different solvent extraction.

As for the separation by genotype, the samples of white sorghum (WSA and WSE) were located in the left quadrant, while those of sorghum with tannins (TSA and TSE) were in the right quadrant.

Considering the separation by solvent type, a clear separation between the sorghum extracts with tannin (TSA and TSE) can be noted. However, the white sorghum extracts (WSA and WSE) had only slight differences between the extractors used. Although both variables impact the phenolic profile found, the higher value of PC1 means that it had a greater impact on the differentiation of phenolic compounds than PC2, meaning greater separation by genotypes than by solvent type. Phenolic compounds of different genotypes would cause greater separation and, therefore, differentiation. However, we highlight that the use of warm water and ethanol/water solvents also caused a differentiation — even if minor for white sorghum extracts — to obtain characteristic phenolic profiles.

Table 2 shows the most abundant phenolic compounds found in the phenolic extracts; some are common to all extracts or to genotypes only. The WSA extract showed a high relative ion abundance of the phenolic acids 4-hydroxybenzoic acid, dicaffeoylquinic acid isomer, and m-coumaric acid, in descending order. The presence of phenolic acids in this sample is observed, consistent with what was expected due to water use in the extraction process. Overall, daidzin/puerarin (not precisely identified) was the phenolic compound found in greater relative abundance, present in all four extracts. However, in the WSE and TSE samples, it was the compound with the highest relative abundance, which may indicate its greater affinity for the less polar solvent ethanol/water. Other compounds present in greater abundance in the WSE extract are tetrahydroxyisoflavone isomer and trihydroxyflavanone isomer VII, from the flavonoid class.

Regarding tannin sorghum extracts, TSA showed dihydroquercetin, kaempferol naringenin 7-O-glucoside, and trihydroxyflavanone isomer VII as the compounds with the highest abundance, the first two being flavonols and the last two flavanones. The TSE sample showed a high abundance of the compounds naringenin 7-O-glucoside, dihydroquercetin as well as the TSA extract, but also of the compounds tetrahydroxyisoflavone isomer and methylgalangin, an isoflavone and a methylated flavone, respectively. It is possible to observe from the relative abundance of each extract the phenolic profile that the different solvents were able to generate. For example, even though methylgalangin is present in the two tannin sorghum extracts, TSE (ethanol/water as solvent) managed to extract it in greater abundance. On the other hand, kaempferol was better extracted by warm water than by ethanol/water, with greater relative abundance in TSA than in TSE.

Some of the compounds in Table 2 were also reported in the study by D'Almeida et al. (2021) as the most abundant for sorghum BRS 305 extrudate extracted by 80 % (v/v) ethanol solvent, such as daidzin, caffeic acid, methylgalangin, puerarin, and kaempferol. D'Almeida et al. (2021) also reported the presence of daidzin in sorghum for the first time, which until then was an isoflavone attributed only to soybeans. Our findings again show this compound in both genotypes and may help clarify the phenolic composition of sorghum. Isoflavones have a significant role in plants as signaling molecules to hinder pathogen attacks, and they are produced as a response to environmental stress (Cederroth et al., 2012).

To better understand which are the phenolic compounds that impact this differentiation between genotypes and extractors, volcano plots were prepared (Figure S2) comparing the following samples: a) extractor impact (TSA *vs.* TSE and WSA *vs.* WSE); b) impact of genotype (TSA *vs.* WSA and TSE *vs.* WSE). We found some common phenolic compounds among the samples, but there were 51 differentially expressed compounds. These 51 compounds are listed in Table S3 and where they are up/down regulated, of which 35 are flavonoids, 10 are phenolic acids, 2 other polyphenols, and 4 of unassigned class.

The comparison between TSA and TSE (Fig. S2A) showed the highest number of differentially abundant phenolic compounds, with a total of 37 differentially expressed: 31 down-regulated and 6 up-regulated. In this same comparison, for example, the phenolic compounds trihydroxyflavone isomer and methylgalangin, both down-regulated, are mainly responsible for the separation in PC2. On the other hand, the comparison between white sorghum extracts WSA *vs.* WSE (Fig. S2B) showed 10 differentially expressed phenolic compounds, among them the dicaffeoylquinic acid isomer, the only one which is up-regulated.

Regarding the confrontations of the influence of genotypes, we found 12 differentially expressed phenolic compounds in TSA *vs.* WSA (Fig. S2C). A down-regulated compound not fully identified (kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3 -O-rhamnosyl-galactoside/quercetin 3-O-rutinoside III) was responsible for the greatest difference in this comparison. Furthermore, 22 compounds, such as up-regulated vanillic acid, were differentially expressed when confronting TSE *vs.* WSE. Although some of these compounds were present in all four extracts, meaning they are common to sorghum grains, they were expressed in different relative ion abundances, allowing separation between samples.

A heatmap was created to better visualize the 51 up and down-regulated phenolic compounds (Figure S3), where the differential profile of each sample can be observed. It is observed in Fig. 2 that, although the TSA and TSE samples are from the same sorghum genotype, the presence of ethanol in the extraction led to different abundances of phenolic compounds from the sorghum matrix, which did not happen with only heated water. On the other hand, m-coumaric acid was up-regulated in this same comparison, with a higher expression in the TSA sample than in the TSE. This shows that heated water also generates extracts with distinct and interesting characteristics to be explored. Likewise, the dicaffeoylquinic acid isomer was up-regulated in WSA.

The 51 phenolic compounds in the heatmap can be subdivided into three groups per dendrogram (Fig. 2). Group I (Fig. 2A) comprises the first compounds shown in the heatmap, composed mainly of phenolic acids, such as those of the subclass of hydroxycinnamic and hydroxybenzoic acids, and isoflavonoids, which were more expressed in the WSA and WSE, respectively. As previously stated, WSA was expected to be richer in phenolic acids due to the type of solvent used, as well as isoflavonoids. In addition to containing the phenolic acids present in the WSA, WSE showed a great relative abundance of ions from isoflavonoids that were probably extracted by the ethanol/water solvent.

Group II (Fig. 2B) was formed due to the presence of hydrocinnamic acids, isoflavonoids and flavanones, which showed greater total relative abundance and were more expressed in the TSA and TSE samples. Once again, it is possible to notice the solvent effect in the extract composition, where TSA had a higher relative abundance of hydrocinnamic acids than TSE. Regarding isoflavonoids and flavanones from group II, both solvents could extract these phenolic compounds with close total relative abundances.

Group III (Fig. 2C) showed high relative abundance for several flavonoid types, such as isoflavonoids, flavanols, and flavones, which were overexpressed in the TSE samples. This high relative abundance of isoflavonoids may be due to the presence of daidzin/puerarin, the most abundant compound in TSE and WSE (Table 2), which was clustered in this group. Furthermore, the relative abundance of dihydroflavonol can be attributed to dihydroquercetin, the second most abundant compound found in the TSA and TSE. These analyses allowed us to detect differences and similarities among extracts. It was also possible to verify which profiles are due to the genotype and which are due to the type of extractor solvent and thus attribute any biochemical activity it may have to its composition.

3.4. Biological activities of toasted sorghum flour phenolic extracts

3.4.1. In vitro cytotoxicity

The cytotoxicity and antiproliferative effects of sorghum extracts were tested in EA.hy926, A549 and HCT-8 cells (Fig. 3). Sorghum extracts reduced the cell viability for all cell lines and the lower IC_{50} values for HCT-8 cells were from the WSE (21.49 μg GAE/mL) and TSE (19.89 μg GAE/mL); and for A549 cells were WSA (33.17 μg GAE/mL) and WSE (20.97 μg GAE/mL). The results of the WSE and TSE extracts in cancer cells behaved interestingly in a dual face way: cell proliferation was

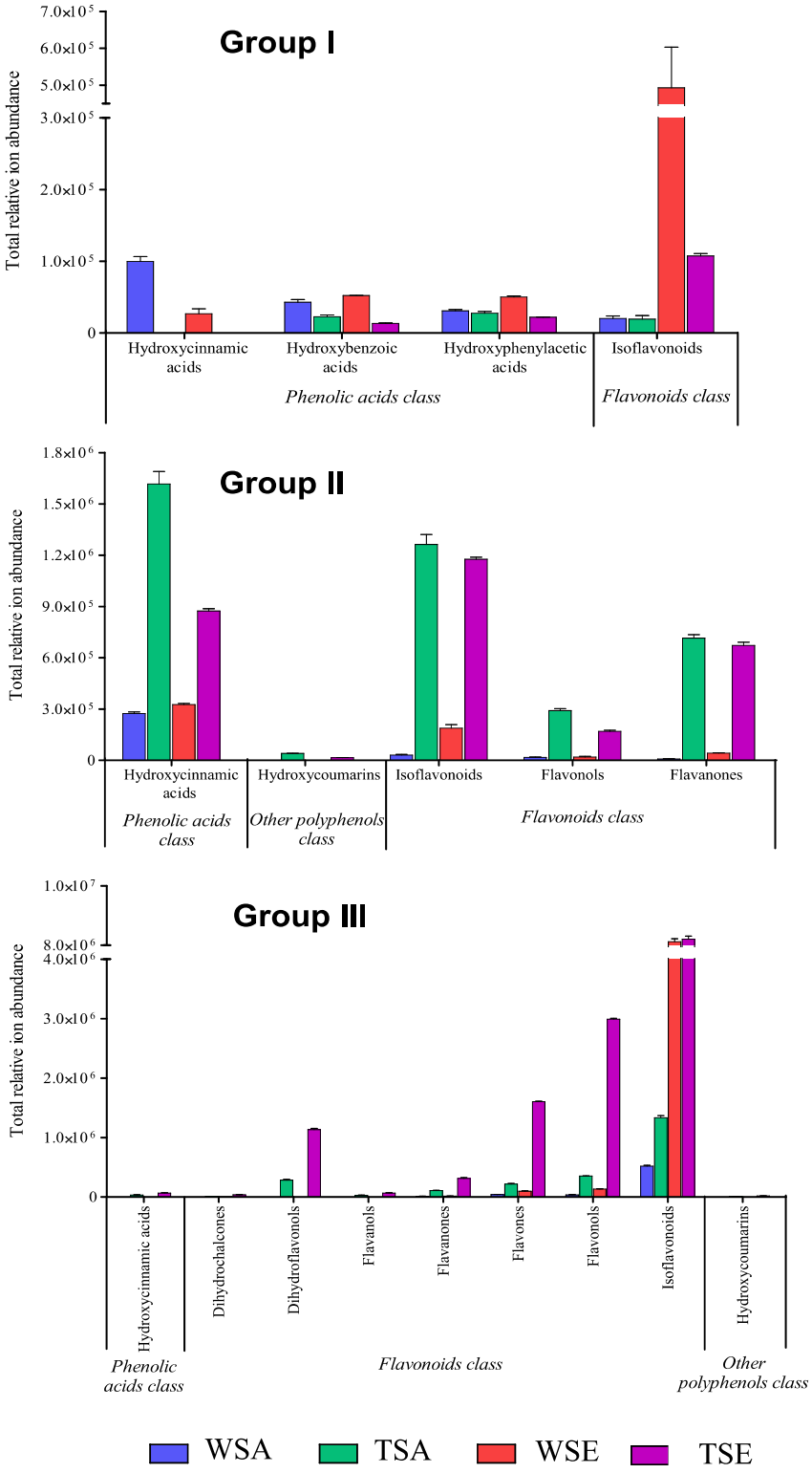


Fig. 2. Total relative ion abundance of the classes of phenolic compounds in the (A) group I, (B) group II, and (C) group III from heatmap.

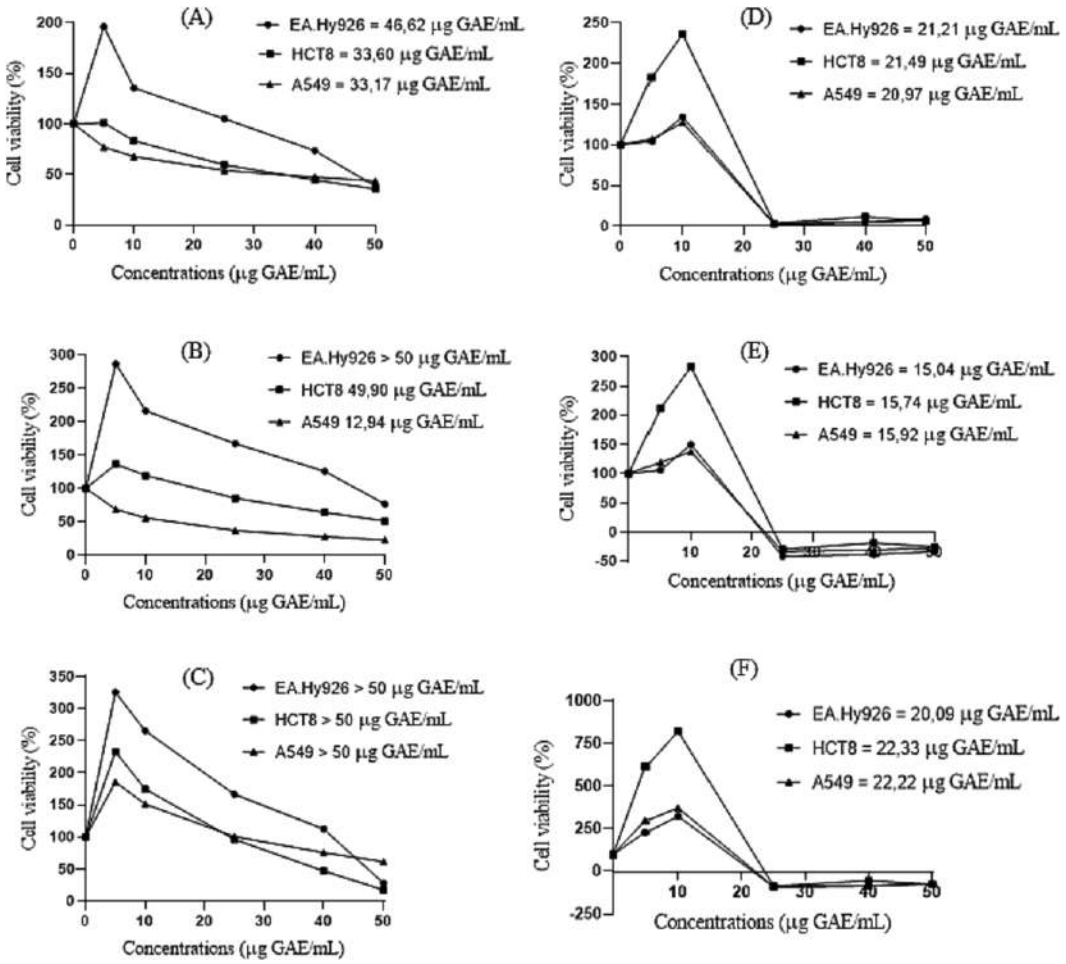


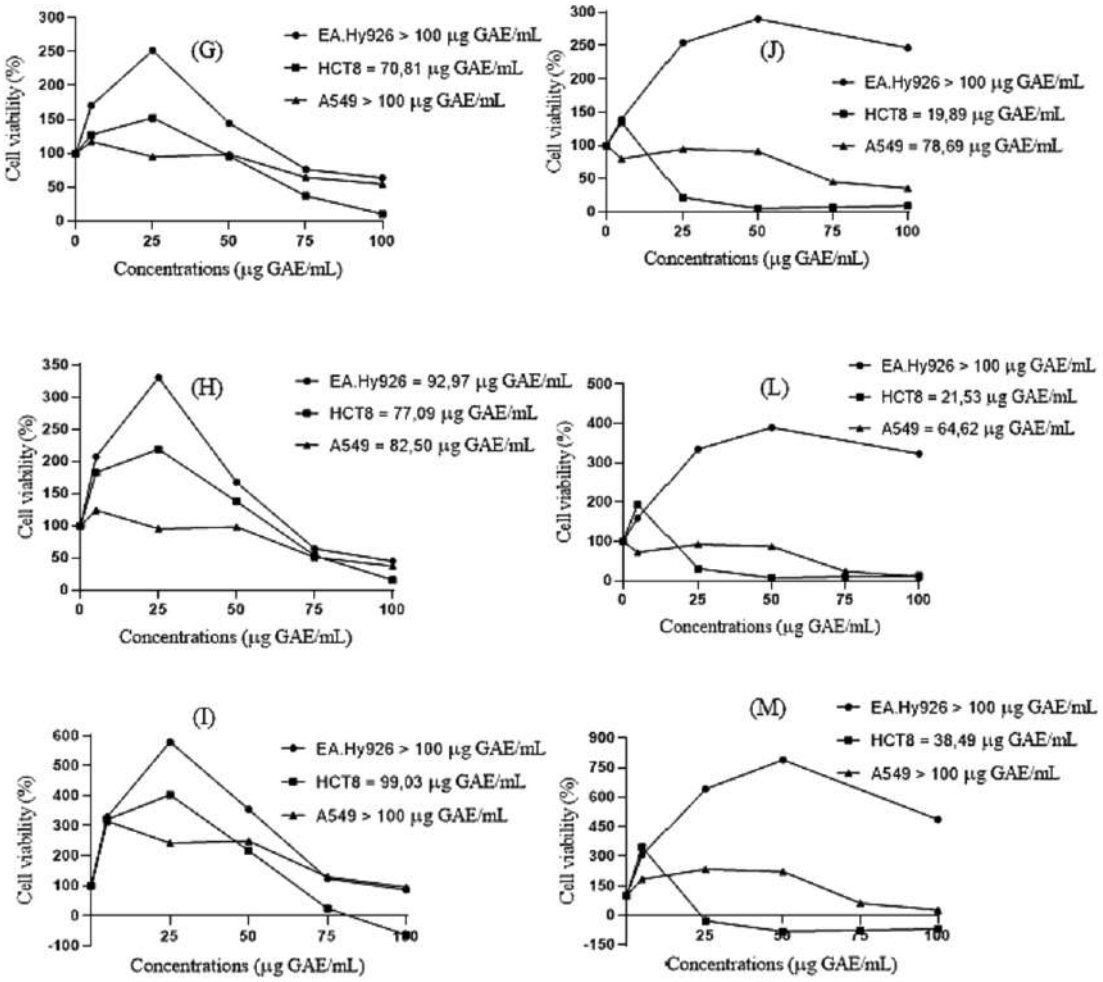
Fig. 3. Cytotoxicity and proliferation inhibition of EA.hy926, A549 and HCT-8 cells after 48 h of treatment with sorghum phenolic extracts, with (A) half inhibitory concentration – IC_{50}, (B) inhibition of growth by 50 % – GI_{50}, and (C) concentration of the agent resulting in a net loss of 50 % of cells – LC_{50} of the WSA sample, (D) half inhibitory concentration – IC_{50}, (E) inhibition of growth by 50 % – GI_{50}, and (F) concentration of the agent resulting in a net loss of 50 % of cells – LC_{50} of the WSE sample. Cytotoxicity and proliferation inhibition of EA.Hy926, A549 and HCT-8 cells after 48 h of treatment with sorghum phenolic extracts, with (G) half inhibitory concentration – IC_{50}, (H) inhibition of growth by 50 % – GI_{50}, and (I) concentration of the agent resulting in a net loss of 50 % of cells – LC_{50} of the TSA sample; (J) half inhibitory concentration – IC_{50}, (L) inhibition of growth by 50 % – GI_{50}, and (M) concentration of the agent resulting in a net loss of 50 % of cells – LC_{50} of the TSE sample.

stimulated at low concentrations (5 – 10 µg GAE/mL), while at higher concentrations (25 – 100 µg GAE/mL) the extracts were able to reduce the cell viability. This dual face behavior was also observed in the studies by Fidelis et al., (2020) and Rietjens et al., (2005), both of which tested extracts containing phenolic compounds in cancer cells.

Regarding the antiproliferative activity, HCT-8 cells exhibited lower susceptibility to WSA and TSA than WSE and TSE (GI_{50} = 49.90, 77.09, 15.74, 21.53 µg GAE /mL, respectively). On the other hand, A549 cells were more resistant to tannin sorghum samples (GI_{50} TSA = 82.50 µg GAE/mL; GI_{50} TSE = 64.62 µg GAE/mL). In the case of EA.hy946 cells, their growth remained unaffected even at the highest tested concentrations of the WSA, TSA, and TSE extracts. In other words, these sorghum extracts exhibited no cytotoxicity to the cells. This behavior indicates that these samples seemed to possess specificity and safety in *in vitro* tests, once they acted reducing the cell viability only against cancer cells. Similar findings were reported by Cardoso et al. (2020) in their evaluation of kombucha derived from black and green tea, where malignant cells (CACO-2, A549, and HCT-8) experienced growth inhibition, while normal cells (IMR90) remained unaffected.

Surprisingly, our results for white sorghum extracts (WSA and WSE) had lower values of IC_{50} (IC_{50} from 20.9 µg GAE/mL to 46.6 µg GAE/mL), differing from the findings (Awika et al., 2009; Smolensky et al., 2018), where the action of the latter was lower or non-existent. In contrast, Awika et al. (2009) investigated the effect of eight sorghum extracts of different genotypes on colon carcinoma cells (HT-29), where the IC_{50} of white sorghum extract was higher (389 ± 23 µg/mL) compared to sorghum extracts with tannins (ranging from 54.8 ± 4.8 to 65.4 ± 3.9 µg/mL).

The target fishing analysis was performed on the most abundant phenolic compounds in the extracts (Table 2) using the similarity ensemble approach (SEA), which can help explain cytotoxicity mechanicalistically. Herein, sorghum extracts led to cell death (LC_{50} from 20.9 µg GAE/mL to > 100 µg GAE/mL) especially against cancer cells. Phenolic compounds that lacked tentatively determined identification and isomers were excluded; the results are presented in Table S4. Some of the most abundant phenolic compounds present in sorghum extracts, such as naringenin 7-O-glucoside and 5-caffeoylquinic acid, did not exhibit predicted targets against colon and lung cancer cells. In



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addition, dihydroquercetin, caffeic acid, and 4-hydroxybenzoic acid showed the highest similarity predicted targets (Tanimoto coefficient MaxTC = 1.00) and were predicted to inhibit carbonic anhydrases (CA) 2, 4, 7, 9, and 12. These zinc-binding enzymes play a vital role in facilitating the reversible hydration of CO_2 to bicarbonate, a reaction essential for the pH homeostasis of the body (Viikilä et al., 2016). Along with various other proteins like ion transporters, they help tumor cells maintain a neutral intracellular pH (Parks et al., 2013).

Both kaempferol and eriodictyol were predicted to target cytochrome P450 1B1 (CYP1B1), with a similarity index greater than 0.70 but less than 1 (Table S4). CYP1B1 is an enzyme that exhibits higher expression in tumor cells, particularly in the colon and lung, compared to the surrounding normal tissue (Gibson et al., 2003; Li et al., 2017). Li et al. (2017) highlighted that the inhibition of this enzyme, which is involved in xenobiotic metabolism and overexpressed in cancer cells, can potentially contribute to reducing the risk and progression of cancer.

Similarly, kaempferol was also predicted to target xanthine dehydrogenase/oxidase (XDH/XOR) and death-associated protein kinase 1 (DAPK1), which are involved or expressed in cancer pathway (Linder et al., 2009; Michie et al., 2010). DAPK1, a pro-apoptotic serine/threonine protein kinase, can be inhibited by kaempferol (Yokoyama et al., 2015), which may have influenced the cytotoxicity results since this enzyme levels critically modulate cell fate decisions, integrating signals from apoptotic and autophagic pathways and thereby influencing the outcome of survival or death (Michie et al., 2010; Bovellan et al., 2010). The other phenolic compound-targets predictions showed a low similarity index (MaxTC > 0.70), suggesting that they have little effect on cytotoxicity against cancer cells.

The performance of aqueous extracts WSA and TSA in cytotoxic tests is noteworthy, as they exhibit antiproliferative activity against cancer cells and are relatively safe at the tested concentrations. The variation in biological activity results between samples of the same sorghum genotype but with different solvents highlights the significance of chemical composition and extraction methods in extract production. Furthermore, the similarity in actions observed among the samples suggests the cytotoxic potential of phenolic compounds they have in common.

3.4.2. *Clonogenic assay*

A clonogenic assay was performed to examine the effect of sorghum extracts on the proliferation dynamics of HCT-8 cells. Our results showed that WSA, WSE, TSA and TSE have an antiproliferative effect on this cell line, as the abundance of colonies was lower in the groups treated with sorghum extracts compared to the control (Fig. 4). WSA significantly reduced it at the 2 µg/ml concentration, while the other samples achieved the same result only at a higher concentration. At the highest concentrations tested, we observed a reduction of almost 100 % in colonies formed in TSA and TSE, while it was reduced completely in WSA and WSE.

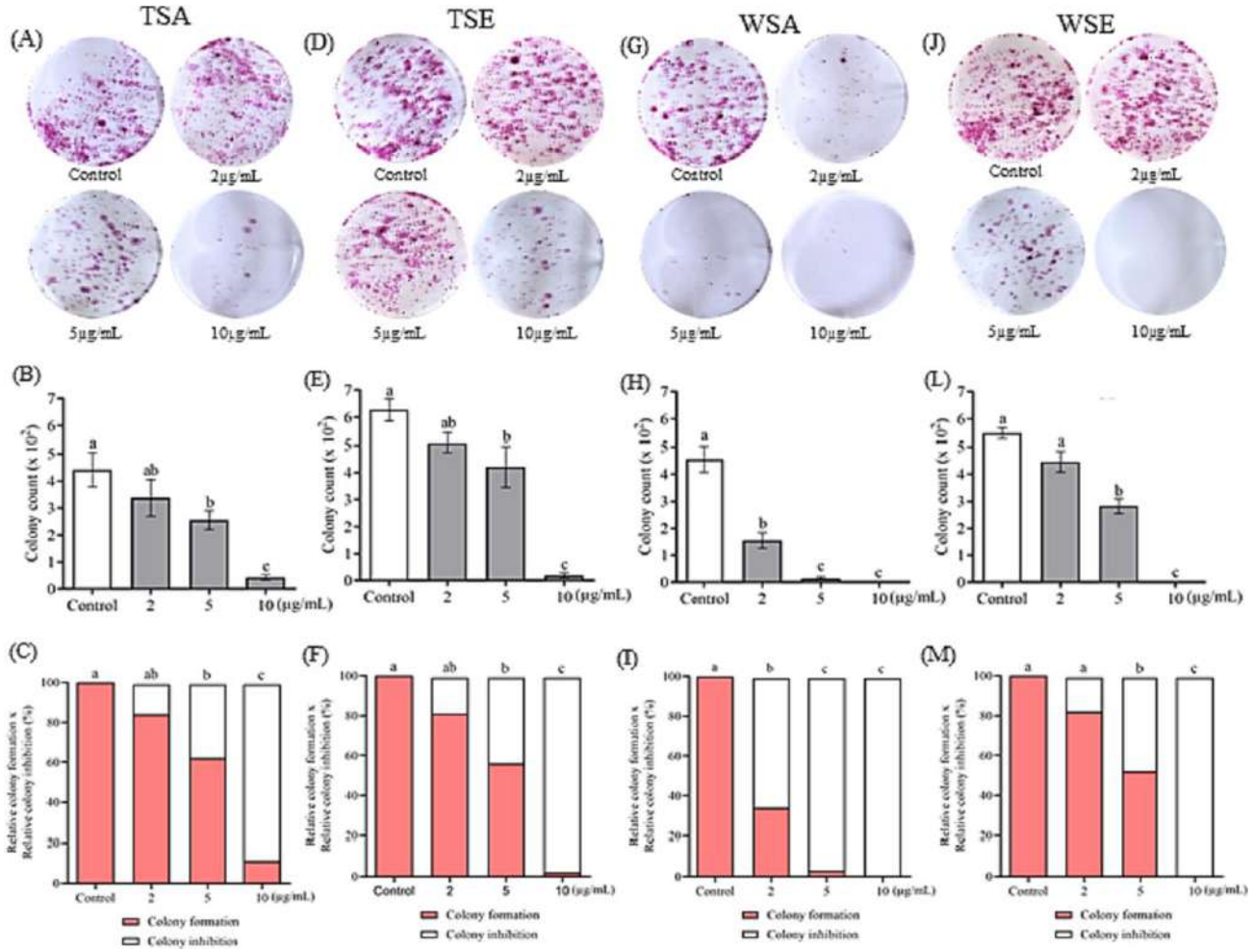


Fig. 4. Effects of sorghum extracts WSA, WSE, TSA, and TSE on colony formation of HCT-8 ileocecal adenocarcinoma cells. (A, D, G, and J) Photomicrography showing the formation of HCT-8 colonies after treatment with 2, 5 or 10 µg/mL of TSA, TSE, WSA or WSE for 14 days. After that, the colonies were fixed with methanol, stained with crystal violet solution, and counted (B, E, H and L) The bar graph represents the number of colonies formed and counted after 14 days of assay (C, F, I and M). The relative percentage of colonies formed *versus* the relative percentage of colony inhibition. Mean ± SEM. Different letters (a,b) denote statistical difference among the groups ($p < 0.05$) by Tukey's test.

Regarding the phenolic composition of sorghum extracts, it has been observed that phenolic acids found in all extracts, such as caffeic acid, exhibit not only antiproliferative activities but also inhibit colony formation in colon cancer cells (Jaganathan, 2012). Although the WSA extract did not exhibit the highest relative abundance of phenolic compound ions compared to the other extracts, it had excellent results in preventing colony formation. This could be attributed to the presence of specific phenolic compounds in WSA, rather than their abundance, that may be responsible for impairing cell clone formation.

Although most likely, this anti-clonogenic effect is influenced by the simplest phenolic compounds, such as the phenolic acids identified in all extracts, other classes of phenolics have also have shown this effect. Other studies showed that kaempferol and dihydroquercetin, both present in TSA and TSE extracts, significantly reduced the number of colon cancer cells by more than 50 % compared to the control (Wu et al., 2021; Razak et al., 2018). The observed impact on clonogenic activity could also be attributed to unidentified phenolic compounds or potential synergistic interactions among them.

3.4.3. *In vitro cell-based reactive oxygen species (ROS) generation*

Considering the presence of phenolic compounds in extracts of toasted sorghum flour, which have been reported to exert both antioxidant and pro-oxidant effects and exhibit a close relationship with cytotoxicity (do Carmo et al., 2018), the present study aimed to assess the intracellular generation of reactive oxygen species (ROS) in cell lines exposed to various concentrations of sorghum phenolic extracts (Fig. 5A-L).

In EA.hy926 cell line (Fig. 5A-D), the WSA extract showed antioxidant activity in all tested concentrations, with and without hydrogen peroxide. WSE and TSE ethanolic extracts induced ROS generation, however, it is worth noting that the TSE protected the cells against oxidative stress, while WSE exerted pro-oxidant effect. TSA extract did not interfere on basal ROS production and, when added with H_2O_2, it acts as an antioxidant agent.

High levels of intracellular ROS without meaning greater lethality can be explained by healthy cells displaying specialized mechanisms to cope with the detrimental ROS effects, such as a balanced production of these molecules, robust antioxidant defense, and efficient cellular repair (Moloney & Cotter, 2018). Consequently, these cellular adaptations lead

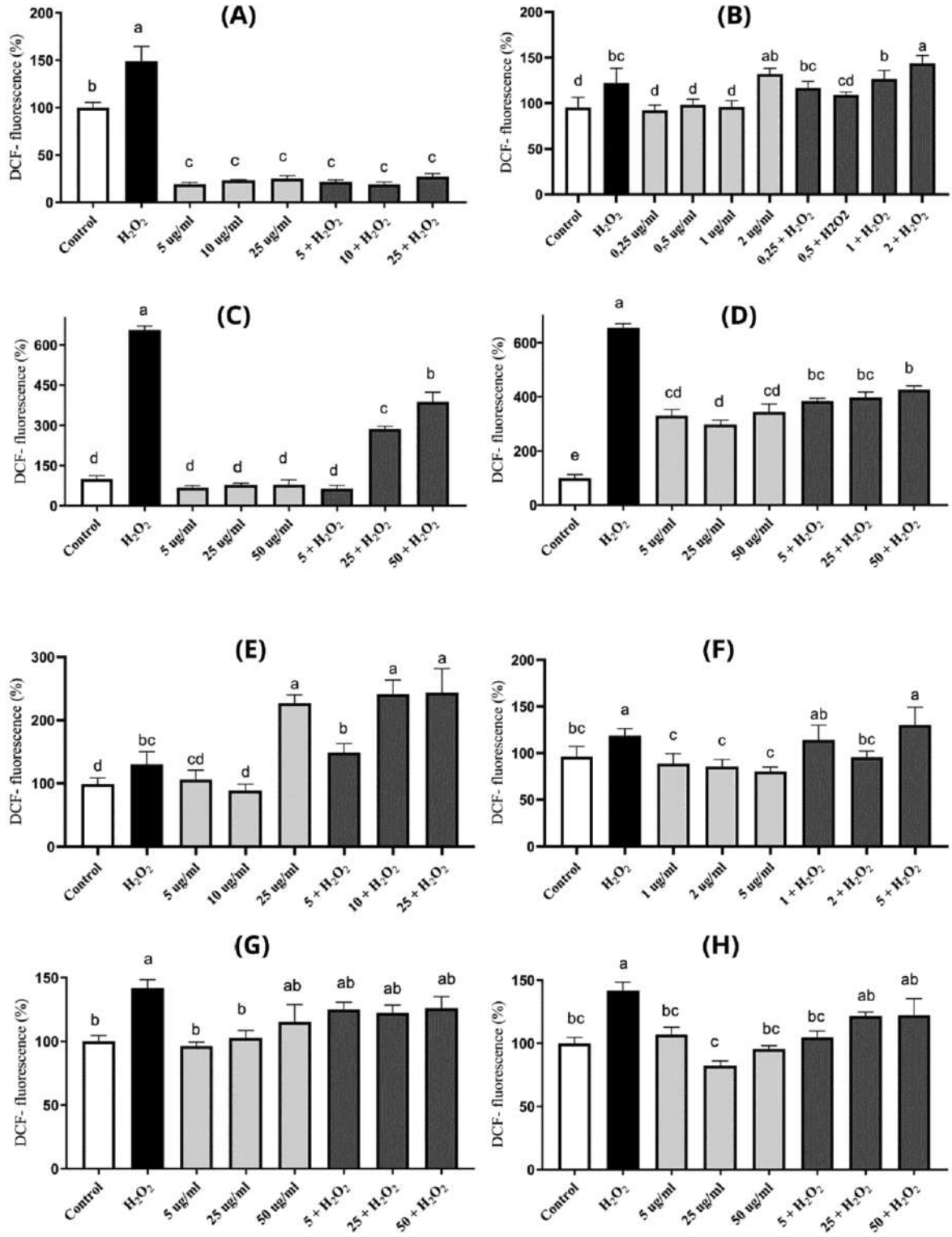
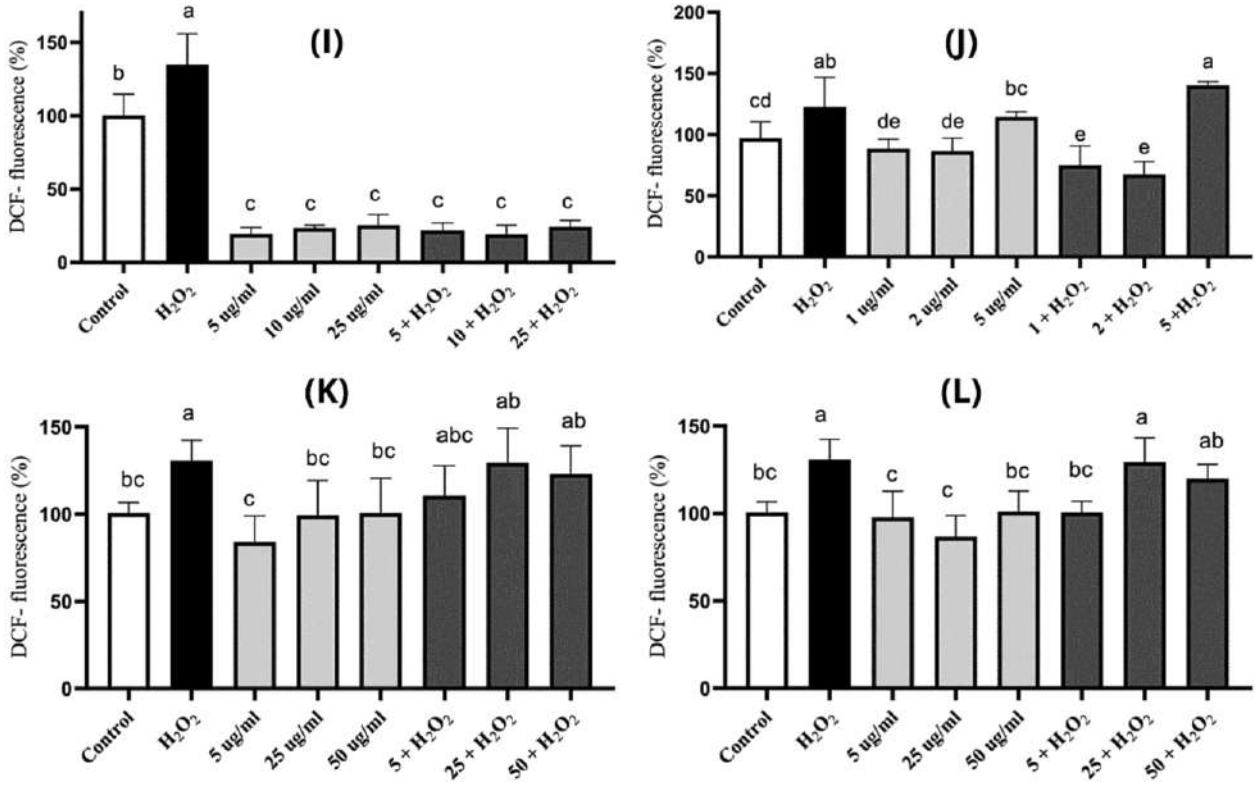


Fig. 5. Result of the activities on Reactive Oxygen Species (ROS) in EA.Hy926 normal cells by (A) WSA, (B) WSE, (C) TSA, and (D) TSE sorghum extracts; in A549 cancer cells by (E) WSA, (F) WSE, (G) TSA, and (H) TSE sorghum extracts. Result of the activities on ROS in HCT-8 cancer cells by (I) WSA, (J) WSE, (L) TSA, and (M) TSE sorghum extracts.



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to the generation of non-toxic concentrations of ROS in normal cells (do Carmo et al., 2018). However, when the cellular system fails to neutralize these free radicals, such as superoxide dismutase, glutathione reductase, and glutathione peroxidase, cell death is a common outcome (Pressete et al., 2019), as seems to happen in the WSE extract.

WSA extract in HCT-8 cancer cells acted as antioxidant, in the same way as in EA.hy926 cells. In general, for A549 e HCT-8 cells, sorghum extracts did not induce ROS generation above basal cell levels. However, in contrast, they did not protect the cells against the ROS production induced by H_2O_2, thus exhibiting a pro-oxidant effect. This pro-oxidant activity can be attributed to phenolic compounds content (WTSF = 1.31 ± 0.04, TTSF = 45.34 ± 1.44) and this effect may activate apoptotic pathways due to ROS overproduction. Cancer cells, which frequently suffer from oxidative stress and accumulate an excessive amount of ROS, fail to maintain cellular homeostasis and ultimately succumb to cell death (Glasauer & Chandel, 2014), as observed in the cell viability assay. However, the overconcentration of intracellular ROS is not the only stress stimulus that can trigger apoptosis. In fact, other factors such as cell membrane damage, mitochondrial dysfunction (Peixoto et al., 2017), generation of tumor necrosis factor α (TNFα), high concentration of glutamate/calcium (Fulda, 2013).

From the computational point of view, some targets related to the reported activities were suggested according to similarity to other compounds reported on the literature (expressed as Tanimoto coefficient) and the probability of prediction from the SEA calculations. Complete results can be found in the Table S4. Phenolic compounds were suggested to be involved in ROS generation pathways, such as NADPH oxidase 4 (NOX4), which is predicted to be targeted by kaempferol. Tumor initiation or progression induced by oxidative stress may occur due to the excessive ROS production by members of the NADPH oxidase (NOX) family (Hussain et al., 2003). NOX4 specifically shows high expression in lung cancer cells and tissues (Boudreau et al., 2014; Zhang et al., 2014), making it a target for treating and preventing lung cancer related to oxidative stress (Han et al., 2016). Moreover, specific phenolic compounds identified in the extracts could be attributed to the ROS effects, and indeed, many of them have had their antioxidant activity related to cytotoxic properties by increasing ROS levels (Han et al., 2015; Ahmad et al., 2020) or protecting against cytotoxic ROS (Wang et al., 2016).

3.4.4. Adhesion and invasion properties

Cancer cells exhibit certain activities that contribute to metastasis, such as the ability to form colonies, as mentioned earlier, as well as the ability to adhere and invade (Bouzaiene et al., 2015; Chiang & Massagué, 2008). Therefore, we investigated their effects on the adhesion and invasion of HCT-8 cells to assess the impact of phenolic-rich sorghum extracts on these characteristics.

Cell adhesion without Matrigel® decreased after 12 h of treatment with TSA, TSE, and WSA sorghum extracts at all concentrations, compared to the control treatment (Fig. 6A, B, and C). WSE extract also exhibited significantly reduced cell adhesion, but only at 10 μg/mL (Fig. 6D). Thus, cell adhesion with Matrigel® was significantly decreased at 10 μg/mL for all sorghum extracts (Fig. 6E, F), without statistical difference among them.

Within complex multicellular organisms, cellular adhesion, including cell-to-cell and cell-to-extracellular matrix interactions, is crucial in facilitating organization, structural integrity, communication, and cohesiveness (Hynes & Zhao, 2000). One of the apoptotic pathways in cells is the intrinsic pathway, which involves the activation of cellular stress signals, including ROS and loss of cell adhesion (Lefort & Blay, 2012; Pereira & Amarante-Mendes, 2011; Frisch & Francis, 1994). In this context, our findings indicating that phenolic-rich sorghum extracts can reduce cancer cell adhesion are encouraging in the quest for new therapies. Bouzaiene et al. (2015) demonstrated that phenolic acids,

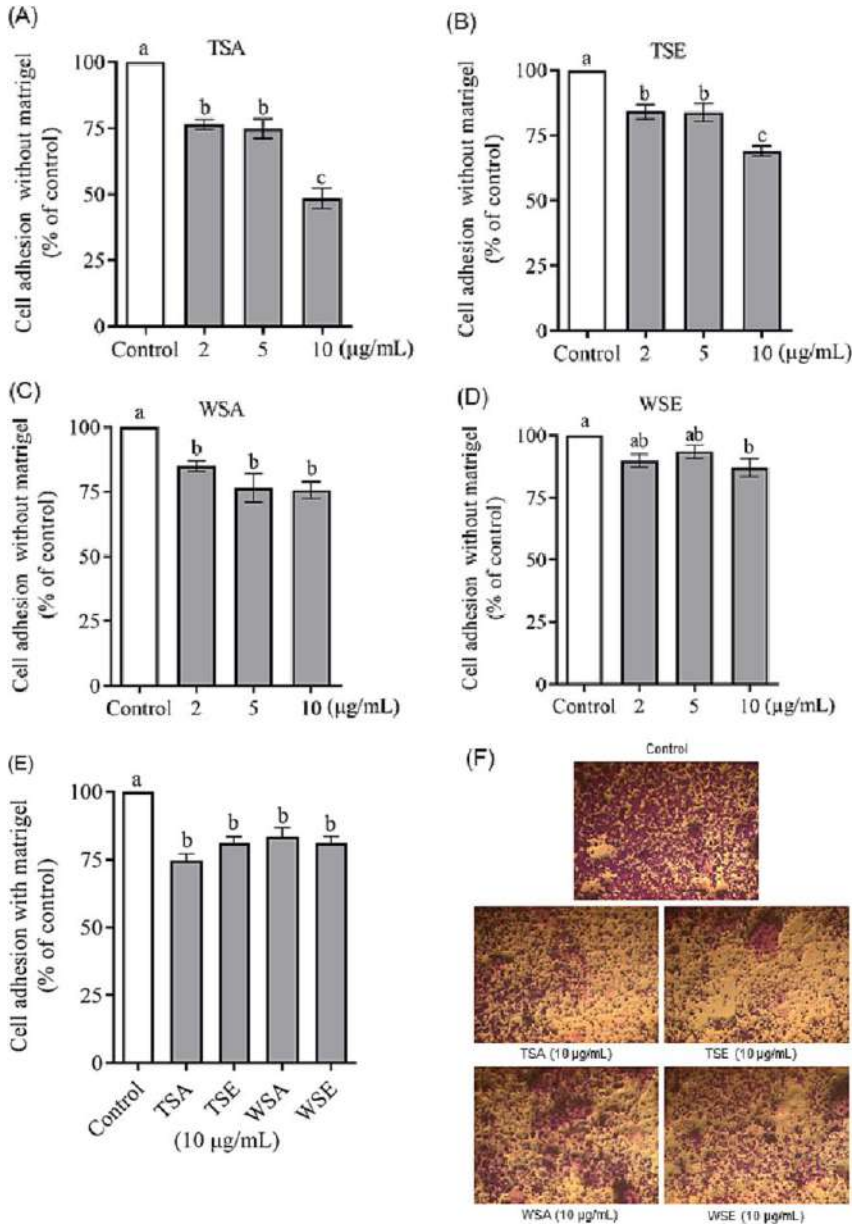

Fig. 6. Effects of sorghum extracts WSA, WSE, TSA, and TSE on adhesion of HCT-8 ileocecal adenocarcinoma cells. (A, B, C and D) The bar graph represents the adhered cell number without Matrigel® that was treated with 2, 5 or 10 µg/mL of TSA, TSE, WSA or WSE. (E) The bar graph represents the adhered cell number with Matrigel® that was treated with 10 µg/mL of TSA, TSE, WSA or WSE. (F) Photomicrography representing the adhered cell number with Matrigel® that was treated with 10 µg/mL of TSA, TSE, WSA or WSE (100 x magnification).

such as caffeic, ferulic, and coumaric acid, reduced the adhesion of HT29-D4 colon cancer cells dose-dependently. Regarding flavonoids, apigenin has shown activity against both adhesion and cell invasion, partly by inhibiting matrix metalloproteases and cell adhesion molecules (CAMs), such as cadherins and integrins (Lefort & Blay, 2012; Desgrosellier & Cheresh, 2010). Furthermore, catechin, epicatechin, and naringenin, found in TSA and TSE extracts, exhibited cytotoxic effects on colon cancer cells and reduced the expression of CAMs involved in metastasis (Dükel et al., 2021).

The key distinguishing characteristic of a malignant tumor is its capability to infiltrate adjacent normal tissues through a complex sequence of events involving the action of proteolytic enzymes that degrade components of the extracellular matrix (ECM) (Lefort & Blay, 2013; Friedl & Wolf, 2003). In this test, Matrigel® was used to simulate the ECM; the results indicated decreased invasive ability of HCT-8 cells treated with phenolic-rich sorghum extracts compared to the control (Fig. 7A), which was also visually evident in the microscopy analysis (Fig. 7B). Similar to the cell adhesion tests, we found no significant difference in the invasion percentage among the tested extracts.

The high polyphenol sorghum extracts obtained through extraction with 70 % ethanol and 5 % citric acid (v/v) by Lee et al. (2020) for their effects on the invasion of HCT15, SW480, HCT116, and HT-29 colon cancer cells. Despite using a different sorghum genotype and solvent, their results were consistent with the present findings, showing a

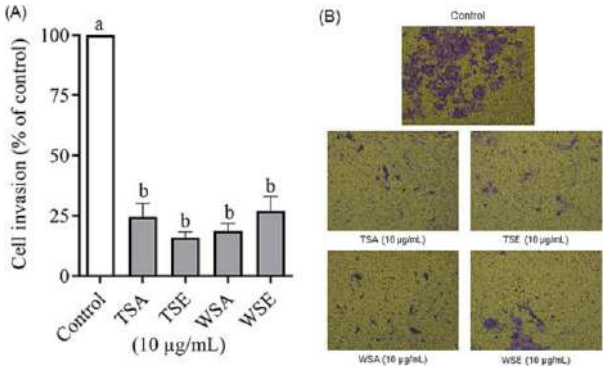

Fig. 7. Effects of sorghum extracts TSA, TSE, WSA and WSE on invasion of HCT-8 ileocecal adenocarcinoma cells. (A) The bar graphs represent invasive cell percentage that was treated with 10 µg/mL of TSA, TSE, WSA or WSE for 24 h. (B) Photomicrography representing the invasive cell that was treated with 10 µg/mL of TSA, TSE, WSA or WSE (100X magnification).

consistent reduction in cell invasiveness. Furthermore, various phenolic compounds found in our extracts, including caffeic acid (Secme et al., 2023), daidzein (Chan et al., 2018), dihydroquercetin (Li et al., 2019), naringenin (Han et al., 2018; Chen et al., 2019), and kaempferol (Hung et al., 2017), have already displayed anti-invasive effects against a range of cancer cells.

Lastly, target fishing results (Table S4) related to invasion showed that matrix metalloproteinase-9 (MMP-9) is a predicted target for caffeic and m-coumaric acids, with a similarity index of 1 and 0.63, respectively. It is important to mention that Tanimoto coefficient = 1 means that this compound is equal (100 % similar) to a particular one previously reported in the literature. During tumor metastasis and invasion, cancer cells express MMP-9, a proteolytic enzyme responsible for degrading type IV collagen in the ECM (Nelson et al., 2000; Park et al., 2005; Alam et al., 2022). Therefore, the documented inhibitory effect of caffeic acid on MMP-9 suggests that this phenolic acid may play an important role in suppressing cell invasion and, consequently, inhibiting cell metastasis (Park et al., 2005; Alam et al., 2022).

3.4.5. *Antimalarial activity of sorghum phenolic extracts*

As the antimalarial properties of sorghum have not been investigated to date, we explored whether these different extracts can combat *P. falciparum* responsible for causing the disease. Sorghum extracts exerted toxicity against both strains W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) of *P. falciparum* (Fig. 8), of which WSE stands out, since it required the lowest concentration to reach the IC_{50} in both strains (8.0 µg GAE/mg and 22.9 µg GAE/mg, respectively). Even the samples that required a higher concentration of phenolics to reach the IC_{50} showed excellent results for the W2 (WSA = 90.7 µg GAE/mg) and 3D7 strains (TSE = 67.8 µg GAE/mg). One hypothesis is that the antimalarial action is due to the common phenolic compounds of sorghum, regardless of the genotype or type of extraction.

Extracts rich in flavonoids have been linked to antimalarial activity in previous studies (Carmo et al., 2020; Enechi et al., 2021; Noronha et al., 2022). Carmo et al. (2020) found that a smaller amount of food is needed to reach IC_{50} value, that is, to reduce parasitemia by 50 %, when phenolic compounds such as epicatechin, catechin, and quercetin are present at high concentrations. These three compounds are present in the two sorghum extracts with tannins (TSA and TSE); however, these were not present in the white sorghum extracts (WSA and WSE), and are therefore related to the genotype. Additionally, caffeic acid and its derivatives have already been tested *in vitro* against the 3D7 strain of *P. falciparum*, with an IC_{50} of 20 µM (Alson et al., 2018). This phenolic acid is present in all extracts, but with a greater relative abundance in TSA, presenting the second-highest toxicity for the chloroquine-resistant strain. Abdulah et al. (2017) investigated the antimalarial effect of (+)-catechin, the main active compound of the Indonesian medicinal plant *Garcinia celebica*, and found that the chloroquine-resistant *P. falciparum* had its growth inhibited in both the trophozoite and schizont stages. The presence of these compounds in sorghum phenolic extracts with tannins may explain their action, which, despite requiring a higher concentration of phenolics, was still effective in reducing parasitemia.

Regarding white sorghum extracts, WSA had less activity against the W2 strain, as expected, due to its lower phenolic content. Surprisingly, the WSE sample exhibited the highest toxicity against *P. falciparum* despite not having the highest abundance of phenolic compounds. Two hypotheses can explain the efficacy of WSE against both strains. Firstly, the phenolic profile of the WSE contains compounds that, although shared with other samples, are present in sufficient quantities to be toxic to the plasmodium. Secondly, its toxicity may be attributed to unique compounds exclusive to WSE. The WSE extract contained two distinct phenolic compounds, although their exact identification remains uncertain: a flavanone or isoflavanone (hesperetin/homoeriodictyol/4′-methoxy-2′,3,7-trihydroxyisoflavanone), and a phenolic acid (vanillic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxymandelic acid). Flavanones have already been reported in terms of their antimalarial activity, with an IC_{50} of up to 1.6 µg/mL against a multidrug-resistant strain of *P. falciparum* (Portet et al., 2007; Khaomek et al., 2008).

Target fishing results using SEA (Table S4) show that some compounds did not have any target related to plasmodium activity, such as caffeic acid and 4-hydroxybenzoic acid. On the other hand, compounds, such as methylgalangin, kaempferol and eriodictyol, with a predicted target, were mainly related to enzymes involved in the synthesis of fatty acids (FAS), including enoyl reductases. Fatty acids are synthesized through enzymatic processes involving multiple rounds of elongation reactions, including condensation, dehydration, and reduction. Parasites possess a unique FAS pathway known as FAS-II, which consists of a series of specific and highly conserved enzymes, including β-ketoacyl-ACP-reductase (FabG), β-hydroxyacyl-ACP-dehydratase (FabZ), and enoyl-ACP-reductase (FabI) (Colizzi et al., 2008). FabI is a crucial

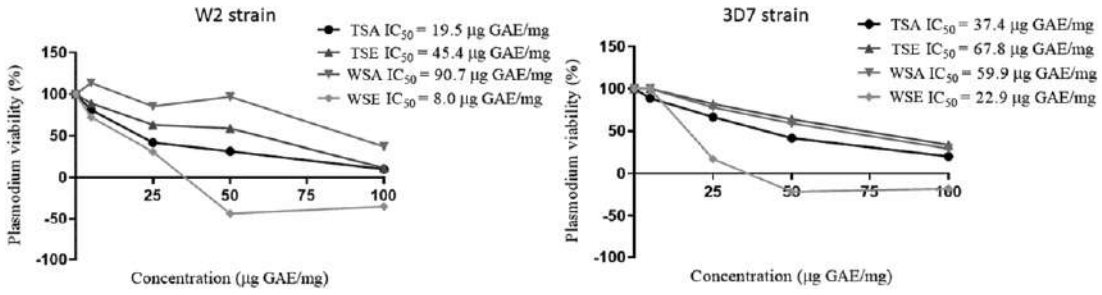

Fig. 8. Antimalarial activity of sorghum phenolic extracts against chloroquine resistant strain (W2) and chloroquine sensitive strain (3D7). Concentrations are expressed in µg/mL of extract.

regulator of fatty acid biosynthesis and has been identified as a promising drug target for developing antimalarial agents (Tasdemir et al., 2006). Tasdemir et al. (2006) showed that flavonoids, including kaempferol and catechin, improve inhibition of *P. falciparum* enoyl reductase enzymes. Catechins, in particular, have been shown to act through these mechanisms in previous studies (Mamede et al., 2020; Tasdemir et al., 2006).

In addition to the enzymes involved in FAS, phenolic compounds were also predicted to interact with proteinases and histone deacetylase (HDAC1) (Table S4). HDAC1 derived from *P. falciparum* is specifically found within the nucleus of the parasite and is transcribed in various stages of its life cycle, including asexual intraerythrocytic stage parasites, gametocytes, and sporozoites, during infection in the human host (Andrews et al., 2012). The inhibition of this enzyme can affect various aspects, from protein-protein interactions related to gene expression in the parasite (Andrews et al., 2012). Thus, the predictions of phenolic compounds and their antimalarial activity are based on impaired metabolism of fatty acid synthesis and inhibition of proteases, leading to rupture of the plasmodium membrane.

We acknowledge that the antimalarial activity observed in the samples may be attributed to unidentified compounds and various interactions between these compounds and their targets. Numerous phenolic compounds derived from plants have been extensively studied for their potential against malaria, emphasizing the significance of ongoing research to enhance our understanding and to discover new potential antimalarial drugs.

Thus, computational predictions of molecular targets showed to be useful, suggesting potential mechanisms of action to *in vitro* ROS generation, as well as anticancer and antiplasmodial activities (sections 3.4.1–3.4.5). In this sense, the relationship between probabilities of prediction (measured as p-values and maximum Tanimoto coefficient values) and the relative abundancy suggest that among all predicted targets, flavonoids present in WSE are majorly related to targets responsible for membrane synthesis; and flavonoids as well as phenolic acids present in TSE are majorly related to targets from carbonic anhydrase class (Fig. 9). However, the chemical profile of tested samples is highly complex and SEA predictions suggested compounds with several potential targets against all tested diseases models, on the other hand, compounds with low abundancy were not included in the *in silico* study and should not be discarded to play a role in the mechanism of action of the samples. Biochemical assays could be carried out in further studies to elucidate the proposed mechanism of action of phenolic compounds in sorghum extracts. Furthermore, the target fishing method is limited to the existing knowledge and, therefore, should not be used to exclude other hypotheses. Therefore, the multitarget concept of mechanism of action should be considered in further studies, given the nature of samples as natural product sources (Koeberle & Werz, 2014; Kaur et al., 2022).

4. Conclusion

Toasted sorghum flour extracts, obtained using GRAS solvents (water and ethanol/water), varied in their phenolic composition according to the extraction method and genotype. These sorghum phenolic extracts displayed dose-dependent antiproliferative activity against A549 (lung adenocarcinoma epithelial cells) and HCT-8 cells (human colon carcinoma). Among them, the tannin sorghum ethanolic extract was shown to be safe for normal cells. The observed cytotoxic activity could be associated with ROS generation, where a pro-oxidant effect of the extracts possibly leads to apoptosis in cancer cells. The results of

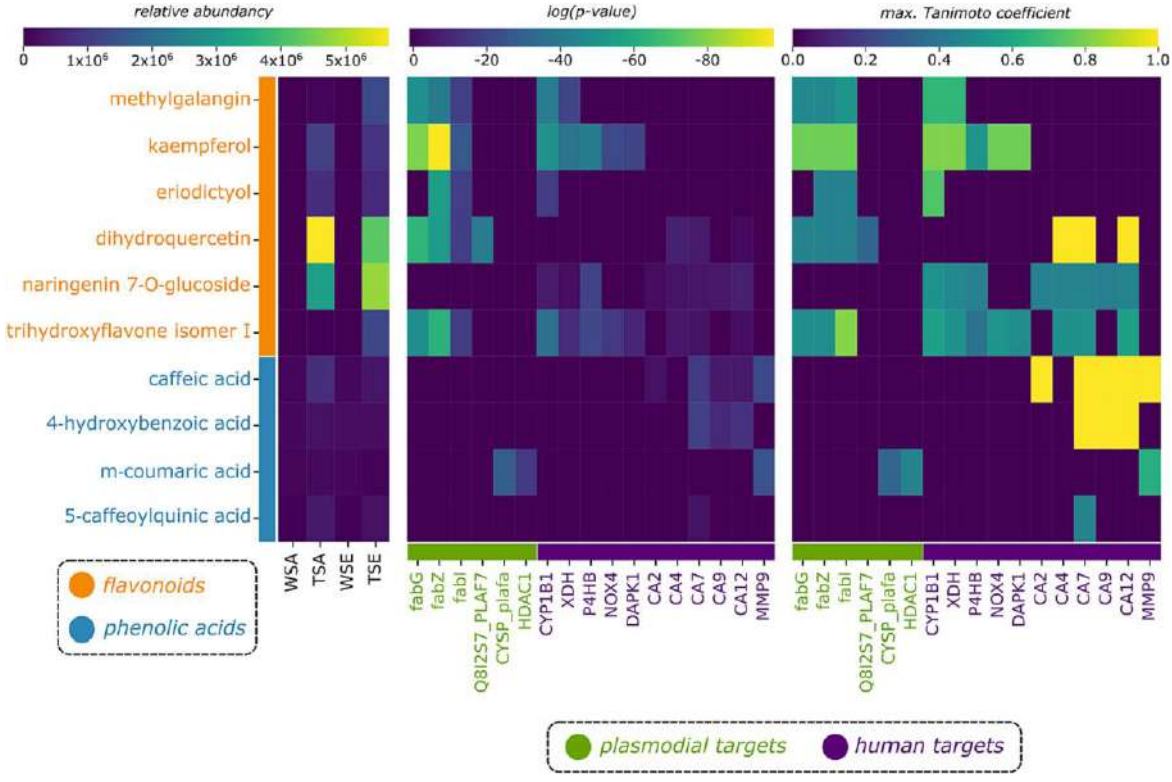

Fig. 9. Heatmaps of relative abundancy of compounds with higher concentration in the four tested samples (left); heatmap of the *p*-values obtained from SEA (center) for most abundant compounds and predicted targets; and heatmap of max Tanimoto coefficient reported by SEA (right) for most abundant compounds and predicted targets. Figure generated with Seaborn package in python.

cytotoxicity and ROS generation, combined with the findings on anti-clonogenic, anti-adhesive, and anti-invasive activities, indicate that these extracts exhibit antimetastatic properties and are predicted to target multiple pathways. Additionally, our study revealed promising antimalarial activity of the sorghum extracts against both chloroquine-resistant and sensitive strains, particularly the toasted white sorghum ethanolic extract, which was rich in phenolic acids and flavonoids.

Thus, incorporating toasted white and tannin sorghum flours into products such as *farofas* can offer potential health benefits through the consumption of these phenolic compounds alongside other nutrients. Another potential application is the production of phenolic extracts from toasted sorghum flours for use as antioxidant supplements.

CRediT authorship contribution statement

Laise Trindade Paes: Formal analysis, Investigation, Methodology, Data curation, Writing – original draft, Writing – review & editing. **Carolina Thomaz dos Santos D'Almeida:** Methodology, Formal analysis, Data curation. **Mariana Araújo Vieira do Carmo:** Methodology, Formal analysis, Writing – review & editing. **Laura da Silva Cruz:** Methodology, Formal analysis. **Amanda Bubula de Souza:** Methodology, Formal analysis. **Leonara Martins Viana:** Methodology, Formal analysis. **Vinicius Gonçalves Maltarollo:** Data curation, Writing – review & editing. **Hércia Stampini Duarte Martino:** Writing – review & editing. **Graziela Domingues de Almeida Lima:** Methodology, Formal analysis. **Mariana Simões Larraz Ferreira:** Methodology, Formal analysis. **Luciana Azevedo:** Methodology, Funding acquisition, Writing – review & editing. **Frederico Augusto Ribeiro de Barros:** Methodology, Writing – review & editing, Funding acquisition, Supervision, Funding acquisition, Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Brazil, Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) - Brazil, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – Brazil for their financial support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113739.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Total identification of phenolic compounds in the sorghum extracts by UPLC-MS^E.

Name of compound	Molecular formula	m/z	RT (min)	Score (%)	FS (%)	Error (ppm)	IS (%)	Class	WSA	TSA	WSE	TSE
Sesamin	C20H18O6	353.1064	0.62	39.7	20.0	9.57	89.17	L	5.80E+03			
Rosmarinic acid	C18H16O8	359.0766	0.63	38.6	0.0	-1.75	95.06	PA	5.15E+03	6.20E+03		
Dicaffeoylquinic acid isomer	C25H24O12	515.1233	0.73	35.6	0.0	7.45	86.38	PA	9.96E+04	2.71E+04		
4-Hydroxybenzaldehyde	C7H6O2	121.0284	1.53	38.6	5.2	-8.74	97.37	OP			2.85E+04	
Benzoic acid	C7H6O2	121.0284	3.71	37.4	0.0	-9.40	97.44	PA	4.13E+04			
Caffeoylquinic acid isomer	C16H18O9	353.0853	4.65	36.1	0.0	-7.14	88.66	PA	1.35E+04	1.10E+04		
Hydroxybenzoic acid isomer/protocatechuic aldehyde/sesamol	C7H6O3	137.0232	4.91	55.2	86.2	-8.69	99.47	NI	2.76E+04	1.04E+04	1.47E+04	
4-Hydroxybenzoic acid	C7H6O3	137.0232	5.23	56.9	95.2	-8.49	98.81	PA	7.15E+05	7.20E+05	1.09E+06	8.15E+05
Procyanidin dimer B-type	C30H26O12	577.1338	5.57	54.7	82.2	-2.27	93.89	F	1.31E+05	2.57E+05	1.85E+05	1.93E+05
Esculin	C30H26O12	339.0708	5.73	54.7	82.2	-2.27	93.89	OP		9.51E+04		1.19E+05
5-O-Galloylquinic acid	C14H16O10	343.0662	5.84	38.0	4.4	-2.57	88.45	PA		5.05E+04		2.79E+04
Procyanidin trimer C-type	C45H38O18	865.1963	5.85	37.2	0.0	-2.56	89.18	F				1.00E+04
p-Coumaric acid 4-O-glucoside	C15H18O8	325.0914	5.92	56.9	98.0	-4.46	91.71	PA		1.10E+05		1.89E+05
Hydroxyphenylacetic acid isomer I	C8H8O3	151.0387	5.96	36.8	0.0	-8.94	94.03	PA	4.95E+04	2.63E+04		1.71E+04
5-Caffeoylquinic acid	C16H18O9	353.0864	6.02	54.7	80.7	-4.11	97.67	PA	2.44E+04	2.68E+04	8.74E+04	1.93E+04
(+)-Catechin	C15H14O6	289.0703	6.05	50.8	64.1	-4.92	95.84	F		3.82E+05	2.54E+05	3.01E+04
Vanillic acid	C8H8O4	167.0344	6.19	38.1	0.0	-3.34	94.26	PA	3.06E+04	1.26E+05	1.97E+05	
Dihydroxybenzoic acid isomer	C7H6O4	153.0179	6.24	36.3	0.0	-9.53	91.85	PA	1.23E+04	1.23E+04	5.36E+03	
Caffeic acid	C9H8O4	179.0337	6.42	57.5	96.3	-7.23	99.54	PA	9.18E+04	1.04E+04	1.85E+04	3.39E+04
eriodictyol 7-O-glucoside/phloretin 2'-O-glucuronide I	C21H22O11	449.1081	6.44	38.5	4.4	-1.94	90.35	F	7.41E+04	1.20E+05	7.95E+03	
Feruloyl glucose	C16H20O9	355.1027	6.57	57.2	95.5	-2.13	92.96	PA	1.03E+04		3.28E+05	
Eriocitrin/neoeriocitrin isomer I	C27H32O15	595.1658	6.57	36.8	0.0	-1.76	86.01	F	3.11E+04		8.52E+03	
eriodictyol 7-O-glucoside/phloretin 2'-O-glucuronide II	C21H22O11	449.1098	6.68	47.7	49.0	2.01	91.79	F	1.74E+04		6.69E+04	
Phenylacetic acid	C8H8O2	135.0439	6.73	36.8	0.0	-9.49	94.18	PA	3.09E+04	2.77E+04	2.79E+04	
Hydroxycoumarin isomer I	C9H6O3	161.0232	6.73	52.6	77.0	-7.47	94.52	NI	5.14E+04	4.56E+04	5.02E+04	
6''-O-Malonylglycitin	C25H24O13	531.1100	6.73	40.5	28.0	-8.26	83.81	F	6.47E+03	8.98E+03	8.34E+04	3.74E+04
Hesperidin/neohesperidin	C28H34O15	609.1798	6.85	37.5	0.0	-4.37	92.71	F				8.86E+03
Dihydroxyphenylacetic acid/hydroxymandelic acid isomer	C8H8O4	167.0333	6.87	36.3	0.0	-9.75	91.94	PA		3.14E+04		7.71E+03
Hydroxyphenylacetic acid isomer II	C8H8O3	151.0387	6.92	36.2	0.0	-9.27	91.06	PA	1.04E+04	2.44E+04		
4-p-Coumaroylquinic acid	C16H18O8	337.0918	6.98	38.1	0.0	-3.31	94.15	PA		8.20E+04		7.61E+04
(-)-Epicatechin	C15H14O6	289.0707	7.01	46.2	36.5	-3.81	99.22	F		6.60E+03		1.45E+04
Datiscin/Pucrarin	C21H20O9	415.1042	7.01	39.5	7.9	1.78	91.87	F				7.45E+03
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside I	C21H20O11	447.0926	7.16	54.1	85.9	-1.43	86.33	F		1.52E+04		1.67E+04
Eriocitrin/neoeriocitrin I	C27H32O15	595.1652	7.17	37.3	3.7	-2.84	86.26	F		1.61E+04		2.01E+04
p-anisaldehyde isomer I	C8H8O2	135.0439	7.23	36.5	0.0	-9.57	92.96	NI	2.33E+04	1.12E+04	3.82E+04	1.32E+04
Coumaric acid isomer	C9H8O3	163.0387	7.27	36.6	0.0	-8.59	92.46	PA		6.84E+03		
Vanillin	C8H8O3	151.0387	7.30	37.1	0.0	-9.23	95.57	OP	8.99E+03		8.62E+03	
Feruloylquinic acid isomer	C17H20O9	367.1021	7.35	58.3	96.4	-3.55	99.11	PA	2.91E+04	2.08E+05	2.29E+04	1.75E+05
Phloridzin	C21H24O10	435.1282	7.39	56.3	86.6	-3.31	98.57	F		2.38E+04		2.28E+04
eriodictyol 7-O-glucoside/phloretin 2'-O-glucuronide III	C21H22O11	449.1083	7.43	49.0	48.4	-1.36	98.35	F		3.99E+05		7.31E+05
Eriocitrin/neoeriocitrin II	C27H32O15	595.1647	7.44	48.3	58.3	-3.65	87.62	F		3.12E+04		3.29E+04
Cinnamoyl glucose	C15H18O7	309.0989	7.53	40.0	11.9	2.90	91.58	PA		6.46E+03		6.55E+03

Apigenin 7-O-apiosyl-glucoside	C26H28O14	563.1391	7.55	41.0	9.9	-2.79	98.49	F		3.09E+05		3.44E+05
Procyanidin dimer B-type	C30H26O12	577.1334	7.57	38.2	0.0	-3.11	94.44	F		2.16E+04		5.16E+04
Chrysoeriol 7-O-glucoside/tectoridin	C22H22O11	461.1076	7.59	38.2	0.0	-2.92	94.55	F		7.03E+03		1.77E+04
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside II	C21H20O11	447.0936	7.62	46.2	43.9	0.73	88.17	F				8.39E+03
m-Coumaric acid	C9H8O3	163.0387	7.64	37.9	0.0	-8.29	98.71	PA	9.23E+04	1.50E+05	1.12E+05	6.14E+04
Kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside I	C27H30O16	609.1816	7.67	54.7	96.2	4.65	82.61	F		1.22E+05		1.28E+05
Esculetin	C9H6O4	177.0179	7.75	42.9	24.0	-7.99	99.23	OP		7.79E+03		1.67E+04
Kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside II	C27H30O16	609.1489	7.75	54.7	96.2	4.65	82.61	F		1.59E+04		3.03E+04
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/ hesperetin-O-glucuronide isomer I	C22H22O12	477.1003	7.78	44.9	36.3	-7.43	96.38	F				1.56E+05
Narirutin	C27H32O14	579.1668	7.84	37.8	15.6	-8.84	83.01	F		2.17E+04		2.22E+04
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside III	C21H20O11	447.0924	7.91	57.7	96.7	-2.05	94.35	F		8.50E+04		2.25E+05
Kaempferol 3-O-glucosyl-rhamnosyl-galactoside/kaempferol 3-O-glucosyl-rhamnosyl-glucoside/quercetin 3-O-rhamnosyl-rhamnosyl-glucoside	C33H40O20	755.2036	7.91	55.3	91.4	-0.53	85.59	F		8.00E+03		6.64E+03
Trihydroxyflavone isomer I	C15H10O5	269.0444	7.93	52.6	70.0	-4.09	97.83	F		2.86E+04		1.20E+06
Verbascoside	C29H36O15	623.1932	7.96	41.2	24.6	-7.98	90.14	PA		1.34E+04		9.52E+03
Trihydroxyflavanone isomer I	C15H12O5	271.0595	7.98	56.2	94.1	-6.39	94.34	F		1.02E+05		4.53E+04
Hydroxyphenylacetic acid isomer III	C8H8O3	151.0386	8.00	36.5	2.1	-9.83	91.06	PA	8.95E+03		1.52E+04	
Eriocitrin/neoeriocitrin isomer II	C27H32O15	595.1630	8.01	53.1	75.1	-6.37	97.44	F		6.90E+04		7.56E+04
Umbelliferone	C9H6O3	161.0234	8.05	36.6	0.0	-6.40	90.11	OP				8.81E+03
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/ 6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/ kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside IV	C21H20O11	447.0923	8.07	38.3	0.0	-2.12	94.01	F		1.48E+05		1.24E+05
Dihydroxy-methoxyisoflavone isomer/ 2'-hydroxyformononetin	C16H12O5	283.0598	8.12	56.3	95.4	-5.03	92.11	F				5.30E+03
Apigenin 6-C-glucoside/dihydrodaidzein 7-O-glucuronide I	C21H20O10	431.0970	8.12	44.1	28.4	-3.07	95.71	F		6.81E+04		1.21E+05
Isoferulic acid	C10H10O4	193.0493	8.14	45.1	35.1	-6.61	97.94	PA	3.68E+04	1.36E+05	4.12E+04	5.45E+04
Kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside III	C27H30O16	609.1453	8.14	40.0	6.1	-1.34	95.72	F	1.71E+04	2.91E+05	1.92E+04	1.69E+05
Resveratrol	C14H12O3	227.0696	8.16	39.4	20.4	-7.74	85.28	S				8.80E+03
Trihydroxyflavanone isomer II	C15H12O5	271.0601	8.16	57.5	93.3	-3.88	98.80	F		2.12E+05		3.25E+05
Naringenin 7-O-glucoside	C21H22O10	433.1136	8.16	56.3	83.9	-1.07	99.12	F		3.19E+06		4.79E+06
Apigenin 6,8-di-C-glucoside/chrysoeriol 7-O-apiosyl-glucoside/luteolin 7-O-rutinoside/ kaempferol 3-O-galactoside 7-O-rhamnoside/kaempferol 3-O-rutinoside	C27H30O15	593.1498	8.17	40.9	11.8	-2.30	95.38	F	3.92E+04	1.64E+05	9.63E+04	2.95E+05
Dihydroquercetin 3-O-rhamnoside	C21H22O11	449.1073	8.23	56.9	90.1	-3.69	98.59	F		1.27E+05		5.37E+05
Myricetin 3-O-rhamnoside	C21H20O12	463.0868	8.23	56.4	91.7	-2.98	93.92	F		2.84E+04		1.30E+05
6-Geranylnaringenin	C25H28O5	407.1896	8.35	35.3	0.0	7.96	85.19	F		2.44E+04		2.47E+04
Quercetin 3-O-glucoside	C21H20O12	463.0860	8.35	39.5	7.1	-4.64	95.93	F		4.25E+04		8.84E+04
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/ quercetin 3-O-rhamnoside V	C21H20O11	447.0916	8.37	41.4	21.1	-3.87	90.39	F	2.56E+04	7.03E+04	8.24E+04	8.00E+05
Pyrogallol	C6H6O3	125.0232	8.41	42.5	26.8	-9.90	96.32	OP		1.31E+05		9.61E+04
Kaempferol	C15H10O6	285.0391	8.41	47.2	42.2	-4.66	99.36	F		1.10E+06		8.50E+05
Dihydroquercetin	C15H12O7	303.0495	8.41	51.0	61.5	-4.98	99.44	F		5.66E+06		4.24E+06
Daidzein/Chrysin	C15H10O4	253.0487	8.44	37.2	0.0	-7.71	94.57	F			1.26E+04	1.14E+05
Paeoniflorin	C23H28O11	479.1523	8.48	36.9	0.0	-7.38	92.64	PA		1.02E+04		1.07E+04

Genistin	C21H20O10	431.0969	8.50	56.4	91.1	-3.44	95.11	F		7.42E+04		1.92E+05
Methylgalangin	C16H12O5	283.0597	8.51	50.6	60.2	-5.20	98.73	F		8.43E+04		1.25E+06
Cirsimaritin	C17H14O6	313.0691	8.51	49.7	68.4	-8.39	89.25	F		1.80E+04		
Luteolin 7-O-glucuronide	C21H18O12	461.0717	8.57	54.9	90.4	-1.79	86.11	F				1.75E+04
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/ hesperetin-O-glucuronide isomer II	C22H22O12	477.1027	8.59	37.9	0.0	-2.38	92.37	F		3.03E+04		8.67E+04
Nepetin/rhamnetin I	C16H12O7	315.0491	8.60	50.5	69.4	-6.09	90.33	F		1.75E+04		2.58E+04
Naringin	C27H32O14	579.1691	8.60	57.4	97.3	-4.84	95.48	F		4.03E+04		3.83E+04
Dicaffeoylquinic acid isomer	C25H24O12	515.1178	8.62	42.4	18.8	-3.32	97.17	PA		1.37E+05		1.75E+05
Trihydroxyflavanone isomer III	C15H12O5	271.0591	8.64	36.0	0.0	-7.84	88.61	F		1.44E+04		1.66E+04
Dihydroxy-dimethoxyisoflavone isomer I	C17H14O7	329.0651	8.71	49.1	54.6	-4.66	96.11	F				5.83E+03
Butein	C15H12O5	271.0596	8.77	56.6	97.6	-5.74	91.90	F				2.86E+04
Eriocitrin/neoeriocitrin isomer III	C27H32O15	595.1651	8.78	57.9	97.8	-2.95	95.42	F		1.26E+04		1.16E+04
Koparin	C16H12O6	299.0544	8.80	52.6	77.3	-5.67	92.42	F				6.88E+03
6''-O-Acetylglycitin	C24H24O11	487.1225	8.82	54.5	87.0	-4.37	90.36	F		2.64E+04		1.86E+04
Dicaffeoylquinic acid isomer	C25H24O12	515.1185	8.82	57.2	98.7	-1.93	89.61	PA		9.32E+04		1.66E+05
Phloretin	C15H14O5	273.0752	8.83	36.8	0.0	-6.16	90.90	F		9.17E+03	6.01E+03	3.79E+04
Violanone	C17H16O6	315.0883	8.87	38.3	0.0	2.74	94.96	F		7.06E+03		
Trihydroxyflavanone isomer IV	C15H12O5	271.0598	8.93	47.1	49.0	-5.25	92.58	F	1.29E+04	2.14E+04	1.51E+04	8.77E+04
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/ hesperetin-O-glucuronide isomer III	C22H22O12	477.1030	8.93	48.7	54.4	-1.72	91.09	F		2.29E+04		6.81E+04
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/ quercetin 3-O-rhamnoside VI	C21H20O11	447.0921	8.98	42.0	28.7	-2.58	84.33	F				3.82E+04
Dalbergin	C16H12O4	267.0648	9.01	57.1	95.3	-5.62	96.71	F				1.11E+05
Luteolin 7-O-malonyl-glucoside	C24H22O14	533.0917	9.04	50.7	71.8	-3.62	85.73	F		1.31E+04		5.27E+04
(+)-Catechin 3-O-gallate/(-)-Epicatechin 3-O-gallate	C22H18O10	441.0807	9.05	37.6	4.0	-4.51	89.21	F				9.76E+03
Dicaffeoylquinic acid isomer	C25H24O12	515.1183	9.05	51.2	62.8	-2.26	96.10	PA		1.11E+05		1.67E+05
6''-O-Acetyldaidzin	C23H22O10	457.1130	9.07	47.7	54.5	-2.26	86.78	F				2.19E+04
eriodictyol 7-O-glucoside/phloretin 2'-O-glucuronide IV	C21H22O11	449.1084	9.10	58.1	92.3	-1.10	99.35	F		1.57E+05		5.96E+05
Isorhamnetin 3-O-rutinoside	C22H22O11	461.1078	9.10	55.9	90.2	-2.54	92.27	F	1.01E+04	1.81E+04	2.99E+04	1.31E+05
Rhoifolin	C27H30O14	577.1530	9.10	54.1	88.9	-5.75	88.17	F		6.84E+04		8.22E+04
p-anisaldehyde isomer II	C8H8O2	135.0439	9.17	36.2	0.0	-9.39	91.26	NI				6.58E+03
Homovanillic acid/Dihydrocaffeic acid/Syringaldehyde	C9H10O4	181.0494	9.17	36.5	0.0	-6.72	89.87	NI				3.03E+04
Trihydroxyflavanone isomer V	C15H12O5	271.0595	9.20	36.1	0.0	-6.09	87.39	F		6.07E+03		1.14E+04
Irilone	C16H10O6	297.0387	9.20	51.6	73.7	-5.77	90.88	F		8.87E+03		1.85E+04
Glycitin	C22H22O10	445.1114	9.23	54.2	88.6	-5.95	89.19	F		2.32E+04		2.75E+04
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/ 6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside VII	C21H20O11	447.0918	9.23	36.6	0.0	-3.38	86.85	F				2.57E+04
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/ hesperetin-O-glucuronide isomer IV	C22H22O12	477.1025	9.23	36.8	0.0	-2.79	87.26	F				5.33E+03
Trihydroxyflavone isomer II	C15H10O5	269.0448	9.28	41.2	20.6	-2.66	88.35	F				7.33E+03
Piceatannol	C14H12O4	243.0646	9.30	40.6	16.1	-6.72	94.51	S		6.21E+03		9.42E+03
Hydroxycoumarin isomer II	C9H6O3	161.0233	9.33	37.6	0.0	-7.03	95.83	OP				6.13E+04
Hydroxyphenylpropionic acid isomer/methoxyphenylacetic acid	C9H10O3	165.0554	9.33	37.6	0.0	-1.93	90.07	PA				8.09E+03
Apigenin 6-C-glucoside/dihydrodaidzein 7-O-glucuronide II	C21H20O10	431.0973	9.39	45.0	44.8	-2.56	83.43	F		2.35E+04	8.12E+03	7.69E+04
Catechol	C6H6O2	109.0285	9.49	36.6	0.0	-9.10	93.26	OP				5.49E+03
Resacetophenone	C8H8O3	151.0390	9.49	47.9	55.9	-6.84	91.06	OP				1.38E+04

Trihydroxyflavanone isomer VI	C15H12O5	271.0601	9.52	37.9	0.0	-3.92	94.13	F		4.86E+04		1.13E+05
Trihydroxyflavone isomer III	C15H10O5	269.0453	9.59	38.0	0.0	-0.76	90.90	F		6.49E+03		3.25E+04
Scopoletin	C10H8O4	191.0336	9.60	36.5	0.0	-7.19	90.82	OP		4.03E+04		1.57E+04
Dihydroxy-dimethoxyisoflavone isomer II	C17H14O7	329.0666	9.64	46.2	40.4	-0.32	90.92	F				1.58E+04
Pseudobaptigenin	C16H10O5	281.0444	9.68	51.8	80.5	-3.97	83.09	F		9.53E+03		1.44E+04
3'-Hydroxymelanettin/isotectorigenin/tectorigenin/hispidulin I	C16H12O6	299.0548	9.68	55.5	94.2	-4.21	88.31	F		6.15E+03		1.70E+04
eriodictyol 7-O-glucoside/phloretin 2'-O-glucuronide V	C21H22O11	449.1071	9.93	56.4	96.5	-3.97	89.98	F				1.66E+04
Salvianolic acid B	C36H30O16	717.1447	10.00	37.8	9.2	-1.92	82.14	OP				1.61E+04
Hydroxycoumarin isomer III	C9H6O3	161.0231	10.02	51.9	76.6	-8.36	92.12	OP			2.44E+04	2.01E+04
Daidzin/Puerarin	C21H20O9	415.1025	10.02	57.0	88.7	-2.35	99.30	F	4.97E+05	8.76E+05	7.23E+06	6.11E+06
Eriodictyol	C15H12O6	287.0551	10.10	53.1	70.1	-3.42	99.27	F	7.62E+03	7.15E+05	4.23E+04	6.73E+05
Isorhamnetin	C16H12O7	315.0492	10.10	51.9	83.3	-5.68	82.68	F				6.99E+03
Pinocembrin/2-dehydro-O-desmethylangolensin/dihydrodaidzein/isoliquiritigenin	C15H12O4	255.0685	10.18	34.9	0.0	8.78	84.17	F			5.10E+03	8.51E+03
6"-O-acetylgenistin	C23H22O11	473.1074	10.21	37.3	0.0	-3.20	90.22	F				5.58E+03
Tetrahydroxyisoflavone isomer	C15H10O6	285.0394	10.26	57.9	94.5	-3.85	99.56	F	2.28E+04	2.87E+05	8.79E+05	1.53E+06
Quercetin	C15H10O7	301.0344	10.28	57.4	91.2	-3.40	99.70	F		4.55E+04		2.67E+05
Nepetin/rhamnetin II	C16H12O7	315.0497	10.34	57.8	97.1	-4.05	96.45	F		3.18E+04	2.15E+04	2.85E+05
Hesperetin/homoeriodictyol/4'-methoxy-2',3,7-trihydroxyisoflavanone	C16H14O6	301.0694	10.91	55.9	96.4	-7.87	91.86	F			1.79E+04	
Trihydroxyflavanone isomer VII	C15H12O5	271.0601	10.92	57.9	94.7	-4.20	99.83	F	3.05E+04	1.16E+06	1.89E+05	1.13E+06
Dihydroformononetin	C16H14O4	269.0835	11.08	36.8	0.0	5.71	90.45	F	7.12E+03		1.26E+04	5.28E+03
Jaceosidin/3,7-Dimethylquercetin I	C17H14O7	329.0652	11.13	37.2	0.0	-4.44	91.12	F		1.14E+04		5.12E+04
3'-Hydroxymelanettin/isotectorigenin/tectorigenin/hispidulin II	C16H12O6	299.0548	11.16	57.9	97.7	-4.37	96.97	F	1.31E+04	1.95E+04	4.81E+05	1.02E+05
Jaceosidin/3,7-Dimethylquercetin II	C17H14O7	329.0654	11.30	38.6	7.1	-4.00	90.73	F				2.31E+04
3'-Hydroxymelanettin/isotectorigenin/tectorigenin/hispidulin III	C16H12O6	299.0547	11.93	46.8	53.7	-4.64	85.79	F				1.66E+04

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids;

Supplementary Table 2. Phenolic compounds tentatively identified and common to all four sorghum phenolic extracts.

Name of compound	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Fragment data	Error (ppm)	IS (%)	Class	WSA	TSA	WSE	TSE
Hydroxybenzoic acid isomer/protocatechuic aldehyde/sesamol[d]	$C_7H_6O_3$	137.0232	4.91	55.2	86.2	136.0147 (44.91%)	-8.69	99.47	NI	7.15E+05	7.20E+05	1.09E+06	8.15E+05
4-Hydroxybenzoic acid[a]	$C_7H_6O_3$	137.0232	5.23	56.9	95.2	93.0335 (56.65%)	-8.49	98.81	PA	1.31E+05	2.57E+05	1.85E+05	1.93E+05
Hydroxyphenylacetic acid isomer I[c]	$C_8H_8O_3$	151.0387	5.96	36.8	0.0	0.0	-8.94	94.03	NI	4.95E+04	2.68E+04	8.74E+04	1.93E+04
5-Caffeoylquinic acid[a]	$C_{16}H_{18}O_9$	353.0864	6.02	54.7	80.7	85.0281 (1.15%); 167.0334 (6.13%); 191.0538 (100%)	-4.11	97.67	PA	2.44E+04	3.82E+05	3.01E+04	2.54E+05
Vanillic acid[a]	$C_8H_8O_4$	167.0344	6.19	38.1	0.0	0.0	-3.34	94.26	PA	3.06E+04	1.23E+04	3.39E+04	5.36E+03
Dihydroxybenzoic acid isomer[b]	$C_7H_6O_4$	153.0179	6.24	36.3	0.0	0.0	-9.53	91.85	PA	1.23E+04	1.04E+04	1.85E+04	7.95E+03
Caffeic acid[a]	$C_9H_8O_4$	179.0337	6.42	57.5	96.3	134.0353 (1.58%); 135.0435 (100%)	-7.23	99.54	PA	9.18E+04	7.41E+05	1.20E+05	3.28E+05
Phenylacetic acid[b]	$C_8H_8O_2$	135.0439	6.73	36.8	0.0	0.0	-9.49	94.18	PA	3.09E+04	2.77E+04	5.02E+04	2.20E+04
Hydroxycoumarin isomer I[c]	$C_9H_6O_3$	161.0232	6.73	52.6	77.0	133.0280 (17.05%); 135.0437 (45.84%)	-7.47	94.52	NI	5.14E+04	4.56E+04	8.34E+04	3.74E+04
p-anisaldehyde isomer I[c]	$C_8H_8O_2$	135.0439	7.23	36.5	0.0	0.0	-9.57	92.96	NI	2.33E+04	1.12E+04	3.82E+04	1.32E+04
Feruloylquinic acid isomer[b]	$C_{17}H_{20}O_9$	367.1021	7.35	58.3	96.4	93.0336 (6.23%); 173.0433 (100%); 193.0487 (18.82%)	-3.55	99.11	PA	2.91E+04	2.08E+05	2.29E+04	1.75E+05
m-Coumaric acid[b]	$C_9H_8O_3$	163.0387	7.64	37.9	0.0	0.0	-8.29	98.71	PA	9.23E+04	1.50E+05	1.12E+05	6.14E+04
Trans-ferulic acid[a]	$C_{10}H_{10}O_4$	193.0493	8.14	45.1	35.1	107.0122 (3.88%); 119.0489 (28.68%); 134.0357 (14.77%); 178.0241 (2.42%)	-6.61	97.94	PA	3.68E+04	1.36E+05	4.12E+04	5.45E+04
Kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside III[c]	$C_{27}H_{30}O_{16}$	609.1453	8.14	40.0	6.1	313.0539 (4.72%)	-1.34	95.72	F	1.71E+04	2.91E+05	1.92E+04	1.69E+05
Apigenin 6,8-di-C-glucoside/chrysoeriol 7-O-apiosyl-glucoside/luteolin 7-O-	$C_{27}H_{30}O_{15}$	593.1498	8.17	40.9	11.8	119.0335 (37.45%); 285.0381 (47.14%)	-2.30	95.38	F	3.92E+04	1.64E+05	9.63E+04	2.95E+05

rutinoside/kaempferol 3-O-galactoside 7-O-rhamnoside/kaempferol 3-O-rutinoside[c]													
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside V[c]	$C_{21}H_{20}O_{11}$	447.0916	8.37	41.4	21.1	253.0682 (4.79%)	-3.87	90.39	F	2.56E+04	7.03E+04	8.24E+04	8.00E+05
Trihydroxyflavanone isomer IV[c]	$C_{15}H_{12}O_5$	271.0598	8.93	47.1	49.0	135.0439 (12.48%); 151.0022 (23.35%)	-5.25	92.58	F	1.29E+04	2.14E+04	1.51E+04	8.77E+04
Isorhamnetin 3-O-rutinoside[b]	$C_{22}H_{22}O_{11}$	461.1078	9.10	55.9	90.2	135.0436 (17.36%); 151.0023 (68.60%); 449.1026 (100%)	-2.54	92.27	F	1.01E+04	1.81E+04	2.99E+04	1.31E+05
Daidzin/Puerarin[b]	$C_{21}H_{20}O_9$	415.1025	10.02	57.0	88.7	135.0437 (37.09%); 161.0227 (69.25%); 179.0331 (52.65%); 253.0693 (100%)	-2.35	99.30	F	4.97E+05	8.76E+05	7.23E+06	6.11E+06
Eriodictyol[b]	$C_{15}H_{12}O_6$	287.0551	10.10	53.1	70.1	107.0127 (8.77%); 149.0229 (5.04%); 151.0021 (100%); 271.0586 (20.90%)	-3.42	99.27	F	7.62E+03	7.15E+05	4.23E+04	6.73E+05
Tetrahydroxyisoflavone isomer[c]	$C_{15}H_{10}O_6$	285.0394	10.26	57.9	94.5	107.0128 (15.31%); 133.0279 (23.33%); 149.0229 (5.48%)	-3.85	99.56	F	2.28E+04	2.87E+05	8.79E+05	1.53E+06
Trihydroxyflavanone isomer VII[c]	$C_{15}H_{12}O_5$	271.0601	10.92	57.9	94.7	65.0025 (3.65%); 93.0335 (11.74%); 107.0126 (21.88%); 109.0489 (75.35%); 151.0020 (100%); 177.0174 (4.80%)	-4.20	99.83	F	3.05E+04	1.16E+06	1.89E+05	1.13E+06
3'-Hydroxymelanettin/isotectorigenin/tectorigenin/hispidulin II[c]	$C_{16}H_{12}O_6$	299.0548	11.16	57.9	97.7	284.0288 (100%)	-4.37	96.97	F	1.31E+04	1.95E+04	4.81E+05	1.02E+05

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids; Identified level I[a], II[b], III[c] and IV[d], according to the Metabolomics Standards Initiative.

Supplementary Table 3. Volcano plot data for the phenolic compounds differentially expressed.

Name of compound	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Fragment data	Error (ppm)	IS (%)	Class	TSA *vs* TSE			WSA *vs* WSE			TSA *vs* WSA			TSE *vs* WSE		
										Log2 Fold-change	p-value	Regulation	Log2 Fold-change	p-value	Regulation	Log2 Fold-change	p-value	Regulation	Log2 Fold-change	p-value	Regulation
Dicaffeoylquinic acid isomer	C25H24O12	515.1233	0.73	35.6	0.0	0.0	7.45	86.38	PA				1.88	0.00	Up-regulated						
Hydroxyphenylacetic acid isomer I	C8H8O3	151.0387	5.96	36.8	0.0	0.0	-8.94	94.03	NI										2.18	0.00	Up-regulated
5-Caffeoylquinic acid	C16H18O9	353.0864	6.02	54.7	80.7	85.0281 (1.15%); 167.0334 (6.13%); 191.0538 (100%)	-4.11	97.67	PA							-3.97	0.00	Down-regulated	-3.08	0.00	Down-regulated
Vanillic acid	C8H8O4	167.0344	6.19	38.1	0.0	0.0	-3.34	94.26	PA	1.20	0.04	Up-regulated				1.32	0.01	Up-regulated	2.66	0.00	Up-regulated
Dihydroxybenzoic acid isomer	C7H6O4	153.0179	6.24	36.3	0.0	0.0	-9.53	91.85	PA										1.22	0.01	Up-regulated
Caffeic acid	C9H8O4	179.0337	6.42	57.5	96.3	134.0353 (1.58%); 135.0435 (100%)	-7.23	99.54	PA	1.18	0.00	Up-regulated				-3.01	0.00	Down-regulated	-1.45	0.00	Down-regulated
Feruloyl glucose	C16H20O9	355.1027	6.57	57.2	95.5	175.0390 (100%)	-2.13	92.96	PA	-1.10	0.02	Down-regulated									
Phenylacetic acid	C8H8O2	135.0439	6.73	36.8	0.0	0.0	-9.49	94.18	PA										1.19	0.00	Up-regulated
Hydroxycoumarin isomer I	C9H6O3	161.0232	6.73	52.6	77.0	133.0280 (17.05%); 135.0437 (45.84%)	-7.47	94.52	NI										1.15	0.01	Up-regulated
Hydroxyphenylacetic acid isomer II	C8H8O3	151.0387	6.92	36.2	0.0	0.0	-9.27	91.06	NI				-1.23	0.01	Down-regulated						
(-)-Epicatechin	C15H14O6	289.0707	7.01	46.2	36.5	93.0336 (6.23%); 173.0433 (100%); 193.0487 (18.82%)	-3.81	99.22	F	-1.13	0.01	Down-regulated									
p-anisaldehyde isomer I	C8H8O2	135.0439	7.23	36.5	0.0	0.0	-9.57	92.96	NI							1.05	0.00	Up-regulated	1.53	0.00	Up-regulated
Feruloylquinic acid isomer	C17H20O9	367.1021	7.35	58.3	96.4	93.0036 (6.23%); 173.0433 (100%); 193.0487 (18.82%)	-3.55	99.11	PA							-2.84	0.00	Down-regulated	-2.93	0.00	Down-regulated
Procyanidin dimer B-type	C30H26O12	577.1334	7.57	38.2	0.0	0.0	-3.11	94.44	F	-1.25	0.00	Down-regulated									
Chrysoeriol 7-O-glucoside/tectoridin	C22H22O11	461.1076	7.59	38.2	0.0	0.0	-2.92	94.55	F	-1.33	0.00	Down-regulated									
m-Coumaric acid	C9H8O3	163.0387	7.64	37.9	0.0	0.0	-8.29	98.71	PA	1.29	0.00	Up-regulated									
Esculetin	C9H6O4	177.0179	7.75	42.9	24.0	71.0127 (1.44%); 149.0230 (46.46%)	-7.99	99.23	OP	-1.10	0.02	Down-regulated									
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside III	C21H20O11	447.0924	7.91	57.7	96.7	253.0687 (22.42%); 285.0378 (100%)	-2.05	94.35	F	-1.40	0.00	Down-regulated									

Trihydroxyflavone isomer	C15H10O5	269.0444	7.93	52.6	70.0	109.0280 (5.20%); 151.0382 (14.27%); 225.0533 (6.25%)	-4.09	97.83	F	-5.40	0.00	Down-regulated								
Trihydroxyflavanone isomer I	C15H12O5	271.0595	7.98	56.2	94.1	151.0026 (14.48%)	-6.39	94.34	F	1.18	0.00	Up-regulated								
Isoferulic acid	C10H10O4	193.0493	8.14	45.1	35.1	107.0122 (3.88%); 119.0489 (28.68%); 134.0357 (14.77%); 178.0241 (2.42%)	-6.61	97.94	PA	1.32	0.00	Up-regulated				-1.89	0.00	Down-regulated		
Kaempferol 3,7-O-diglucoside/kaempferol 3-O-sophoroside/quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside III	C27H30O16	609.1453	8.14	40.0	6.1	313.0539 (4.72%)	-1.34	95.72	F							-4.09	0.00	Down-regulated	-3.14	0.00	Down-regulated
Apigenin 6,8-di-C-glucoside/chrysoeriol 7-O apiosyl-glucoside/luteolin 7-O-rutinoside/kaempferol 3-O-galactoside 7-O-rhamnoside/kaempferol 3-O-rutinoside	C27H30O15	593.1498	8.17	40.9	11.8	119.0335 (37.45%); 285.0381 (47.14%)	-2.30	95.38	F				-1.29	0.02	Down-regulated	-2.07	0.00	Down-regulated	-1.62	0.00	Down-regulated
Dihydroquercetin 3-O-rhamnoside	C21H22O11	449.1073	8.23	56.9	90.1	287.0533 (100%); 301.0324 (13.18%); 303.0479 (26.23%)	-3.69	98.59	F	-2.08	0.00	Down-regulated								
Quercetin 3-O-glucoside	C21H20O12	463.0860	8.35	39.5	7.1	300.0237 (9.50%)	-4.64	95.93	F	-1.06	0.00	Down-regulated								
Naringenin-O-glucuronide isomer/luteolin glucoside isomer/6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside V	C21H20O11	447.0916	8.37	41.4	21.1	253.0682 (4.79%)	-3.87	90.39	F	-3.51	0.00	Down-regulated	-1.69	0.00	Down-regulated	-1.46	0.00	Down-regulated	-3.28	0.00	Down-regulated
Daidzein/Chrysin	C15H10O4	253.0487	8.44	37.2	0.0	0.0	-7.71	94.57	F										-3.19	0.00	Down-regulated
Genistin	C21H20O10	431.0969	8.50	56.4	91.1	240.0391 (39.70%); 268.0337 (43.98%); 269.0415 (84.41%); 283.0572 (100%)	-3.44	95.11	F	-1.38	0.00	Down-regulated								
Methylgalangin	C16H12O5	283.0597	8.51	50.6	60.2	196.0500 (23.33%); 240.0391 (39.70%); 268.0337 (43.98%)	-5.20	98.73	F	-3.89	0.00	Down-regulated								
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/hesperetin-O-glucuronide isomer II	C22H22O12	477.1027	8.59	37.9	0.0	0.0	-2.38	92.37	F	-1.52	0.01	Down-regulated								
Phloretin	C15H14O5	273.0752	8.83	36.8	0.0	0.0	-6.16	90.90	F	-2.05	0.00	Down-regulated							-2.66	0.00	Down-regulated
Trihydroxyflavanone isomer IV	C15H12O5	271.0598	8.93	47.1	49.0	135.0439 (12.48%); 151.0022 (23.35%)	-5.25	92.58	F	-2.03	0.00	Down-regulated							-2.53	0.00	Down-regulated
Isorhamnetin-O-galactoside/isorhamnetin-O-glucoside/isorhamnetin-O-rhamnoside/hesperetin-O-glucuronide isomer III	C22H22O12	477.1030	8.93	48.7	54.4	134.0439 (12.48%); 151.0022 (23.35%); 287.0535 (93.77%); 299.0534 (35.85%); 341.0834 (31.24%)	-1.72	91.09	F	-1.57	0.00	Down-regulated								
Luteolin 7-O-malonyl-glucoside	C24H22O14	533.0917	9.04	50.7	71.8	113.0230 (2.61%); 121.0280 (100%); 173.0436 (29.89%); 489.0976 (89.63%)	-3.62	85.73	F	-2.01	0.01	Down-regulated								
Eriodictyol 7-O-glucoside/phloretin 2'-O-	C21H22O1	449.1084	9.10	58.1	92.3	135.0436 (5.34%);	-1.10	99.35	F	-1.93	0.00	Down-								

glucuronide IV	1					287.0537 (100%)						regulated						
Isorhamnetin 3-O-rutinoside	C22H22O11	461.1078	9.10	55.9	90.2	135.0436 (17.36%); 151.0023 (68.60%); 449.1026 (100%)	-2.54	92.27	F	-2.85	0.00	Down-regulated	-1.57	0.04	Down-regulated	-2.13	0.00	Down-regulated
Irilone	C16H10O6	297.0387	9.20	51.6	73.7	271.0595 (100%)	-5.77	90.88	F	-1.06	0.00	Down-regulated						
Apigenin 6-C-glucoside/dihydrodaidzein 7-O-glucuronide II	C21H20O10	431.0973	9.39	45.0	44.8	309.0590 (100%)	-2.56	83.43	F	-1.71	0.00	Down-regulated				-3.24	0.00	Down-regulated
Trihydroxyflavanone isomer VI	C15H12O5	271.0601	9.52	37.9	0.0	0.0	-3.92	94.13	F	-1.22	0.00	Down-regulated						
Trihydroxyflavone isomer III	C15H10O5	269.0453	9.59	38.0	0.0	0.0	-0.76	90.90	F	-2.32	0.01	Down-regulated						
Scopoletin	C10H8O4	191.0336	9.60	36.5	0.0	0.0	-7.19	90.82	OP	1.36	0.00	Up-regulated						
3'-Hydroxymelanettin/isotectorigenin/tectorigenin/hispidulin I	C16H12O6	299.0548	9.68	55.5	94.2	135.0433 (5.11%); 255.0651 (9.74%); 281.0434 (14.33%); 287.0540 (100%)	-4.21	88.31	F	-1.46	0.02	Down-regulated						

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids.

Supplementary Table 4. Predicted targets for compounds present in highest abundance in the tested sample.

Compound	Predicted Target			p-value	MaxTC[a]
	Key	Name	Description		
FLAVONOIDS					
Methylgalangin	Q965D6_PLAFA	fabG	3-oxoacyl-acyl-carrier protein reductase	1.448e-48	0.47
	Q965D7_PLAFA	fabZ	Beta-hydroxyacyl-ACP dehydratase	6.003e-41	0.47
	CP1B1_HUMAN	CYP1B1	Cytochrome P450 1B1	2.226e-41	0.66
	XDH_HUMAN	XDH	Xanthine dehydrogenase/oxidase	1.523e-20	0.66
	Q965D5_PLAFA	fabI	Enoyl-acyl-carrier protein reductase	5.396e-18	0.52
Kaempferol	Q965D7_PLAFA	fabZ	Beta-hydroxyacyl-ACP dehydratase	7.496e-99	0.78
	Q965D6_PLAFA	fabG	3-oxoacyl-acyl-carrier protein reductase	1.364e-79	0.78
	CP1B1_HUMAN	CYP1B1	Cytochrome P450 1B1	4.491e-50	0.81
	PDIA1_HUMAN	P4HB	Protein disulfide-isomerase	6.101e-41	0.52
	XDH_HUMAN	XDH	Xanthine dehydrogenase/oxidase	4.6e-38	0.81
	Q965D5_PLAFA	fabI	Enoyl-acyl-carrier protein reductase	6.232e-27	0.78
	NOX4_HUMAN	NOX4	NADPH oxidase 4	1.895e-21	0.78
	DAPK1_HUMAN	DAPK1	Death-associated protein kinase 1	6.256e-20	0.78
Eriodictyol	Q965D7_PLAFA	fabZ	Beta-hydroxyacyl-ACP dehydratase	6.604e-55	0.44
	Q965D5_PLAFA	fabI	Enoyl-acyl-carrier protein reductase	5.668e-18	0.44
	CP1B1_HUMAN	CYP1B1	Cytochrome P450 1B1	1.059e-17	0.73
Dihydroquercetin	CAH4_HUMAN	CA4	Carbonic anhydrase 4	9.6e-09	1.00
	CAH7_HUMAN	CA7	Carbonic anhydrase 7	5.223e-07	1.00
	CAH12_HUMAN	CA12	Carbonic anhydrase 12	0.01559	1.00
	Q965D6_PLAFA	fabG	3-oxoacyl-acyl-carrier protein reductase	9.262e-65	0.44
	Q965D7_PLAFA	fabZ	Beta-hydroxyacyl-ACP dehydratase	8.962e-54	0.44
	Q8I2S7_PLAF7	-	3-oxoacyl-[acyl-carrier-protein] reductase	2.644e-41	0.33
	Q965D5_PLAFA	fabI	Enoyl-acyl-carrier protein reductase	1.158e-17	0.44
PHENOLIC ACIDS					
Caffeic acid	MMP9_HUMAN	MMP9	Matrix metalloproteinase-9	1.319e-21	1.00
	CAH12_HUMAN	CA12	Carbonic anhydrase 12	1.696e-06	1.00
	CAH7_HUMAN	CA7	Carbonic anhydrase 7	1.11e-16	1.00

	CAH9_HUMAN	CA9	Carbonic anhydrase 9	1.776e-06	1.00
	CAH2_HUMAN	CA2	Carbonic anhydrase 2	0.0004927	1.00
4-Hydroxybenzoic acid	CAH7_HUMAN	CA7	Carbonic anhydrase 7	8.005e-19	1.00
	CAH12_HUMAN	CA12	Carbonic anhydrase 12	6.406e-14	1.00
	CAH9_HUMAN	CA9	Carbonic anhydrase 9	2.492e-11	1.00
m-Coumaric acid	CYSP_PLAFA		Trophozoite cysteine proteinase	7.463e-29	0.33
	MMP9_HUMAN	MMP9	Matrix metalloproteinase-9	2.458e-25	0.63
	Q9XYC7_PLAFA	HDAC1	Histone deacetylase	3.331e-16	0.45

[a] MaxTC = Maximal Tanimoto coefficient between query compound and compounds from SEA dataset, a similarity measurement ranging from 0 (completely different compounds) to 1 (identical compounds)

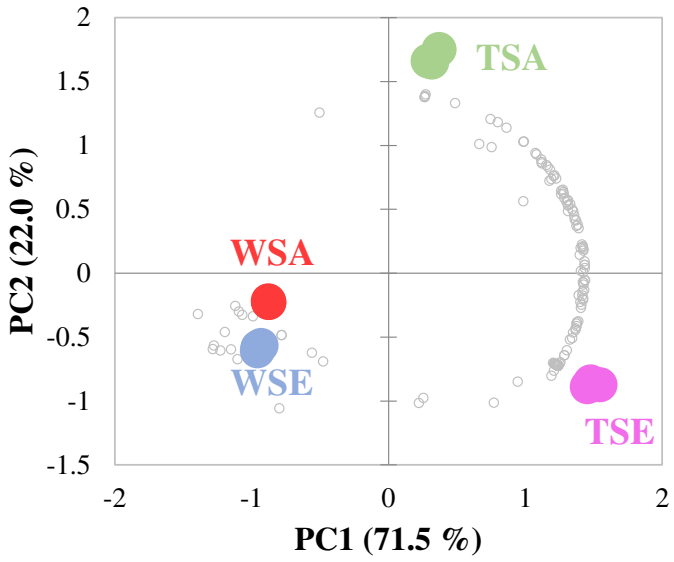

Figure S1. Principal component analysis (PCA) biplot of phenolic compounds relative abundance in sorghum extracts.

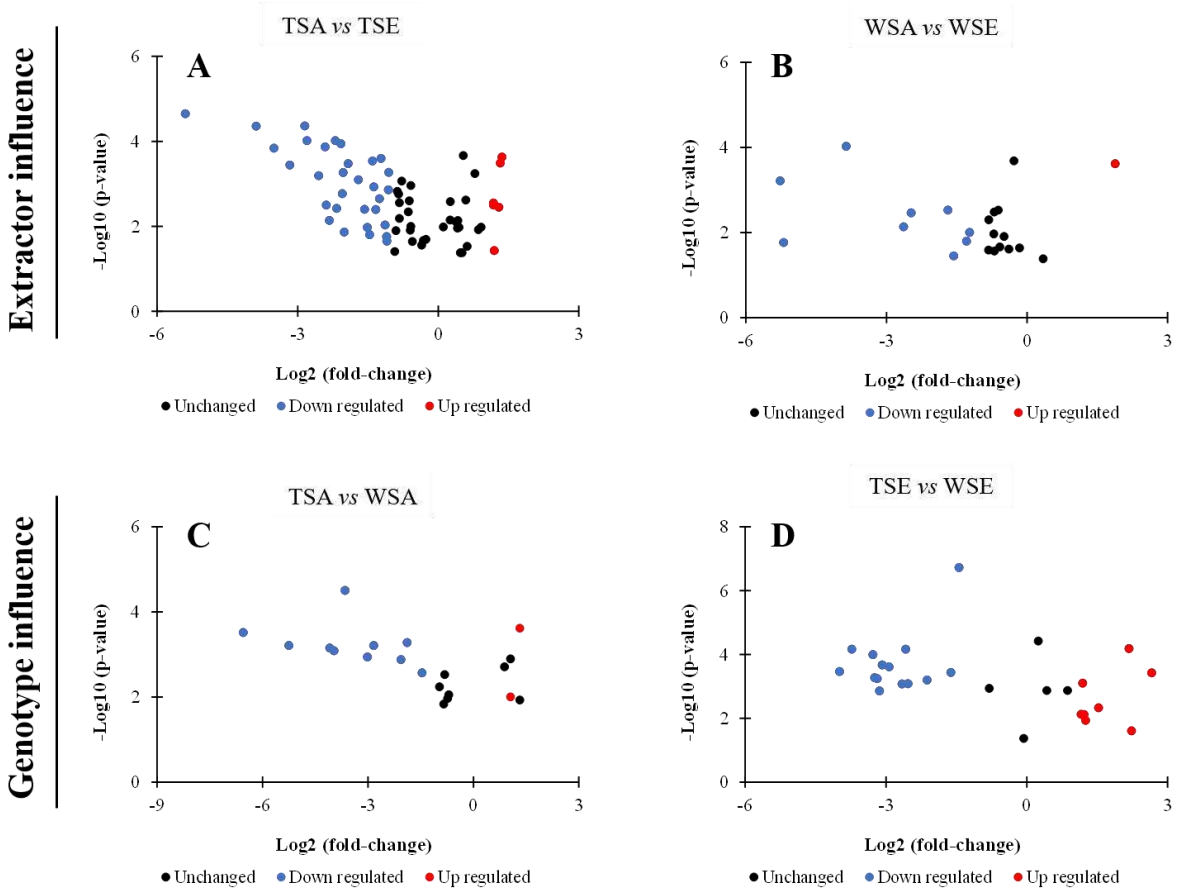

Figure S2. Volcanos-plot to compare the influence of extractors between A) TSA *vs* TSE and B) WSA *vs* WSE, and the genotype influence between C) TSA *vs* WSA and D) TSE *vs* WSE.

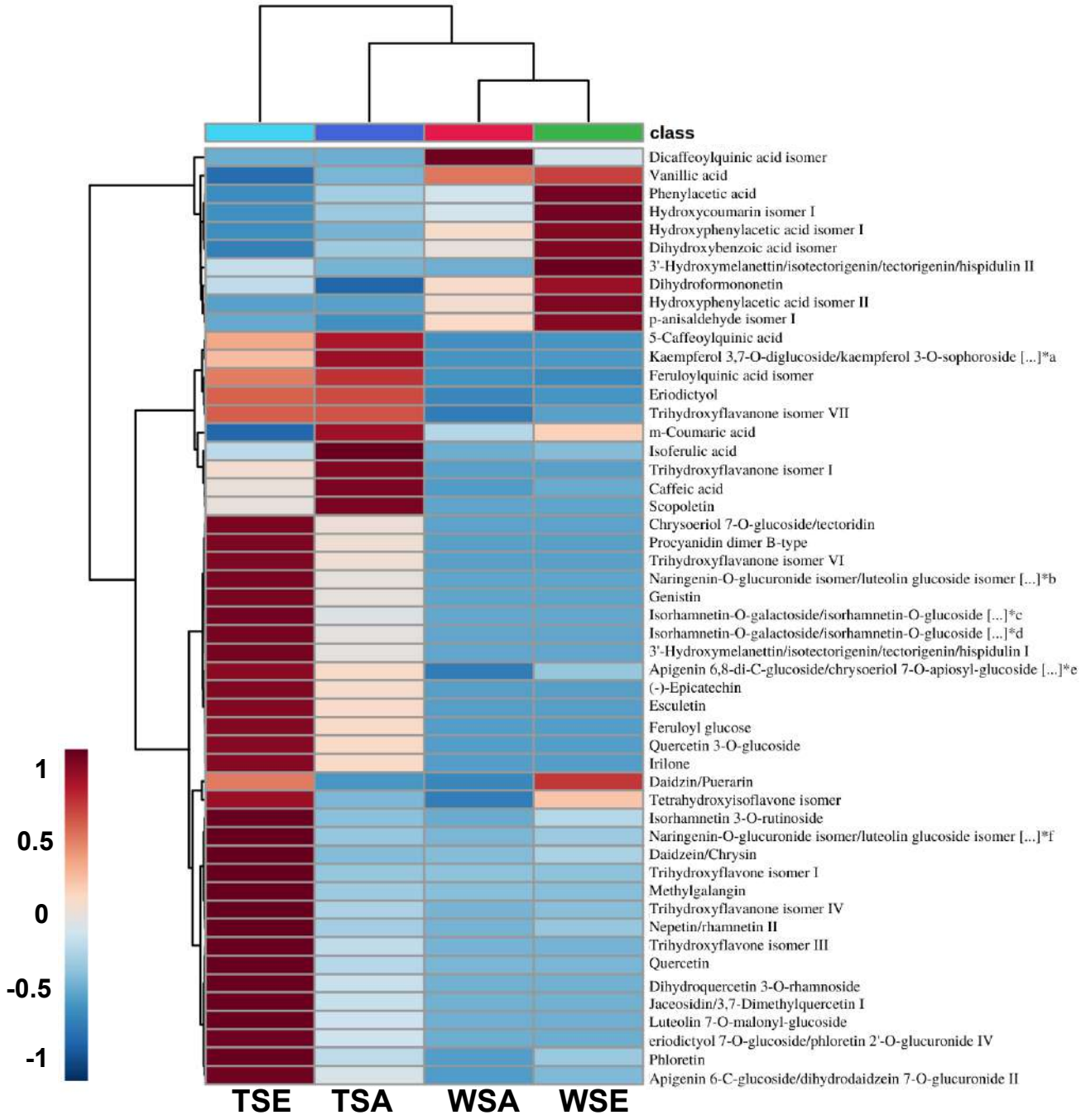

Figure S3. Hierarchical Cluster Analysis (HCA) and Heatmap of the phenolic extracts of sorghum. Continuation of compounds: ***a** = quercetin 3-O-galactoside 7-O-rhamnoside/quercetin 3-O-rhamnosyl-galactoside/quercetin 3-O-rutinoside III; ***b** = 6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside III; ***c** = isorhamnetin-O-rhamnoside/hesperetin-O-glucuronide isomer III; ***d** = isorhamnetin-O-rhamnoside/hesperetin-O-glucuronide isomer II; ***e** = luteolin 7-O-rutinoside/kaempferol 3-O-galactoside 7-O-rhamnoside/kaempferol 3-O-rutinoside; ***f** = 6-hydroxyluteolin 7-O-rhamnoside/kaempferol 3-O-galactoside/kaempferol 3-O-glucoside/quercetin 3-O-rhamnoside V.

7. CHAPTER 3: Toasted Sorghum Flours Enhance Defense Against Oxidative Stress and Reduce Aberrant Crypt Foci in Early-Stage 1,2-Dimethylhydrazine-Induced Colon Carcinogenesis in Rats

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ABSTRACT

The rising incidence of colon cancer globally underscores the urgent need for preventive strategies. Sorghum, rich in dietary fibers and phenolic compounds, offers promising health benefits. This study investigates the potential protective effects of toasted sorghum flours against early-stage dimethylhydrazine (DMH)-induced colon cancer in rats. Forty male rats were divided into four groups and subjected to various diets, including control (G1), tannin and white toasted sorghum flour supplementation with DMH colon induction (G2, G3, respectively), and DMH only (G4). Body weight and food intake was not significant between groups. Biochemical analyses revealed no significant differences in renal and hepatic functions among the groups. However, oxidative stress assessments indicated an enhanced activity of superoxide dismutase (SOD) in the liver of rats fed with tannin-rich sorghum flour (G2: 0.55 ± 0.19 U/mg protein), suggesting improved defense against oxidative stress induced by DMH. Histomorphometric analysis revealed a reduction in the longitudinal muscle length of the colon in DMH-treated groups, indicating DMH-induced alterations. Moreover, aberrant crypt foci (ACF) assays demonstrated a lower incidence of ACF in rats supplemented with sorghum flours (G2: 51.75 ± 26.84, G3: 64.38 ±18 ACF ≤ 3 crypts), suggesting an attenuating effect against colon carcinogenesis. Additionally, determination of short-chain fatty acids (SCFAs) revealed higher acetate levels observed in the group supplemented with white sorghum flour (G3: 36.12 ± 7.01 mM) compared to other groups, and higher propionate levels observed in the same group (G3: 10.51 ± 1.43 mM) compared to the negative control (G1). These findings highlight the promising role of sorghum flours in mitigating early-stage colon carcinogenesis, warranting further exploration of their preventive and therapeutic potentials.

KEYWORDS: Phenolic compounds; gut microbiota; intestinal health; whole grain.

7.1 INTRODUCTION

The incidence of colon cancer has been increasing worldwide, even among younger individuals, and is more common in men than in women (Bray et al., 2018). Some reasons for this are related to our lifestyle, which includes rapid urbanization and the adoption of a Westernized diet, consequently increasing the consumption of high-fat and high-protein foods while decreasing the intake of fiber-rich foods and antioxidant compounds (Venkatachalam et al., 2020).

In this context, sorghum (*Sorghum bicolor* L. Moench) emerges as a cereal rich in dietary fibers and phenolic compounds, offering various health benefits, as evidenced by studies involving cells, animals, and humans (de Oliveira & de Alencar Figueiredo, 2024). Notably, previous *in vitro* investigations have highlighted the efficacy of sorghum phenolic compounds against cancer cells, including colon cancer (Chen et al., 2021; Cox et al., 2019; Yang et al., 2012). Specifically, Yang et al. (2012) demonstrated the estrogenic activity of black and white sorghum extracts, suggesting their potential role in inducing apoptosis in damaged nonmalignant colonocytes. In addition, Cox et al. (2019) highlighted the underexplored anticancer properties of high polyphenol sorghum varieties, emphasizing the need for improved extraction methods to unlock their therapeutic potential. Similarly, Paes et al. (2024) comprehensively evaluated the antiproliferative, antioxidant, and antimetastatic activities of phenolic extracts from toasted white and tannin sorghum flours, revealing diverse biological effects in colon cancer (HCT-8) and liver cancer (A549) cells. These studies not only corroborate the potential of sorghum phenolics in inhibiting cancer cell growth but also highlights the importance of different sorghum varieties in exerting such effects.

Moreover, *in vivo* studies have shown promising results. A previous study by our group showed that toasted flours from white sorghum and tannin sorghum were able to improve oxidative stress by reducing the concentration of alanine aminotransferase (ALT) in rats (Silva et al., 2020). Findings by Ritchie et al. (2017) revealed that bran diets, particularly those rich in polyphenols such as black and sumac sorghum bran, upregulated repair mechanisms and short-chain fatty acid (SCFA) transporter expression, suggesting potential protective effects against colitis-induced inflammation. Additionally, Lee et al. (2021) demonstrated the anti-cancer potential of high-phenolic sorghum bran extracts in a genetic colon cancer rodent model. Their study revealed that treatment with high-phenolic sorghum bran extracts inhibited proliferation,

induced apoptosis, and suppressed tumor formation in mice, suggesting the therapeutic application of sorghum bran as a functional food for colon cancer prevention.

However, while there is existing *in vitro* data on the effects of sorghum flours on colon cancer, more *in vivo* studies are needed to further elucidate these effects on carcinogenesis. White sorghum and tannin sorghum not only have distinct phenolic profiles but also different dietary fiber contents, such as resistant starch, driving the investigation into the differences in biological responses to their consumption (Silva et al., 2020; Paes et al., 2024). Hence, the present study marks the first investigation into the consumption of toasted flours from sorghum BRS 501 and BRS 305 genotypes and their potential impact on colon cancer development. Therefore, our aim was to assess the protective effects of toasted sorghum flours against the early stages of DMH-induced colon cancer in rats, filling a crucial gap in current research.

7.2 MATERIALS AND METHODS

7.2.1 Toasted sorghum flours preparation

Sorghum flours were prepared according to Silva et al. (2020) with the genotypes of white sorghum (BRS 501) and sorghum with tannins (BRS 305) selected from the germplasm bank of “Embrapa Milho e Sorgo”, located in Sete Lagoas - MG. Briefly, after grinding in a knife mill with an opening of 1.7 mm followed by the standardization of granulometry, these flours were submitted to the toasting process in direct heat (200 °C/6 minutes).

7.2.2 Proximate and phenolic composition of the toasted sorghum flours

Proximate composition analyzes of the toasted sorghum flour were carried out in accordance with the AOAC (1998) to determine protein, lipid, ash, and moisture content. The total, soluble and insoluble fibers of the toasted sorghum flours were determined according to the gravimetric-enzymatic method of Lee, Prosky & DeVries (1992). Resistant starch (RS) content of the sorghum flour samples was measured according to the resistant starch assay kit from Megazyme (AACC method 32-40).

Quantification of total extractable phenolic compounds was performed according to the Folin Ciocalteu method, and the individual phenolic compounds present in the flour were determined by UPLC-MS^E following all the same parameters described by Paes et al. (2024). Data processing utilized Progenesis QI (Waters), and identification and annotation were conducted based on standard run parameters, including the isotope distribution of neutral mass, exact mass, retention time, and MS/MS fragments spectra. To aid non-targeted annotation,

PubChem was utilized, leveraging MetaScope, an integrated search tool. Additional parameters were applied in decreasing order of importance within the software, including exact mass error (<10 ppm), isotopic similarity (>80%), score (>30), and fragmentation score. Annotation of unknown compounds was facilitated using the phenol explorer database, published literature data, and chemical features of the molecule. Compounds were considered tentatively identified only if present in all three technical replicates (3/3), where each vial contains a pool of three true replicates and CV <30%. For annotating the features potentially corresponding to phenolic compounds, we employed a tailored database compiled from PubChem and Phenol Explorer for chemical alignments. Additionally, certain compounds were screened and omitted based on their occurrence in the blank, ionic relative abundance below 5,000 (set as our chromatogram baseline), isotopic similarity below 80%, and compounds exhibiting fragmentation data inconsistent with the literature and/or databases.

7.2.3 Biological essay

7.2.3.1 Experimental Design

Forty male rats (*Rattus norvegicus*, albinus, SHR lineage), aged 30 days, were sourced from the Animal Laboratory of the Biological Science and Health Center, Universidade Federal de Viçosa, Brazil, for this study. The animals were divided into four groups of ten (Figure 1), ensuring homogeneity in body weight for equal distribution among groups. They were individually housed in cages, maintained at 21 ± 3 °C with a 12-hour controlled photoperiod, and had access to distilled water *ad libitum*. All experimental procedures were conducted in compliance with the Ethics Committee for Animal Research of the Universidade Federal de Viçosa (approval number 13/2022, supplementary material 1).

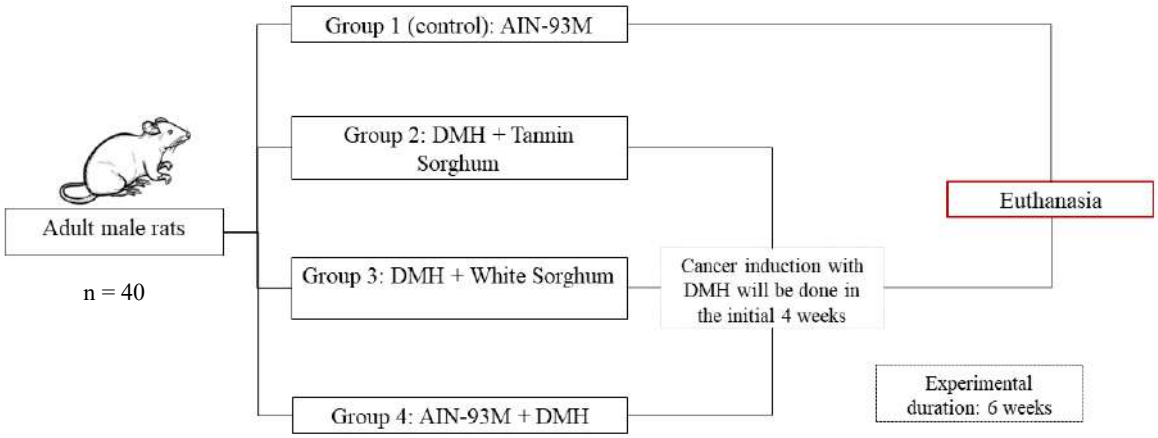

Figure 1. *In vivo* experimental design. AIN-93M: Standard diet for rodents. DMH: 1,2-dimethylhydrazin.

The experimental diets are presented in Table 1 for each group, including the amount of toasted sorghum flours in their diets. The amount of sorghum flours was stablished to achieve the recommended fiber value for rodents while also calculating the other macronutrients to ensure isocaloric diets among the groups. In this way, the diets were adjusted to have the same amount of carbohydrates, proteins, lipids, and dietary fibers.

Table 1. Composition of the experimental diets (g/100 g).

Ingredients	**G1/G4[a]**	**G2[b]**	**G3[c]**
Cellulose	5	-	-
Albumin	20	17.74	14.46
Corn Starch	39.75	26.51	8.31
Dextrinized starch	13.2	13.2	13.2
Sucrose	10	10	10
Soybean oil (mL)	7	5.63	5.43
Mineral mix	3.5	3.5	3.5
Vitamin mix	1	1	1
L-cystine	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25
Toasted white sorghum flour	-	-	43.55
Toasted tannin sorghum flour	-	21.87	-

[a] AIN-93M: Standard diet for rodents; [b] Standard diet for rodents + 100% substitution of cellulose for toasted tannin sorghum flour; [c] Standard diet for rodents + 100% substitution of cellulose for toasted white sorghum flour.

The control group (group 1) received only the AIN93-M diet for rodents (Reeves et al., 1993), and EDTA (Ethylenediaminetetraacetic acid) injections in order to minimize stress-related differences among groups. Groups 2 and 3 also received DMH injections but differed in their diets, with group 2 receiving tannin toasted sorghum flour and group 3 receiving white toasted sorghum flour. Lastly group 4, a positive control group, was fed the AIN93-M diet and treated with 1,2-dimethylhydrazine (DMH) for colon cancer induction. All diets began to be administered in week 1, concurrently with the EDTA or DMH injections, and continued throughout the 6-week experiment. The animals were induced to colon cancer by receiving four injections of DMH (40 mg/kg b.w.), once a week for four weeks (Bird, 1995). Following the 4-week cancer induction, additional 2 weeks were allowed for early colon cancer to develop as

aberrant crypt foci, which start to appear 2 weeks after DMH injection (Bird & Good, 2000). Weekly monitoring of the animals' weight and food intake was carried out during this period. Following a 12-hour fasting period, the animals were anesthetized with 100% isoflurane (Isoforine, Cristália®) and euthanized by cardiac puncture. Afterward, cecal feces, liver, colon, and blood were collected and stored at -80 °C.

7.2.3.2 Biochemical Analysis

The collected blood was centrifuged in test tubes at 4 °C and 2865 × *g* for 10 min to obtain serum. Renal function (creatinine, urea), hepatic function (alanine aminotransferase - ALT, aspartate aminotransferase - AST), and C-reactive protein (CRP) were analyzed in the Laboratory of Clinical Analysis of the Health Division of UFV, Viçosa, MG, Brazil.

7.2.3.3 Oxidative Stress and Antioxidant Defenses in Liver Tissue

Liver samples (200 mg) were macerated in microtubes, followed by the addition of 800 μL of 50 mM phosphate buffer (pH 7.4). The samples were then centrifuged at 10,000 × g and 4 °C for 15 minutes. The supernatants were collected and stored in an ultra-freezer until analysis. Malondialdehyde (MDA) in liver homogenates was determined using the thiobarbituric acid reactive substances (TBARS) assay (Kohn & Liversedge, 1944; Pyles, Stejskal, & Einzig, 1993). Total protein in the liver homogenate was determined by the Bradford method (Bradford, 1976). The results were expressed as mM MDA per milligram of protein (mM MDA/mg protein). Superoxide dismutase (SOD) activity was determined following the Marklund (1985) methodology, and the absorbance was measured at 570 nm. The results were expressed as U of SOD/mg protein, with one unit of SOD defined as the amount of enzyme that inhibits the oxidation rate of pyrogallol by 50%. Additionally, catalase activity (CAT) was measured using the Aebi et al. (1984) method, and nitric oxide concentration was determined in liver tissue following the Griess protocol (Hinson et al., 2002). Glutathione (GSH) analysis was performed according to Habig et al. (1976) and Cataneo et al. (2003). Briefly, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), and liver homogenate were added to a quartz cuvette to react. Readings were taken at 0, 30, 60, and 90 seconds at a wavelength of 340 nm. Results are expressed in nmol/min/mg of protein.

7.2.3.4 Histomorphometry Analysis of Colonic Tissues

Fragments of the distal colon were immersed in histological fixative (10% buffered formalin, pH 7.2) for 24 h, dehydrated in ethanol, diaphanized in xylene, and embedded in

paraffin. Histological sections (5 μm thick) were obtained using a rotary microtome. Subsequently, sections were stained with hematoxylin and eosin (H&E) for general microstructural analysis. In order to avoid analyzing the same area, one in each of the histological sections was collected. After the observation of sections, digital images were captured using a bright-field photomicroscope. Ten microscopic fields for each animal were sampled with a 10 × objective lenses so that a total area of 23,1 x 106 μm^2 was analyzed for each group. The crypt thickness, crypt height and circular and longitudinal muscle layers were measurement and expressed in μm. A total of 1000 goblet cells per group were randomly chosen to measure the area and the result was expressed in μm^2. The total area of inflammatory infiltrate in the mucous, submucosal, and muscular layers was evaluated and expressed in μm^2. All the measurements were carried out with the aid of the ImagePro-Plus application® version 4.5 (Media Cybernetics, Rockville USA).

7.2.3.5 Preneoplastic Colon Lesions: Aberrant Crypt Foci Assay (ACF)

Preneoplastic lesions in DMH-rats were analyzed by assessing the induced colon lesions and ACF. The colon was excised, opened longitudinally, washed with water, trapped in polystyrene plates, and fixed in 10% formaldehyde solution for 24 h. Then, colon was stained with 0.1% methylene blue in phosphate-saline buffer for 20 min. Then, the excess dye was washed with distilled water and the intestines were analyzed, with the mucosa side facing up, under a light microscope (10x) (Haven, São Paulo) and the ACF number was determined. The number of ACFs per focus was recorded and the ACFs were categorized by determining the observed frequency of aberrant crypts for each focus. The established categories included foci with up to three or fewer crypts (ACF ≤ 3) and foci with more than three crypts (ACF > 3) (Bird & Good, 2000).

7.2.3.6 Determination of Short-Chain Fatty Acids

The analysis of short-chain fatty acids was conducted following the methodology described by Cantu-Jungles et al. (2018). Briefly, 100 mg of feces were diluted into a mixture containing 50 mM 4-methyl-valeric acid, a internal standard for SCFA. An aliquot was injected into a gas chromatography equipment (GC-FID 7890 A; Agilent Technologies Inc.) on a fused silica capillary column (Nukon Supelco no. 40369-03A; Bellefonte, PA). The following conditions were used: injector temperature set at 230°C, initial oven temperature set at 100°C, and a temperature ramp of 8°C/min to 200°C with a 3-minute hold at the final temperature. Also, helium was utilized as the carrier gas at a flow rate of 0.75 ml/min. Quantification was

carried out by assessing the relative peak areas, utilizing external standards of acetate (A38S), propionate (A258), and butyrate (AC108111000), along with an internal standard of 4-methylvaleric acid (AAA1540506) sourced from Fisher Scientific (Hampton, NH).

7.2.3.7 Gut Microbiota Analysis

Gut microbiota analysis was performed according to Cantu-Jungles et al. (2021). Frozen fecal samples were thawed and diluted in MilliQ water, then centrifuged at 13,000rpm for 15 minutes, after which the supernatants were discarded. The DNA extraction of the resulting precipitates was automated using the QIAcube Connect instrument (Qiagen, Germantown, MD) with the QIAamp PowerFecal Pro DNA kit (Qiagen), following the manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3') (Walters et al., 2016). These primers contained common sequence tags (referred to as common sequence 1 and 2 [CS1 and CS2]). Each well was assigned a unique 10-base barcode using primer pairs obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; catalog no. 100-4876). Demultiplexing of reads was performed on the instrument. Library preparation, pooling, and sequencing were carried out at the University of Illinois at Chicago Genome Research Core (GRC) within the Research Resources Center (RRC).

The entire 16s rRNA amplicon dataset from this project consisted of 6 samples per group, for each of the four experimental groups (G1: EDTA + AIN-93M diet, G2: DMH + tannin sorghum, G3: DMH + white sorghum, and G4: DMH + AIN-93M diet) for a total of 24 samples.

7.2.3.8 Bioinformatics

Bioinformatic analysis was carried out using QIIME2 version 2022.8 (Bolyen et al., 2019). Demultiplexed, paired-end sequence files were imported using the import plugin and primers were removed from the 5' ends of the reads using cutadapt. Paired-end reads were filtered, denoised, merged, and chimeras were removed using dada2 denoise-paired (Callahan et al., 2016), yielding exact sequence variants (ESVs). Alignment of sequences and construction of a phylogenetic tree was carried out using phylogeny align-to-tree-mafft-fasttree. Taxonomy was assigned to ESVs using a naïve Bayes classifier trained on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database (McDonald et al., 2012), which was implemented using the plugin feature-classifier classify-sklearn (Bokulich et al., 2018).

A total of 692,275 reads were available in the raw, demultiplex reads from all 24 experimental samples of which 609,075 were retained after quality filtering using DADA2. To reduce the effect of sequencing depth on the results, samples were rarefied to 17,000 reads per sample using the diversity alpha-rarefaction plugin. This rarefaction depth did not influence the richness or evenness of the samples. A total of 447 ESVs were identified, with a minimum, median, and maximum frequency of 17,413, 26,998, and 30,303 ESVs per sample, respectively.

7.2.3.9 Statistical Analysis

Statistical analysis of variance (ANOVA) one-way followed by Newman-Keuls ($p < 0.05$) was used for the data in the GraphPrism® software.

7.3 RESULTS AND DISCUSSION

7.3.1 Proximate and phenolic composition of sorghum flours

The proximate composition of the white sorghum toasted flour (WSTF) and tannin-containing sorghum flour (TSTF) were analyzed to determine the macronutrient content for adjusting the rats' diet and providing isocaloric diets. The outcomes depicted in Figure 2 illustrate the comprehensive chromatogram displaying detected peaks in the QC samples through the UHPLC-ESI-QTOF platform, emphasizing the top 10 most predominant phenolic compounds in both white and tannin sorghum. Peaks excluded following the aforementioned filters were disregarded.

Table 3. Proximate and phenolic compounds composition of the toasted sorghum flours

Nutrients/Compounds	**WSTF**	**TSTF**
Moisture	6.29 ± 0.07^{a}	5.84 ± 0.08^{a}
Protein	12.92 ± 0.40^{a}	10.33 ± 0.09^{a}
Carbohydrate	75.54^{a}	75.33^{a}
Lipid	3.59 ± 0.03^{a}	3.48 ± 0.11^{a}
Resistant starch	7.60 ± 1.21^{a}	35.90 ± 2.53^{b}
Total dietary fiber	11.48 ± 0.04^{a}	22.86 ± 0.40^{b}
Ash	1.66 ± 0.01^{a}	2.21 ± 0.13^{b}
Total phenolics (mg GAE/g)	1.31 ± 0.04^{a}	45.34 ± 1.44^{b}

Proximate composition results are expressed in percentage of dry basis, except moisture content. Total phenolic compounds are expressed in mg of Gallic Acid Equivalent/g. WSTF = White sorghum toasted flour; TSTF = Tannin sorghum toasted flour. Same letters in a row means significantly equal, with ANOVA followed by Tukey ($p > 0.05$)

Both toasted sorghum flours have similar macronutrient contents, such as proteins, lipids, and carbohydrates, but with a significant difference in total dietary fiber, especially resistant starch. In fact, the high RS content of this tannin sorghum genotype, as demonstrated in previous studies (Silva et al., 2020; Martinez et al., 2021), highlights why BRS 305 has been investigated, especially when combined with its significantly higher free total phenolic content (45.34 ± 1.44 mg GAE/g) compared to white sorghum (1.31 ± 0.04 mg GAE/g). Even though the diets were calculated to have the same total dietary fiber content, the proportion of fiber types in each diet may vary. Thus, the AIN-93M diet of groups G1 and G4 contains cellulose as the main fiber, while sorghum flours offer fibers such as resistant starch, cellulose, arabinoxylan, among others. These distinct dietary fibers may influence the production of SCFA and the modulation of the intestinal microbiota (Yao, Chen & Lindermann, 2020; Cantu-Jungles et al., 2021) and, therefore, promote specific intestinal effects, including attenuation of inflammation (Akhtar et al., 2022).

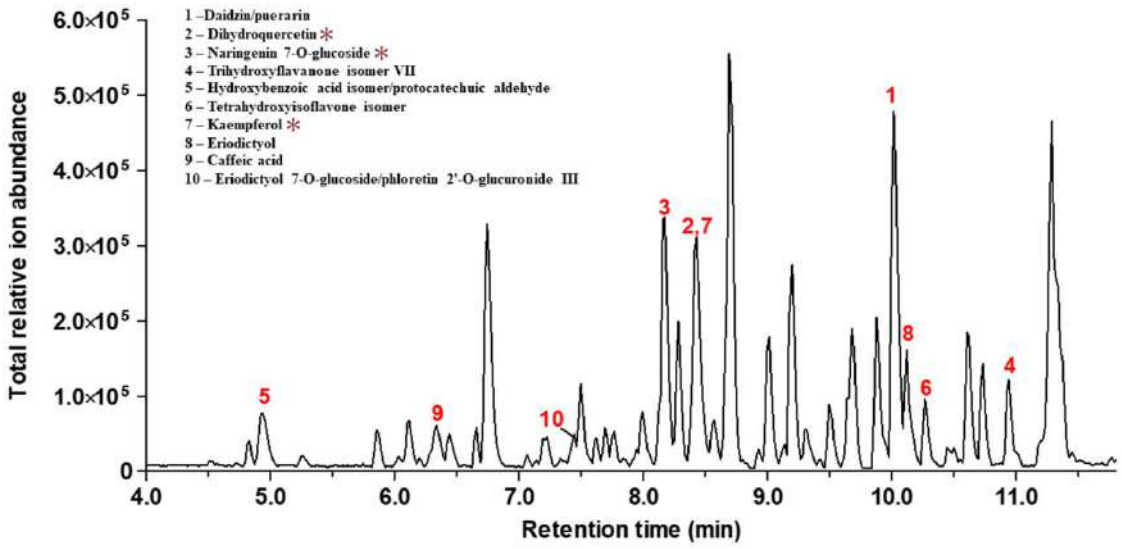

Figure 2. Chromatogram with peaks of phenolic compounds of higher relative abundance identified. * Means that the compound is present only in tannin sorghum extract.

Regarding phenolic composition, daidzein and/or puerarin, trihydroxyflavanone isomer VII, hydroxybenzoic acid and/or protocatechuic aldehyde, tetrahydroxyisoflavone isomer, eriodictyol, and caffeic acid are among the most abundant phenolic compounds in white sorghum flour. On the other hand, tannin sorghum flour contains, in addition to all these, dihydroquercetin, naringenin 7-O-glucoside, and kaempferol. It is observed that TSTF has a higher content of free phenolics, mainly due to the presence of tannins (Table 3). WSTF is richer in phenolic acids, while TSTF exhibits a higher relative abundance of flavonoids (Paes et al.,

2024). Although there is a notable difference in the phenolic content and composition between the two sorghum genotypes, the *in vivo* study by Silva et al. (2020) with the same toasted sorghum flours showed that the quantity may not be the most important factor, and the bioavailability of these compounds should also be considered.

In addition to the phenolic compounds presented in Figure 2, which are the free ones, there are also those bound to the cellular structure and may be released during digestion in the rat's intestine. D'Almeida et al. (2021) showed that caffeic acid ranked as one of the most abundant phenolic compounds in the bound extract from sorghum flour, followed by trans-ferulic acid and p-coumaric acid, with phenolic acids accounting for 78% of the phenolic compounds. The fate of these compounds once released during the utilization of fibers by the intestinal microbiota is still somewhat unclear, with studies showing their utilization by microorganisms, generating new products, their absorption, or even their excretion (Kasprzak-Drozd et al., 2021).

***In vivo* study**

7.3.2 Body Weight, Food Intake and Biochemical Analysis

Table 4 shows the initial and final weight of the animals in each group, as well as weight gain, food intake and biochemical analysis results. At the beginning of the experiment, the rats weighed an average of 160.99 ± 12.95 g and at the end they reached an average weight of 298.95 ± 18.23 g. There was no significant difference among the weight of the 4 groups during the experimental weeks. In addition to evaluating weight change, the animals' diet consumption was also checked, wherein there was no significant difference among groups. These results show that neither sorghum toasted flours nor DMH influenced the amount of food intake by the rats. This was expected, since the diets were balanced in terms of carbohydrates, proteins, lipids, and total fibers. Moreover, weight loss is more common in the progression of colon cancer stages due to the aggressiveness of the disease, leading to reduced food intake and consequent weight reduction (Venkatachalam et al., 2020).

Table 4. Body weight, food intake and serum biochemical results in control and toasted sorghum flour treated group.

Parameters	G1	G2	G3	G4
Initial weight (g)	161.15 ± 13.61[a]	161 ± 13.52[a]	160.73 ± 13.62[a]	161.06 ± 13.14[a]
Final weight (g)	302.10 ± 17.33[a]	294.70 ± 22.43[a]	301.80 ± 16.12[a]	297.20 ± 18.23[a]
Weight gain (g)	140.95 ± 13.21[a]	133.70 ± 14.20[a]	141.07 ± 9.93[a]	136.14 ± 23.15[a]
Food intake (g)	123.70 ± 11.64[a]	118.20 ± 9.87[a]	122.30 ± 12.34[a]	123.30 ± 10.16[a]

ALT (U/L)	25.10 ± 4.51[a]	29.20 ± 7.66[a]	26 ± 3.56[a]	28.10 ± 4.72[a]
AST (U/L)	91.30 ± 16.90[a]	91.22 ± 19.74[a]	86.40 ±10.64[a]	90.30 ± 7.97[a]
Creatinine (mg/dL)	0.37 ± 0.05[a]	0.36 ± 0.035[a]	0.35 ± 0.02[a]	0.37 ± 0.05[a]
Urea	37.20 ± 5.80[a]	33.10 ± 4.90[a]	33.50 ± 4.4[a]	36.40 ± 8.90[a]
CRP (mg/L)	< 0.20	< 0.20	< 0.20	< 0.20
MDA (μM/mg protein)	9.22 ± 3.19[a]	10.17 ± 2.39[a]	10.91 ± 4.30[a]	10.33 ± 4.20[a]
SOD (U SOD/mg protein)	0.44 ± 0.12[ab]	0.55 ± 0.19[a]	0.38 ± 0.16[ab]	0.28 ± 0.09[b]
CAT (μmol/ml)	0.30 ± 0.09[a]	0.32 ± 0.11[a]	0.42 ± 0.36[a]	0.37 ± 0.23[a]
GSH (μM/mg protein)	38.93 ± 6.91[a]	43.10 ± 5.91[a]	37.98 ± 5.19[ab]	34.09 ± 1.92[b]

G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH. ALT = Alanine aminotransferase; AST = aspartate aminotransferase; CRP = C-reactive Protein; MDA = Malondialdehyde is expressed as μM/mg of protein; SOD = superoxide dismutase is expressed as U SOD/mg of protein; CAT = Catalase activity; GSH = Glutathione. Same letter in the row means not significantly different. Analyzed using one-way ANOVA with Newman-Keuls test ($P < 0.05$).

Regarding to the biochemical analyses conducted, it can be observed that the blood concentrations of AST and ALT showed no significant difference among the 4 groups, as well as creatinine, urea, and C-reactive protein. This suggests that the induction of colon cancer by DMH for only 4 weeks, which was performed to monitor the initial stages of carcinogenesis, did not cause major hepatic or renal alterations compared to the control. Metabolism of DMH in the liver to various compounds, including azomethane, azoxymethane, and methylazoxymethanol (MAM), can influence liver function, highlighting the significance of assessing ALT and AST levels (Venkatachalan et al., 2020). However, significant differences in the levels of these enzymes may not have been observed due to the short duration of the experiment.

Additionally, the MDA, SOD and CAT analysis were performed to assess the oxidative stress and oxidative defenses of the liver. There was no significative difference in the MDA and CAT results among groups. In the SOD analyses, G2 showed higher activity of this enzyme in the liver, significantly different from the positive control (G4), which showed the lowest activity. The initial defense against free radicals is initiated by SOD, which eliminates the superoxide radical (O_2 .−) by speeding up its conversion to hydrogen peroxide (H_2O_2). Subsequently, CAT in the peroxisomes converts H_2O_2 into water and O_2, aiding in the elimination of H_2O_2 generated by oxidase activity within these organelles (Sangeetha et al., 2010; Dincer et al., 2006). Therefore, an improvement in SOD activity in the liver of rats in G2 may indicate that the diet with tannin-rich sorghum flour enhanced the defense against oxidative

stress caused by DMH, while in G4, which received a standard diet and DMH, the enzymatic activity was lower despite the damage by DMH. Devasena et al., 2002 suggested that a reduced SOD activity in the liver, possibly resulting from oxidative stress induced by DMH metabolism, indicate the liver's susceptibility to oxidative damage during colon carcinogenesis.

The glutathione (GSH) result indicates that the group receiving sorghum flour with tannins (G2) exhibited GSH activity similar to the negative control (G1) and statistically differed from the group receiving DMH without sorghum flour (G4). On the other hand, the group receiving white sorghum flour (G3) did not significantly differ from any of the other groups. GSH undergoes spontaneous reactions or is catalyzed by glutathione-S-transferases to interact with various activated carcinogens, facilitating their elimination and reducing their toxicity (Mandel et al., 1993). In addition, Manigandan et al. (2014) found that dihydroquercetin, present in tannin sorghum, was able to enhance the activity of SOD, CAT, and GSH enzymes in DMH-induced colon cancer mice, indicating the action of this compound on oxidative stress. Thus, the reduced antioxidant defense, as evidenced by decreased SOD and GSH levels, might have impacted the ACF count, as observed with the higher number of ACF ≤ 3 in G4, as shown below.

7.3.3 Histomorphometry of Colon

Histological analyses of the rats' colon, shown in Table 5, reveal that there was no significant difference between the parameters of intestinal crypt thickness and height, as well as the count of goblet cells among the groups. The reduction of goblet cells, influenced by the increase of ACF (Venkatachalam et al., 2016), and the potential alteration of intestinal mucosa by affecting mucinase activity due to exposure to carcinogens (Venkatachalam et al., 2020) are common in animals treated with DMH, although not observed in this study.

Table 5. Crypts measurements and goblet cells of the colon tissue.

Parameters (μm)	**G1**	**G2**	**G3**	**G4**
Crypt height	144.20 ± 10.50[a]	153.20 ± 11.90[a]	147.60 ± 16.80[a]	147.80 ± 20.60[a]
Crypt thickness	21.41 ± 2.10[a]	22.21 ± 1.10[a]	22.48 ± 2.80[a]	22.44 ± 3.10[a]
Goblet cells	35.93 ± 7.20[a]	34.81 ± 5.90[a]	36.66 ± 9.30[a]	38.99 ± 6.50[a]

G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH. Analyzed using one-way ANOVA and Newman-Keuls test ($P < 0.05$). Same letters mean that is not significantly different.

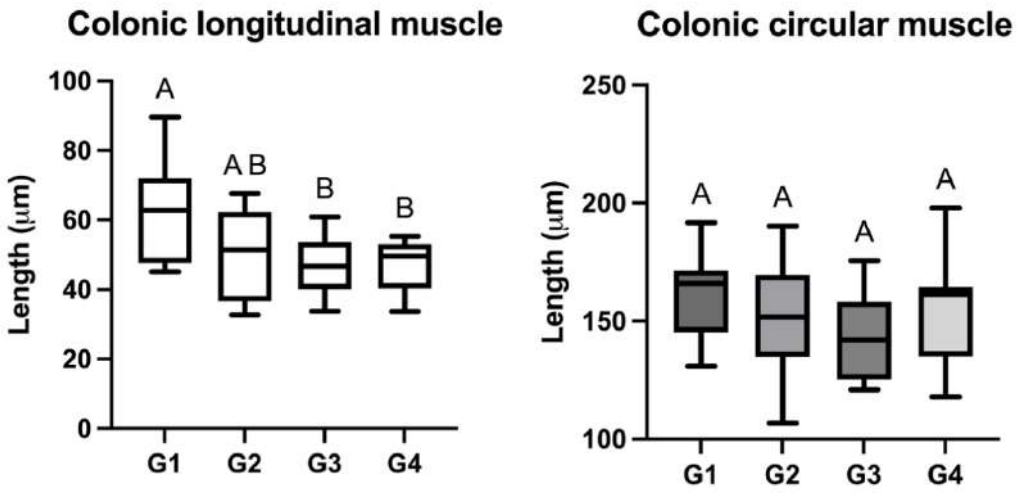

Figure 3. Transverse and circular colonic muscle length measurement. Analyzed using one-way ANOVA and Newman-Keuls test ($P < 0.05$). Same letters mean that is not significantly different. G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH.

The measurements of the longitudinal and circular muscles of the colon are presented in Figure 3. The length of the circular muscle did not show a significant difference among the groups, while there was a significant difference in the length of the longitudinal muscle. A decrease was observed when comparing the negative control group G1 with the group that received WSTF (G3) and the positive control (G4). The inflammation in the colon can lead to a decrease in the area of the longitudinal muscle (Blennerhassett et al., 1992). Chronic inflammation of the intestinal wall can result in alterations to the structure and function of the colon muscle, including changes in muscle contractility and colon motility (Snape Jr et al., 1991; Vermillion et al., 1993; Tanović et al., 2006). Thus, this result suggests that DMH may have caused a reduction of longitudinal muscle length.

7.3.4 Preneoplastic Colon Lesions: Aberrant Crypt Foci Assay (ACF)

Preneoplastic lesions, identified as aberrant crypt foci (ACF), exhibit phenotypic alterations induced by carcinogens but lack crucial characteristics of fully developed tumor cells (Venkatachalam et al., 2020). These lesions were initially recognized topographically as the earliest observable abnormalities in the colons of rodents exposed to carcinogens. Figures 4B-C depict ACF found in the rats' colons and a comparison with normal crypts (Figure 4A). Figures 4D-E present the number of foci and crypts per foci. As expected, there was a significant difference in the number of ACF between the EDTA-treated (G1) and the DMH-treated groups

(G2, G3, and G4). Additionally, the high incidence of ACF in DMH-treated groups indicates induced carcinogenesis.

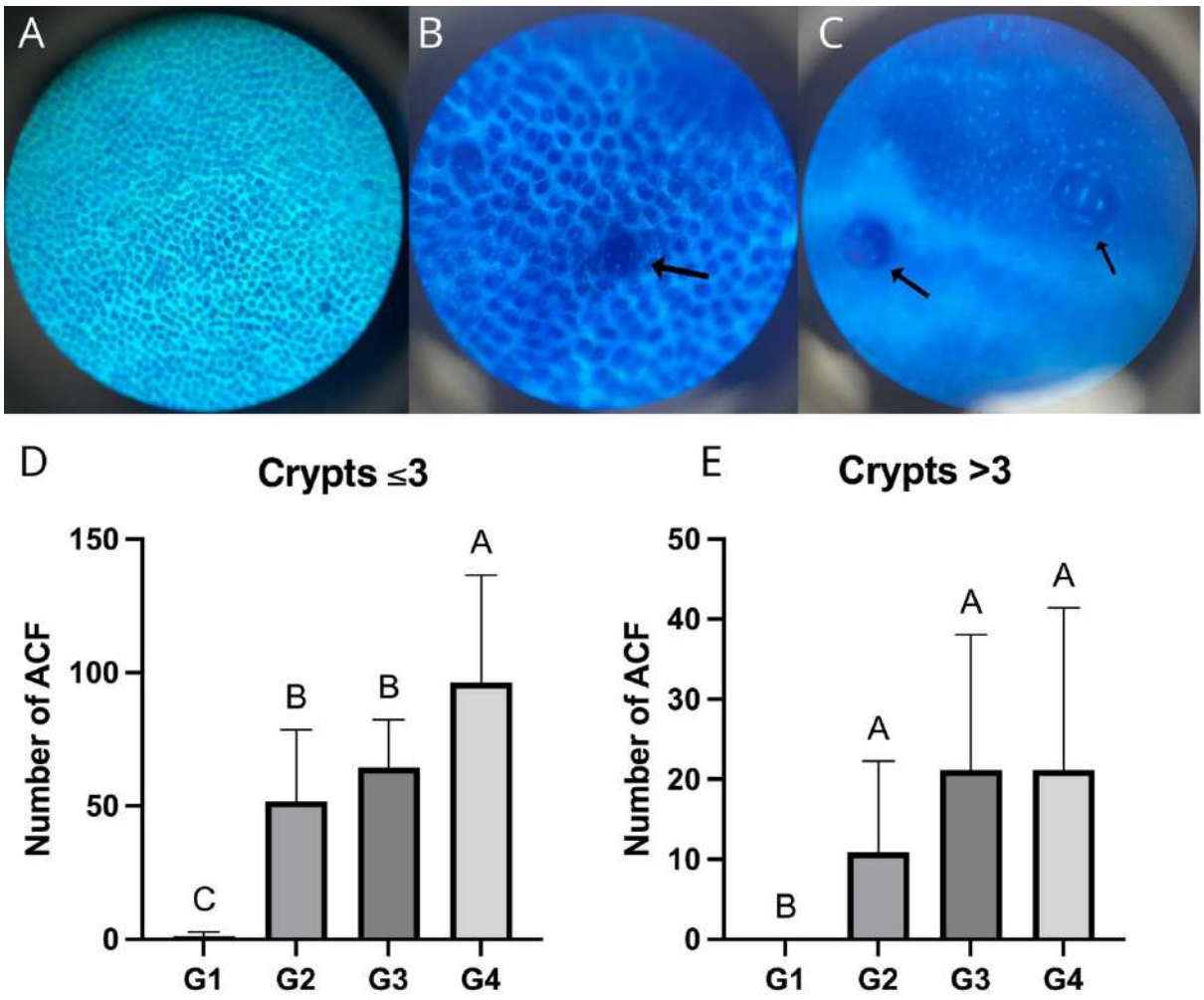

Figure 4. Topography view of the colon mucosa by microscopy; A) non-altered colonic cripts; B-C) Aberrant Crypt Foci-methylene blue stained. Microscopic magnitude: 10X. D) number of ACF less or equal to 3 ($P < 0.05$); E) number of ACF more than 3 ($P < 0.05$). G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH.

Groups G2 and G3, which received treatment with toasted sorghum flours, exhibited a lower count of ACF compared to G4, which received DMH and a standard diet, suggesting a potential preventive effect of sorghum on the initiation of the carcinogenic process. Conversely, the prediction of malignant transformation of these ACF is more closely associated with crypt multiplicity than with the number of foci (Prado-Silva et al., 2014; Renehan et al., 2002). Therefore, groups that received DMH presented numbers of foci with more than 3 crypts statistically similar.

The metabolic process of DMH engages multiple xenobiotic-metabolizing enzymes that activate the procarcinogen. Cytochrome P450 (CYP) is an enzyme involved in the activation of

DMH in the rat liver (Sohn et al., 1991). Previous studies have shown that cytochrome P450 1B1 was a predicted target of the phenolic compounds kaempferol and eriodictyol present in sorghum flours (Paes et al., 2024), which could explain the attenuating action on ACF formation in groups G2 and G3. Furthermore, dihydroquercetin, found in tannin sorghum and also known as taxifolin, has demonstrated efficacy in reducing the incidence of ACF in mice treated with this phenolic compound in the study by Manigandan et al. (2014). This highlights the efficacy of dihydroquercetin in mitigating early-stage cancerous lesions in the colon.

Additionally, dietary fibers can also be an important factor in these results since they are capable of exerting a protective effect against the development of ACF by DMH (do Amaral et al., 2021; Femia et al., 2009). The study by Prado-Silva et al. (2014) demonstrated that diets containing resistant starch type 2 significantly reduced the formation of ACF and aberrant crypts by 60 to 80%, along with a 98 to 100% reduction in mucin-depleted ACF, indicating a protective effect against oxidative stress in rats subjected to DMH-induced colon carcinogenesis.

Not only phenolic compounds and dietary fibers may be involved in the prevention of aberrant crypt formation, but also other bioactive compounds, such as bioactive peptides. The study by Bianco-Gomes et al. (2022) showed that dry heat preserved the protein content in sorghum flour, and its peptides, including those from white sorghum (Castro-Jácome et al., 2020), demonstrated bioactivity *in vitro*. This could be a hypothesis to explain the results for G3 consuming white sorghum, although more investigation is needed to test the bioactivity of the peptides. Thus, both sorghum flours may contribute to the reduction of ACF through different mechanisms or through the same mechanism, such as phenolic acids, for example, which are common to both and have a lower molecular weight for absorption.

7.3.5 Quantification of Short-Chain Fatty Acids

Short-chain fatty acids are products of intestinal microbiota fermentation, mainly acetate, propionate, and butyrate, contributing to the prevention of pathologies through various mechanisms, including their metabolism by colonocytes (Topping & Clifton, 2001). The results of the concentrations of these 3 main SCFAs are presented in Figure 5. It can be observed that the group that received treatment with toasted white sorghum flour (G3) showed a higher concentration of acetate (36.12 ± 7.01 mM), different from the negative control group (G1), the toasted tannin sorghum flour (G2), and the positive control (G4). Regarding propionate, G3 also presented higher concentration than the negative control group (G1) (10.51 ± 1.43 mM and 8.29 ± 1.82 mM, respectively), however it was equal to G2 and G4. Lastly, butyrate

concentration was higher for G2 (3.67 ± 0.69 mM), comparable with G4 that did not receive DMH, and with a difference from the positive control (G4), which showed the lowest concentration (2.80 ± 0.27 mM).

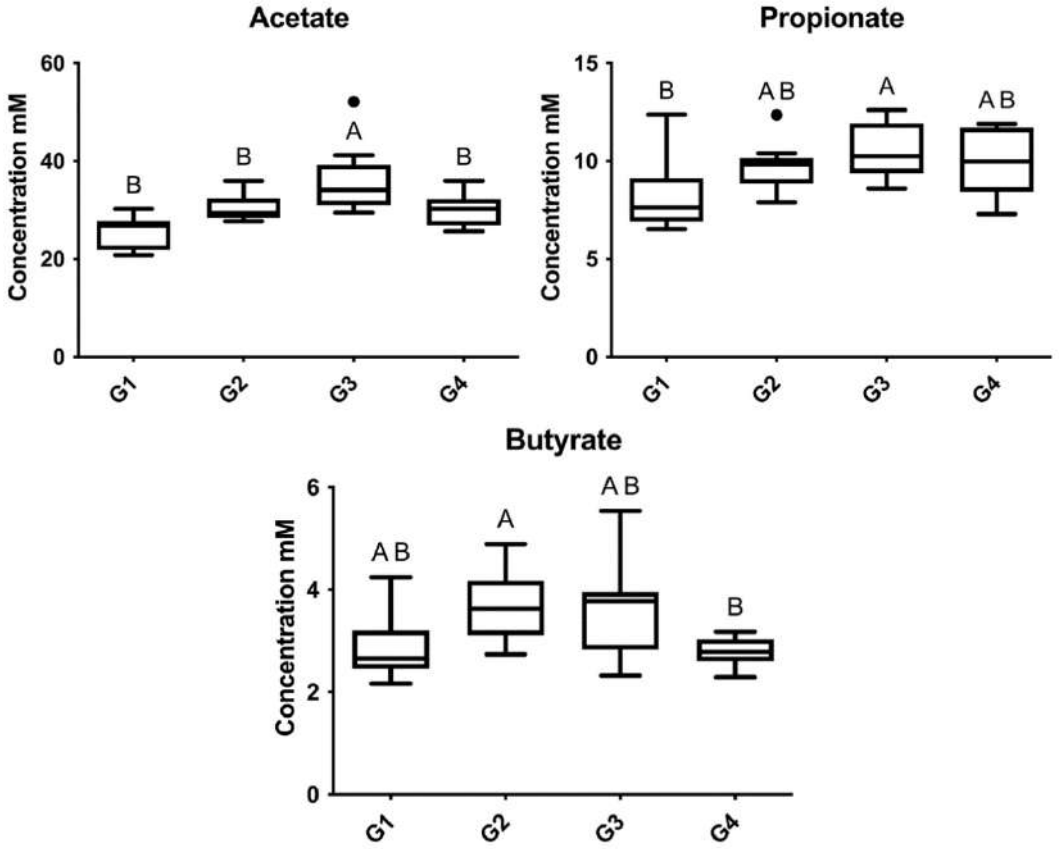

Figure 5. Short-chain fatty acids concentrations (propionate, butyrate, and acetate) in all groups. *P* value < 0.05. G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH.

Sorghum processed in various forms has been shown to have the ability to alter the production of SCFAs in different experimental designs studies (Martinez et al., 2023; Pelpolage et al., 2019). This is due to the dietary fibers present in this cereal, which serve as substrates for microbiota bacteria, while phenolic compounds also influence such modulation. However, even though the white sorghum used has a lower content of total phenolics and total fibers compared to tannin sorghum (Table 3), G3 showed SCFA concentrations statistically similar to G2 for propionate and butyrate. Additionally, although resistant starch has previously been linked to butyrate production (Teichmann & Cockburn, 2021), the high content of resistant starch in tannin sorghum flour did not impact the production of SCFAs, specifically butyrate, as expected.

Furthermore, SCFAs decrease luminal pH, which helps prevent colon cancer (Blachier et al., 2017; Gamet et al., 1992), indicating the possible action observed in the lower count of ACF in G2 and G3 compared to the positive control (G4). As butyrate acts as a fuel, providing energy to colonocytes (Topping & Clifton, 2001), its lower concentration in G4 can help explain the high number of ACF in this group. Additionally, the composition of the microbiota, i.e., the presence or absence of bacteria that utilize resistant starch, can help understand the SCFA data.

7.3.6 Gut Microbiota

Among the commonly employed diversity metrics, alpha diversity assesses the diversity within individual samples, while beta diversity quantifies the dissimilarity or similarity between two distinct microbial communities (Tipton et al., 2019; Cadotte et al., 2010; Lozupone & Knight, 2008). The results of alpha diversity (Figure 6) show a significant difference in Shannon entropy between the control group (G1) and the tannin sorghum group (G2). Shannon entropy, also known as the Shannon index, incorporates microbial data on both richness and evenness based on species (Lozupone & Knight, 2008; Shannon & Weaver, 1949). However, while the Shannon index accounts for species richness, it does not necessarily measure the same quantities as richness itself, making the determination of Chao1 additionally interesting for understanding the microbiota (Roswell et al., 2021). There was no significant difference in the values of richness (Chao1) and observed features between the groups; the latter represents the actual number of taxa observed in the samples.

Cantu-Jungles & Hamaker (2023) note that the lack of significant difference observed between groups regarding richness may be expected, indicating that an increase in the number of species in *in vivo* studies is rarely seen, with reduction being more common. This means that an increase in species diversity does not necessarily translate into health benefits for the rats – as pathogens can increase the richness, for example – highlighting the importance of considering the types of microorganisms present and their abundances.

Additionally, there was a significant difference in Faith phylogenetic diversity among the groups, where the groups treated with toasted sorghum flours (G2 and G3) showed higher values compared to both negative and positive controls (G1 and G4, respectively). Phylogenetic diversity (PD) is a qualitative measure based on divergence, which adds up the total length of branches in a phylogenetic tree leading to each member of a community (Lozupone & Knight, 2008). Therefore, a higher value of Faith PD for groups treated with sorghum (G2 and G3) indicates greater divergence among the taxa within their communities, suggesting that these

communities contain a wider range of evolutionary lineages or branches, reflecting greater phylogenetic novelty or diversity.

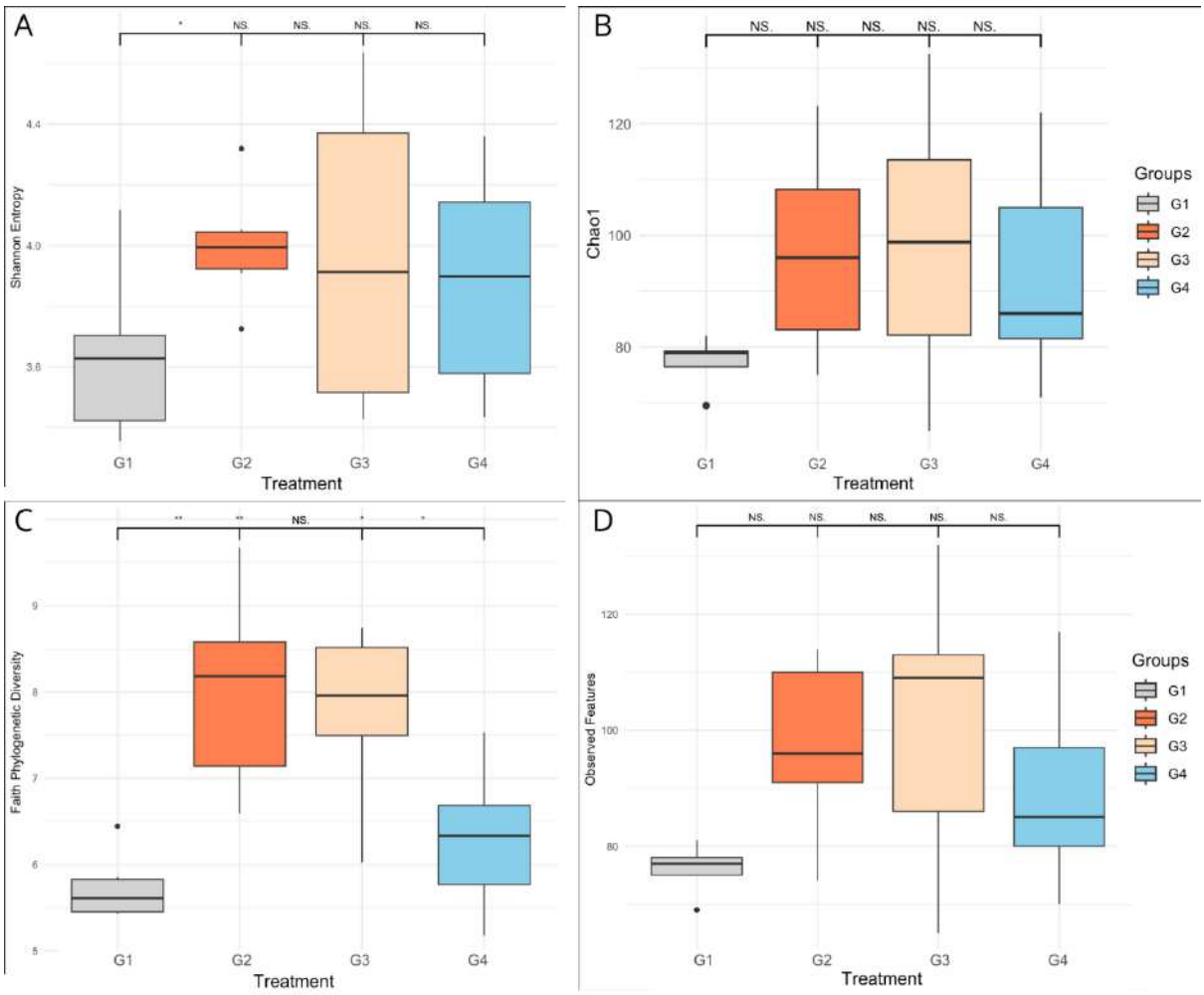

Figure 6. Alpha-diversity plots of A) Shannon entropy, B) Evenness, C) Faith phylogenetic diversity, and D) Observed features of the groups' gut microbiota. G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH.

The taxa bar plots of the phyla, family, and genus of the intestinal microbiota of the groups are presented in Figure 7. Regarding the phyla, Firmicutes was predominant, followed by Actinobacteria. There was a slight increase in the phylum Actinobacteria in the groups treated with DMH (G2, G3, and G4) compared to the group treated with EDTA (G1). Other phyla, such as Bacteroidota and Patescibacteria, were present but in lower abundance. These results differ from those found by Zhu et al. (2020), where Firmicutes and Actinobacteria were significantly less abundant in healthy rats compared to Bacteroidota. This divergence may be due to the characteristics of the animals, such as origin, breeding, diet, among other factors.

Regarding family, there was higher relative abundance of *Bifidobacteriaceae* – more pronounced in the tannin sorghum-treated group (G2) – and *Peptostreptococcaceae*, and a lower abundance in *Erysipelotrichaceae* in the groups treated with DMH (G2, G3, and G4) compared to the control treated with EDTA (G1). The comparison between the negative (G1) and positive controls (G4) highlights that DMH administration induced a change in the gut microbiota. Similarly to our results, the investigation by Silva-Reis et al. (2022) also found a higher abundance in the family *Peptostreptococcaceae* in the DMH-treated groups compared to the EDTA control.

Comparing the sorghum diets (G2 and G3) with the standard diet (G1 and G4), a higher relative abundance *Clostridiaceae* and *Lactobacilaceae* is observed in groups G2 and G3, while the group treated with tannin sorghum (G2) showed an increased abundance in *Turicibacteriaceae*. Silva-Reis et al. (2022) also found an increase in *Clostridiaceae* in the DMH-treated group, while *Lactobacilaceae* increased in the control. Despite the presence of RS being correlated with *Ruminococcaceae* due to their capacity to ferment this fiber (Teichmann & Cockburn, 2021), there was actually a decrease in relative abundance in the group treated with tannin sorghum (G2) compared to the controls (G1 and G4).

Regarding the genera found in the gut microbiota, our findings showed a reduction in *Allobaculum* in the DMH-treated groups compared to the EDTA-treated group. Heatmap analysis of relative abundances (Figure 7D) showed that some genera were associated with the consumption of toasted sorghum flours, such as the genera *Mogibacterium*, *Lactobacillus*, and *Bifidobacterium*. Tannin sorghum group (G2) had an increased relative abundance in *Turicibacter* and a decreased in *Blautia*, *Collinsella*, *Coprobacillus*, and *Dorea*. Several genera were associated with the standard diet of the G1 and G4 groups, such as *Clostridium*, *Dorea*, and also *Streptococcus* (Figure 7D). Meanwhile, *Enterococcus* was more abundant in G4, which received DMH and the standard diet..

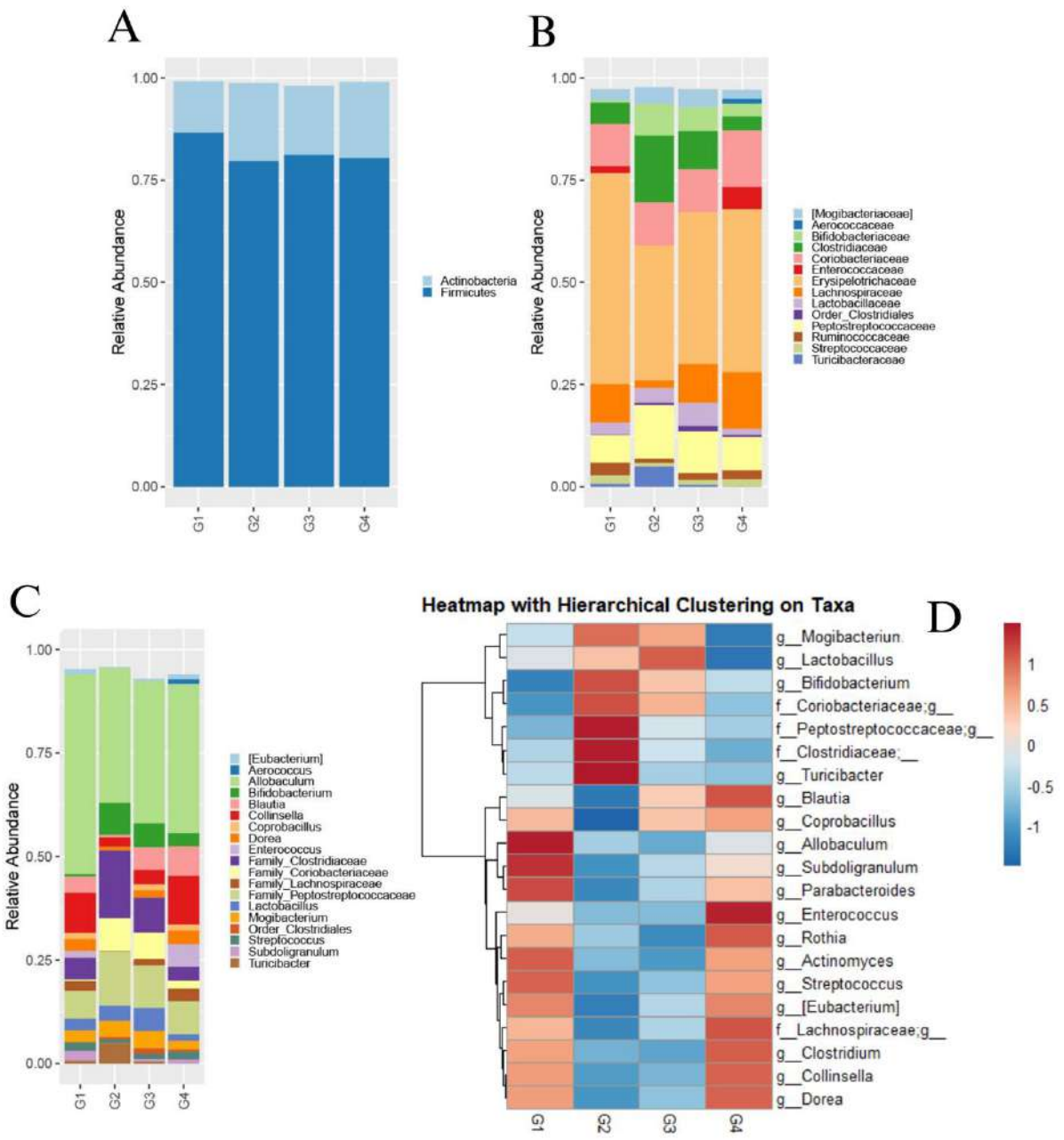

Figure 7. Taxa bar plots of gut microbiota sequencing of A) Phyla, B) Family, and C) Genera. D) Heatmap of gut microbiota genera per group. G1: AIN-93M diet + EDTA; G2: Tannin sorghum toasted flour + DMH; G3: White sorghum toasted flour + DMH; G4: AIN-93M diet + DMH.

Firstly, it is important to note that in studies of gut microbiota and intestinal health, there is a greater abundance of data regarding the association of certain bacterial groups with diseased animals or individuals, rather than clear cause-and-effect relationships, owing to the ecological complexity of the gut microbiota. Analyzing the associations of these microorganisms in studies with DMH-induced colon cancer, a higher abundance of *Blautia* and *Allobaculum* was found in groups with tumors compared to the health group (Zhu et al., 2014). In another study, *Blautia* had been associated with T cell infiltration in colorectal cancer (Luu et al., 2023). On the other

hand, *Allobaculum* is a beneficial genus being linked to SCFA production (Zhang et al., 2012) and, thus, its observed reduction in the DMH-treated groups (G2, G3, G4) can be caused by the carcinogen effect, opposing the results from Zhu et al. (2014).

The specie *Ruminococcus bromii*, belonging to the family *Ruminococcaceae*, and some species of the genus *Bifidobacterium*, such as *B. adolescentis*, are well known to degrade RS (Ze et al., 2012; Duranti et al., 2014; Teichmann & Cockburn, 2021). Therefore, the results show that while there was a decrease in *Ruminococcaceae* in G2, which had a higher content of RS, there was also a higher relative abundance of *Bifidobacterium*, suggesting the degradation and utilization of RS by this genus. Furthermore, the presence of *Bifidobacterium* and *Lactobacillus* is associated to health effects by hindering the colonization of pathogenic bacteria, modulating intestinal immunity, and maintaining the integrity of the intestinal barrier (Loke et al., 2020; Zhu et al., 2014).

7.4 CONCLUSION

In conclusion, our study demonstrated the potential protective effects of toasted sorghum flours against early-stage DMH-induced colon cancer in rats. On one hand, toasted tannin sorghum (BRS 305) flour was effective in increasing the activity of superoxide dismutase (SOD) and glutathione (GSH) enzymes in the liver, which may contribute to the observed reduction in aberrant crypt foci (ACF) formation. On the other hand, toasted white sorghum (BRS 501) flour demonstrated the ability to modulate the production of SCFA, specifically acetate and propionate known for their anti-inflammatory and anti-carcinogenic effects. Thus, both toasted sorghum flours showed promise in modulating key markers of colon carcinogenesis. Our findings support the potential of toasted white and tannin sorghum flours as dietary interventions for reducing the risk of colon cancer initiation. However, further research is needed to elucidate the underlying mechanisms and long-term impacts of sorghum consumption on colon health.

CONFLICT OF INTEREST

No conflict of interest

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8. CHAPTER 4: *IN VITRO* FECAL FERMENTATION

INFLUENCE OF SORGHUM PHENOLIC COMPOUNDS AND DIETARY FIBERS ON GUT MICROBIOTA AND SHORT-CHAIN FATTY ACID PRODUCTION

ABSTRACT

Sorghum, a cereal of growing interest for its potential health benefits, is rich in a combination of phenolic compounds and dietary fibers. This study aimed to assess how dietary fibers and phenolic compounds from toasted sorghum flours influence the gut microbiota profile and its production of short-chain fatty acids (SCFA). Two sorghum genotypes (BRS 501 white, without tannins, and BRS 305 tannin sorghum) underwent direct dry heat toasting. Subsequently, the flours were subjected to an *in vitro* gastrointestinal phase followed by an *in vitro* fecal fermentation model. The results revealed a consistent increase in acetate, propionate, and butyrate concentrations from time 0 to 24 hours. Regarding to acetate, the highest concentration was observed in the samples white sorghum phenolic extracts (WSE) and tannin sorghum phenolic extracts (TSE) with fructooligosaccharides (FOS) (WSE + FOS = 37.55 mM ± 4.03; TSE + FOS = 38.02 mM ± 0.66), attributed to the presence of FOS, with a slight enhancement by phenolic compounds. This enhancement was particularly evident in propionate, where the highest concentration was found in WSE + FOS (29.59 mM ± 1.53), followed by TSE + FOS (19.89 mM ± 0.61), compared to FOS alone (17.33 mM ± 0.76). When comparing the results from WSD and TSD, no significant difference in the concentration of all SCFAs was observed. Additionally, for both WSD and TSD alone, when compared to combination with their phenolic extracts, an increase in the concentration of acetate and propionate was observed in the WSD + WSE sample, and no significant difference was noted in TSD + TSE. Alpha diversity analysis revealed that FOS, combined with phenolic extracts, reduced gut microbiota diversity, whereas sorghum flours-maintained diversity levels comparable to the control. Beta diversity showed clear separations in microbiota composition between different treatments, particularly those involving WSE. Taxonomic analysis showed that many SCFA-producer genera were favored, such as *Bacteroides*, *Enterocloster*, *Blautia*, and *Anaerostipes*. Also, it highlighted that sorghum flours and their phenolic extracts modulate gut microbiota in distinct ways, with specific genera like *Megamonas* being favored by WSE. These findings suggest that, despite tannin sorghum flour having a higher dietary fiber content than white sorghum flour, this factor did not influence SCFA production as much as addition of phenolic extracts. Additionally, both toasted sorghum

flours and their phenolic-rich extracts can beneficially modulate gut microbiota and SCFA production, indicating a gut health potential.

8.1 INTRODUCTION

The gut microbial communities are influenced by various factors, including an individual genetic background, physiological status, environmental conditions (such as living conditions and dietary habits), and medications (Shah et al., 2020). Microbiota-host interactions influence not only the digestive system of the host but also play a crucial role in various immunological and physiological responses throughout the body (Sommer & Bäckhed, 2013). Additionally, not only has the composition of the microbiome been linked to health benefits, but also its metabolites, such as short-chain fatty acids (SCFA). SCFAs derived from the gut microbiota promote intestinal health in various ways, modulating metabolic pathways involved in insulin resistance, obesity (Portincasa et al., 2022), cardiovascular diseases (Overby & Fergunson, 2021), and aiding in the prevention of colorectal cancer (Hou et al., 2022).

Diet is a crucial factor influencing the composition of the colonic microbiota. In this sense, dietary fibers are very important, passing through the upper part of the digestive system and reaching the colon, where they are utilized by the gut microbiota (Ye et al., 2022). It has been demonstrated that it is possible to manipulate this gut microbiota to bring benefits to the hosts, where different types of dietary fibers from various sources favor the growth of different groups of bacteria (Scott et al., 2015; Li et al., 2017). Furthermore, the SCFAs acetate, propionate, and butyrate derived from gut microbiota present diverse behavior depending on the dietary fibers, from low to high specificity to microbiota bacteria utilization (Cantu-Jungles et al., 2021).

Sorghum is a cereal that contains dietary fibers such as arabinoxylans, cellulose, and resistant starch (Tuncil et al., 2018), in addition to having a quite interesting profile of phenolic compounds (Awika & Rooney, 2004; Girard & Awika, 2018; Paes et al., 2024). Previous studies have shown that different fractions of sorghum can modulate gut microbiota and short-chain fatty acid production in *in vitro* fecal fermentation (Tuncil et al., 2018; Ashley et al., 2019). The study by Tuncil et al. (2018) showed that sorghum bran produced more total SCFAs than corn bran, but less than wheat and rice brans. Additionally, sorghum bran, as well as brans from these other cereals, increased the relative abundance of operational taxonomic units (OTUs) related to the genus *Bacteroides*. Sorghum bran polyphenols, proanthocyanidins and 3-deoxyanthocyanins included, also have proven to influence gut microbiota in the study by Ashley et al. (2019), increasing the populations of *Bifidobacterium* and *Lactobacillus*, and

independently promoted the growth of *Roseburia* and *Prevotella*, when combined with fructooligosaccharides (FOS).

The tannin sorghum flour BRS 305 was able to increase propionate production in rats on a high-fat and high-glucose diet (Martinez et al., 2023), as well as modulate the intestinal microbiota (de Sousa et al., 2019). Several phenolic compounds present in sorghum have already been shown to be utilized by the gut microbiota and are capable of modulating it, such as ferulic acid (Tian et al., 2022), naringenin (Wu et al., 2024), and kaempferol (Qu et al., 2021). Additionally, tannin extracts from various sources have demonstrated the ability to modulate gut microbiota and consequently influence SCFA production both *in vitro* (Molino et al., 2021) and in clinical trials (Molino et al., 2022). Molino et al. (2022) demonstrated in their study that tannin supplementation led to a significant increase in microbial diversity, including the growth of the taxa *Ruminococcus bicirculans*, *Faecalibacterium prausnitzii*, and *Lachnospiraceae*, which are associated with increased production of short-chain fatty acids (SCFAs).

Additionally, aiming at the application of sorghum in products, toasted sorghum flours – processed with dry heat to maintain the characteristics of phenolic composition and dietary fiber content – have shown potential health benefits both *in vitro* (Paes et al., 2024) and *in vivo* against oxidative stress (Silva et al., 2020). However, there is still a lack of data on the influence of white and tannin sorghum flours on the human intestinal microbiota. Therefore, the present study aimed to investigate the effect of toasted white and tannin sorghum flours and their respective phenolic-rich extracts on SCFA production in *in vitro* fecal fermentation.

8.2 MATERIALS AND METHODS

8.2.1 Toasted sorghum flours and phenolic-rich extracts preparation

The white sorghum (BRS 501) and tannin sorghum (BRS 305) flours were obtained from the Embrapa germplasm bank located in Sete Lagoas, Minas Gerais, Brazil, and were prepared according to Silva et al. (2020). Briefly, after particle size standardization, they were subjected to direct heat toasting in a pan for 6 minutes at 200 ºC. The preparation of phenolic extracts was conducted according to Paes et al. (2024), where the toasted sorghum flours were mixed at a ratio of 1:10 (w/v) with 70% (v/v) alcohol and shaken for 2 hours. Subsequently, the extracts were subjected to a rotatory evaporator (Yamato Scientific America, USA) and lyophilized (Virtis Benchtop K, USA). The toasted sorghum flours underwent the *in vitro* digestion step as described in Method A in the study by Tuncil et al. (2018). The digested flours and the sorghum extracts lyophilized were kept refrigerated at – 4 ºC until application.

8.2.2 Chemical characterization of toasted sorghum flours

Total soluble and insoluble fiber content was measured using the gravimetric-enzymatic method described by Lee, Prosky, and DeVries (1992). Resistant starch (RS) content in the sorghum flour samples was quantified using a resistant starch assay kit from Megazyme (AACC method 32-40). The quantification of total extractable phenolic compounds was performed using the Folin-Ciocalteu method, and individual phenolic compounds in the flour were identified by UPLC-MS^E, following the parameters described by Paes et al. (2024). Data processing was carried out with Progenesis QI (Waters), and identification and annotation were based on standard parameters such as isotope distribution of neutral mass, exact mass, retention time, and MS/MS fragment spectra. PubChem was used for non-targeted annotation through MetaScope, an integrated search tool. Additional parameters, prioritized by importance, included exact mass error (<10 ppm), isotopic similarity (>80%), score (>30), and fragmentation score. Unknown compounds were considered tentatively identified only if they were present in all three technical replicates (3/3), with each vial containing a pool of three true replicates and CV <30%. For annotating features corresponding to phenolic compounds, a tailored database compiled from PubChem and Phenol Explorer was used. Compounds found in the blank samples, with ionic relative abundance below 5,000, isotopic similarity below 80%, or fragmentation data inconsistent with literature and/or databases, were screened and excluded.

8.2.3 Fecal Sample Collection

Fecal samples were acquired from three individuals reported to be in good health, adhering to their regular diets, and abstaining from antibiotics for the previous six months. The donors consisted of two males and one female, aged between 29 and 34 years old, all falling within the normal body mass index (BMI) range (18.5 to 25 kg/m^2). Samples were collected in sterile plastic tubes, promptly sealed, and placed on ice before being transferred to an anaerobic chamber (BactronEZ Anaerobic Chamber; Shel Lab, Cornelius, OR) for *in vitro* fecal fermentation. All samples underwent fermentation within an hour of collection. Ethical approval for human stool collection and use was obtained from the Institutional Review Board at Purdue University (IRB protocol no. 1510016635).

8.2.4 *In vitro* Fecal Fermentation Procedures

In triplicate, the *in vitro* fermentations of digested sorghum flours, sorghum phenolic extracts combined with fructo-oligosaccharides (FOS), and a blank control (without fiber addition) were carried out according to the procedure outlined in the study by Cantu-Jungles et

al. (2018). Initially, carbonate-phosphate buffer was prepared and sterilized via autoclaving at 121°C for 20 minutes. After cooling to room temperature (25 °C), the buffer underwent oxygen removal by carbon dioxide bubbling, with the addition of cysteine hydrochloride (0.25 g/liter of buffer) as a reducing agent. This prepared buffer was then transferred into the anaerobic chamber the day prior to the experiment to achieve complete reduction. The treatments were as follows: 1) white sorghum digested flour (WSD); 2) white sorghum phenolic extract (WSE) + FOS; 3) FOS; 4) white sorghum digested flour (WSD) + white sorghum phenolic extract (WSE); 5) tannin sorghum digested flour (TSD); 6) tannin sorghum phenolic extract (TSE) + FOS; 7) tannin sorghum digested flour (TSD) + tannin sorghum phenolic extract (TSE). The amount of lyophilized phenolic extract was defined according to the total phenolic content, each tube containing 0.5 mg of phenolic compounds.

On the experimental day, freshly collected fecal samples were pooled together and the fecal slurries were homogenized with carbonate-phosphate buffer (1:3 w/v), followed by filtration through four layers of cheesecloth. Subsequently, 1 ml of this fecal inoculum was introduced into Balch tubes (Chemglass Life Sciences, Vineland, NJ), each containing 50 mg of the dietary fiber substrate (sorghum digested flours WSD, TSD, or FOS) and 4 ml of carbonate-phosphate buffer. The tubes were securely closed with butyl rubber stoppers (Chemglass Life Sciences) and sealed with aluminum seals (Chemglass Life Sciences), before being incubated at 37°C in a shaker incubator (150 rpm; MaxQ 6000; Thermo Fisher, Waltham, MA) for 24 hours. Samples were then prepared and stored at -80°C until further analysis for short-chain fatty acid (SCFA) content (0.5 ml) and DNA sequencing (1 ml). All manipulations of the samples were conducted under anaerobic conditions (85% N_2, 5% CO_2, and 10% H_2).

8.2.5 Short-Chain Fatty Acids analysis

Short-chain fatty acids were determined using the methodology by Cantu-Jungles et al. (2018). To quantify SCFA contents, fermented samples (400 µL) were mixed with a 100 µL solution containing 50 mM 4-methyl-valeric acid (used as an internal standard for SCFA and BCFA analysis, No. 277827- 5G, Sigma-Aldrich Inc., St. Louis, Mo., USA), 5% meta-phosphoric acid, and 1.56 mg mL^{-1} copper sulfate. After thorough mixing, the mixture was centrifuged at 13,000 rpm for 10 min. An aliquot (0.2 µL) was injected into a GC-FID 7890A (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a fused silica capillary column (NukolTM, Supelco No. 40369-03A, Bellefonte, Pa.,USA). The oven temperature was initially set at 50 °C for 2 min, then ramped to 70 °C at a rate of 10 °C min^{-1}, to 85 °C at a rate of 3 °C min^{-1}, to 110 °C at a rate of 5 °C min^{-1}, to 290 °C at a rate of 30 °C/min, and finally

held at 290 °C for 8 min. Helium served as the carrier gas at a constant flow rate of 1 mL min^{-1}. Quantification relied on the relative peak area of each short-chain fatty acid in a fatty acid external standard (Volatile Free Acid Mix, 10 mM, 46975-U, Supelco), with adjustments based on the quantity of the internal standard.

8.2.6 DNA sequencing analysis

DNA sequencing for the analysis of gut microbiota was conducted following the method described by Cantu-Jungles et al. (2021). The DNA extraction process for the resulting precipitates was automated using the QIAcube Connect instrument (Qiagen, Germantown, MD) with the QIAamp PowerFecal Pro DNA kit (Qiagen), in accordance with the manufacturer's protocols. The V4 region of the 16S rRNA gene was targeted for amplification using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVHHHTWTCTAAT-3') as described by Walters et al. (2016). These primers included common sequence tags known as CS1 and CS2. Each sample well received a unique 10-base barcode from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; catalog no. 100-4876). Demultiplexing of reads was performed on the instrument. Library preparation, pooling, and sequencing were executed at the Genome Research Core (GRC) within the Research Resources Center (RRC) at the University of Illinois at Chicago.

8.2.7 Statistical Analysis

The SCFA results were analyzed using GraphPad Prism® software, employing analysis of variance (ANOVA) followed by Tukey's multiple comparisons test ($P < 0.05$). For alpha-diversity analysis, Kruskal-Walls was applied ($P < 0.05$).

8.3 RESULTS

8.3.1 Toasted sorghum flours characterization

The results for total fiber composition and resistant starch (RS) content in the toasted white sorghum flour (WSTF) and tannin sorghum flour (TSTF) before *in vitro* digestion are shown in Table 1. WSTF, chosen as a standard comparison for a sorghum genotype, contains approximately half of the insoluble fiber (10.82% ± 0.33 w/w) and the total fiber content (11.48% ± 0.04 w/w) compared to TSTF (21.88% ± 0.28 and 22.86% ± 0.40 w/w, respectively). However, this difference is more pronounced in the RS content, where TSTF has 35.9 % ± 2.53 and WSTF has 7.6 % ± 1.21. These results highlight the BRS 305 tannin sorghum genotype as

particularly notable for its high RS content compared to other cereals and sorghum genotypes (de Carvalho Teixeira et al., 2016; Štěrbová et al., 2016; Nguyen et al., 2023).

Table 1. Dietary fiber composition and total phenolics of the toasted sorghum flours

Nutrients (%)/Compounds	WSTF	TSTF
Resistant starch	7.6 ± 1.21	35.9 ± 2.53
Insoluble fiber	10.82 ± 0.33	21.88 ± 0.28
Soluble fiber	0.66 ± 0.28	0.97 ± 0.12
Total dietary fiber	11.48 ± 0.04	22.86 ± 0.40
Total free phenolics (mg GAE/g)	1.31 ± 0.04	45.34 ± 1.44

Results are expressed in percentage of dry basis. Total phenolic compounds are expressed in mg of Gallic Acid Equivalent/g. WSTF = White sorghum toasted flour; TSTF = Tannin sorghum toasted flour.

Regarding total phenolic compounds, TSTF also presented a high content with 45.34 ± 1.44 mg GAE/g, while WSTF had only 1.31 ± 0.04 mg GAE/g. The data on the characterization of the phenolic extracts from the toasted white sorghum and tannin sorghum flours are presented in Figure 1, showing the most abundant compounds. Daidzein and/or puerarin was the most abundant compound in both sorghum phenolic extracts and, although the exact identification of these compounds was not determined, both have been found in other sorghum genotypes (D'Almeida et al., 2021). Additionally, tannin sorghum extract (TSE) presented many phenolic compounds that were not present in white sorghum extract (WSE), such as naringenin 7-O glucoside, dihydroquercetin, and methylgalangin.

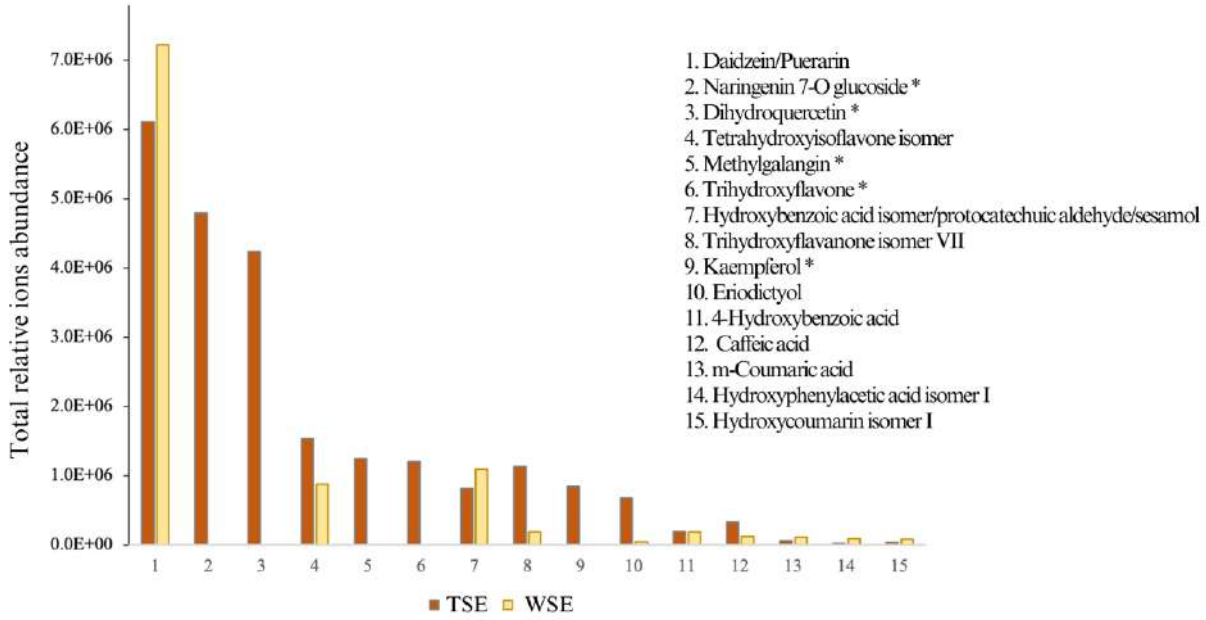

Figure 1. Most abundant phenolic compounds in the toasted sorghum flours. TSE: Tannin sorghum phenolic extract; WSE = White sorghum phenolic extract. * Means that the compounds are present only in tannin sorghum. Compound A/B means that it was not tentatively identified.

Among the most abundant compounds in WSE are phenolic acids such as caffeic acid, 4-hydroxybenzoic acid, and m-coumaric acid, and flavonoids such as daidzein and/or puerarin and eriodictyol. Lastly, it is important to mention that most of the phenolic compounds present in sorghum are bound to the plant cell structure (Awika & Rooney, 2004; D'Almeida et al., 2021), specifically phenolic acids such as ferulic acid (Hahn et al., 1983). Upon reaching the large intestine, fiber-bound phenolics are extensively transformed by the resident microbial community, undergoing many processes before breaking down into lower molecular weight phenolics (Rochetti et al., 2022).

8.3.2 Determination of Short-Chain Fatty Acids

The SCFAs produced in the *in vitro* fecal fermentation were quantified and are presented in Figure 2. The highest concentrations of acetate were observed in the treatments with WSE and TSE phenolic extracts supplemented with FOS, with values of 37.55 mM ± 4.03 and 38.02 mM ± 0.66, respectively. FOS was added to provide the microbiota with a fiber source to utilize, but FOS alone control was included for comparison. However, when comparing the acetate concentrations of WSE+FOS and TSE+FOS with FOS alone, no significant difference was observed. Therefore, this performance was attributed to the presence of this fiber, slightly enhanced by the sorghum phenolics. The addition of phenolic extract significantly increased acetate production in the WSD+WSE treatment compared to white sorghum flour alone (WSD). Furthermore, both sorghum flours (WSD and TSD) were equal regarding this SCFA.

On the other hand, concerning propionate, treatments containing phenolic extracts showed higher concentrations, with WSE+FOS = 29.59 mM ± 1.53 and TSE+FOS = 19.89 mM ± 0.61. In this case, FOS was significantly lower (FOS = 17.33 mM ± 0.76), indicating that these sorghum extracts were responsible for the high concentration. Similarly to acetate, the white sorghum phenolic extract (WSE) increased SCFA production in WSD+WSE (16.89 mM ± 0.30), being statistically equal to FOS; and sorghum flours (WSD and TSD) showed no difference between them.

Finally, butyrate concentrations were higher in treatments containing FOS, indicating that this dietary fiber influenced butyrate production. However, the tannin sorghum phenolic extract (TSE+FOS = 12.92 mM ± 0.29) showed a higher butyrate concentration than the white sorghum extract (WSE+FOS = 9.48 mM ± 1.84). When comparing the two sorghum flours

alone (WSD and TSD) and these flours supplemented with their respective phenolic extracts (WSD+WSE, TSD+TSE), there was no significative difference in any of these cases.

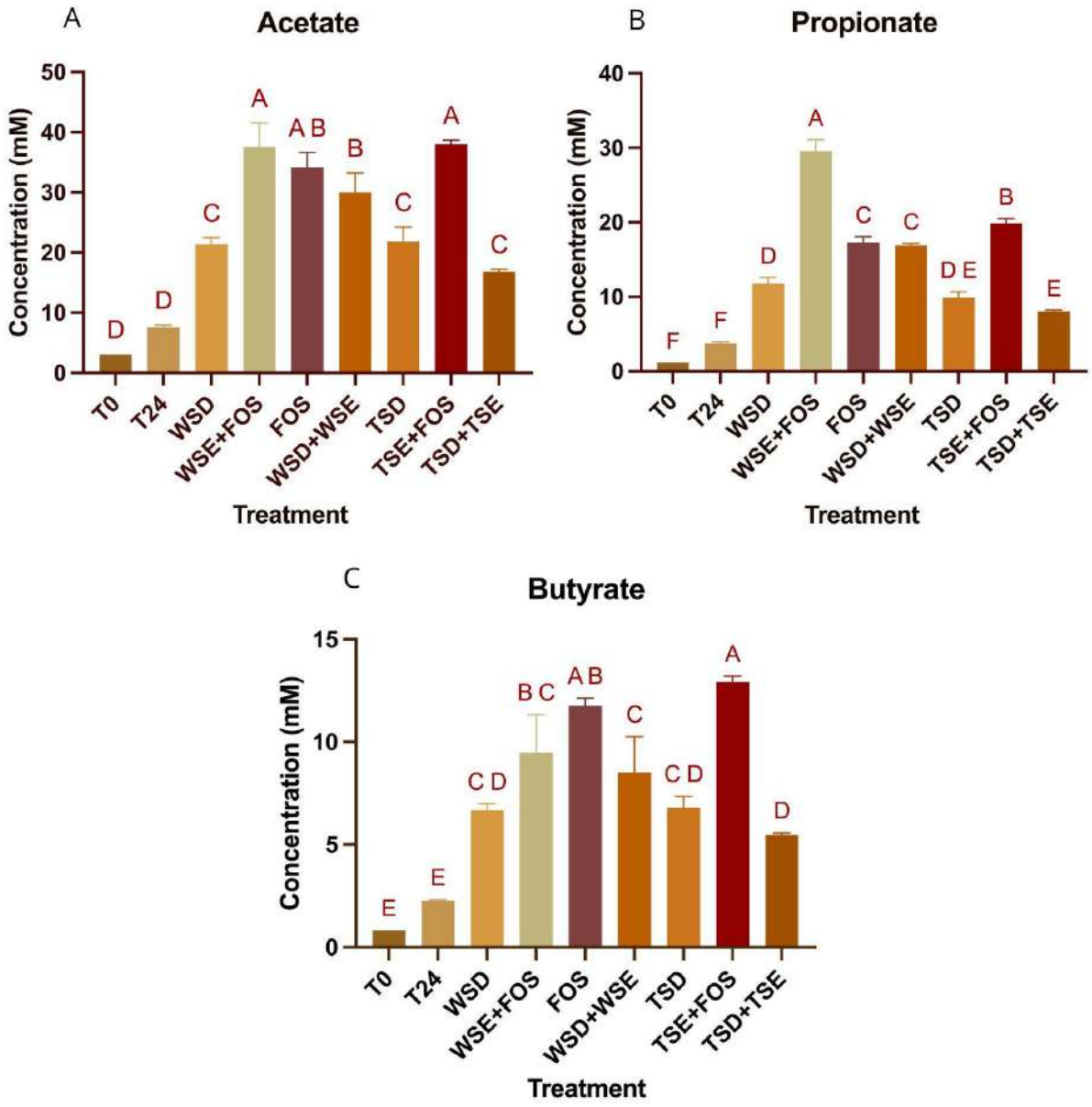

Figure 2. Concentrations of short-chain fatty acids A) acetate, B) propionate, and C) butyrate in different treatments after *in vitro* fecal fermentation. T0: initial timepoint; T24: time 24 hours; WSD: white sorghum digested flour; WSE: white sorghum phenolic extract; FOS: fructo-oligosaccharides; TSD: tannin sorghum digested flour; TSE: tannin sorghum phenolic extract. ANOVA and Tukey test ($P < 0.05$).

8.3.3 Gut Microbiota Analysis

The alpha diversity of the gut microbiota was evaluated for the samples tested in the in vitro fecal fermentation. It can be observed that there were significant differences in the Shannon index between various treatments (Figure 3A), such as the reduction in alpha-diversity seen in the FOS, TSE+FOS, WSD+WSE, and WSE+FOS treatments compared to the control.

On the other hand, the TSD, WSD, and TSD+TSE treatments maintained a Shannon index similar to the control. The WSE+FOS treatment showed the lowest alpha-diversity in the microbiota. Regarding beta diversity through the Bray-Curtis Index analysis, the white sorghum phenolic extract (WSE) showed a similar effect on the microbiota in both the WSE+FOS and WSD+WSE treatments (Figure 3B). Concerning the influence of fiber type, FOS, WSD, and TSD were able to affect this index in distinct ways (Figure 3C), causing clear separation in the principal component analysis.

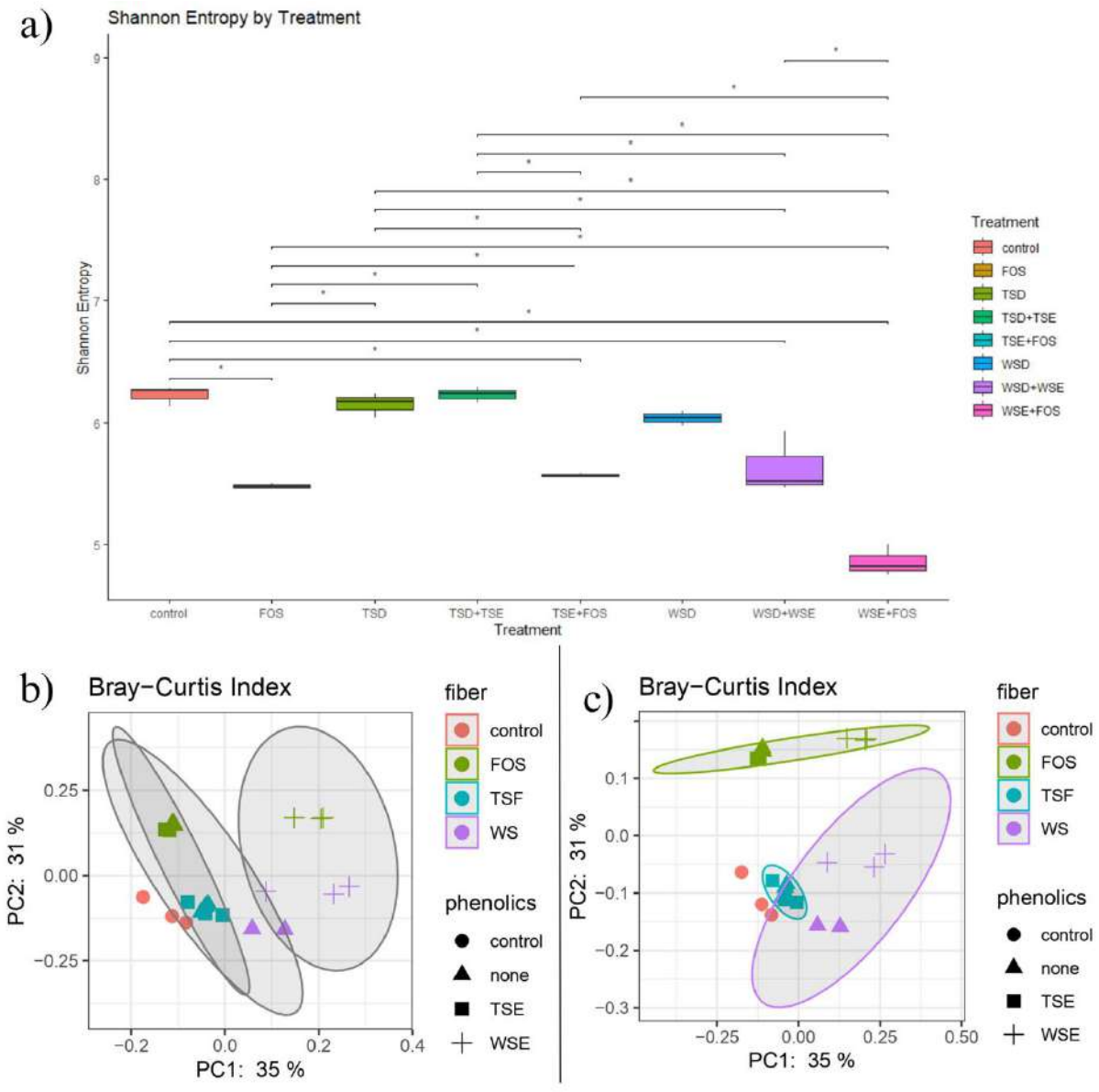

Figure 3. A) Shannon entropy alpha-diversity and Bray-Curtis beta-diversity of gut microbiota in relation to B) phenolic composition and C) fiber. *P*-value > 0.05 for Shannon entropy. WSD/WS: white sorghum digested flour; WSE: white sorghum phenolic extract; FOS: fructo-oligosaccharides; TSD: tannin sorghum digested flour; TSE: tannin sorghum phenolic extract; PC: Principal Component.

The data on the relative abundance of the microbiota in each treatment show that *Firmicutes* was the most abundant phylum in all treatments (Figure 4A), followed by *Bacteroidota* and *Actinobacteria*. The WSE + FOS sample exhibited a higher relative abundance of *Bacteroidota* compared to FOS alone and the other phenolic extract, TSE + FOS. Regarding family (Figure 4B), WSE + FOS showed a higher relative abundance of *Bacteroidaceae*, while the *Bifidobacteriaceae* family was more abundant in the samples containing white sorghum flour (WSD and WSD + WSE). *Ruminococcaceae* family showed a reduction in the treatments compared to the controls (T0 and T24h), with lower relative abundance in the WSD and WSD + WSE samples and higher in the TSE + FOS sample.

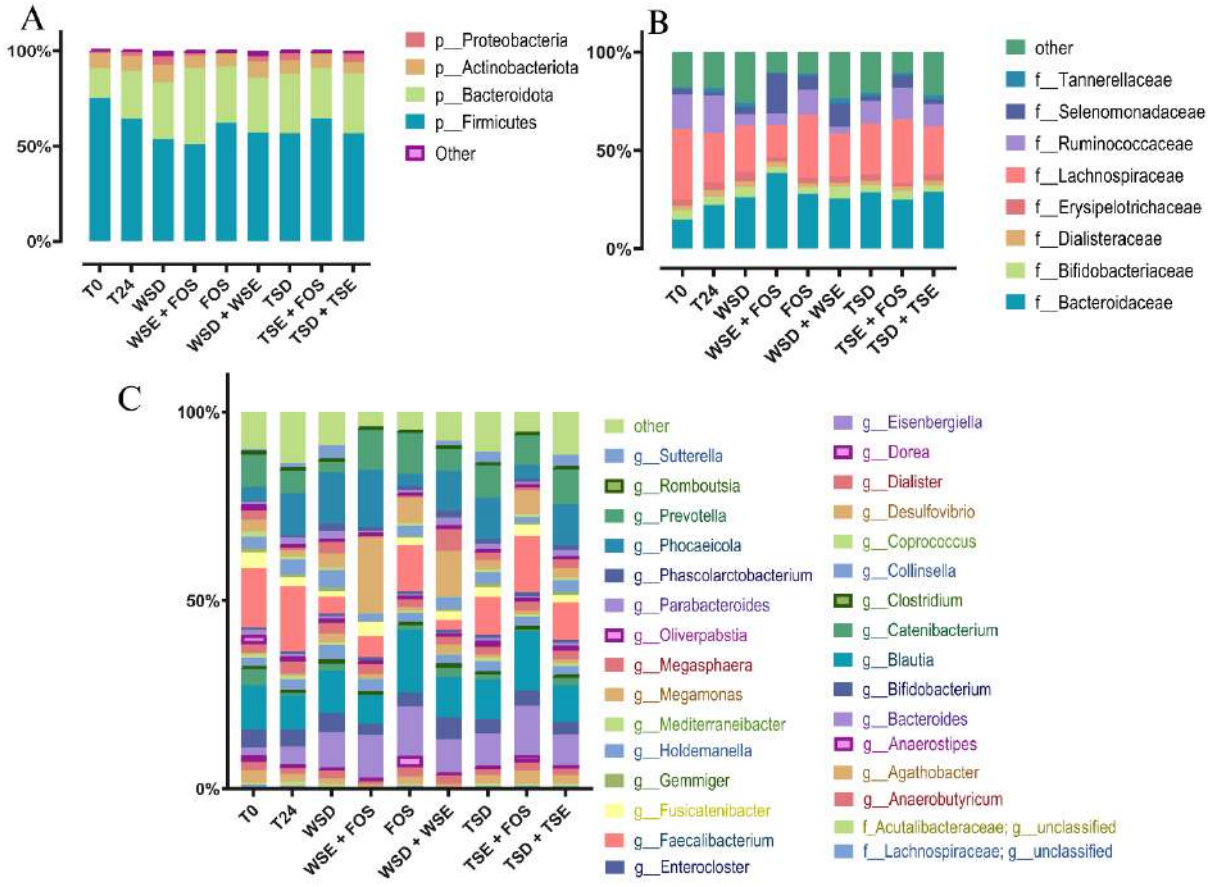

Figure 4. Gut microbiota relative abundances regarding A) Phyla, B) Family, and C) Genera. WSD: white sorghum digested flour; WSE: white sorghum phenolic extract; FOS: fructo-oligosaccharides; TSD: tannin sorghum digested flour; TSE: tannin sorghum phenolic extract.

Delving deeper into the taxonomic levels, the differences in the relative abundance of genera in each treatment (Figure 4C) show that both sorghum flours and phenolic extracts are capable of modulating the gut microbiota in different ways. The genus *Faecalibacterium*

showed a reduction in the white sorghum samples (WSD, WSE + FOS, WSD + WSE) compared to the controls (T0 and T24h), but this decrease in relative abundance was not as pronounced in the tannin sorghum samples (TSD, TSE + FOS, TSD + TSE) and in the FOS. Heatmap analysis (Figure 5) shows a clustering trend of bacterial genera in each treatment, where it can be observed that some genera were more favored in the presence of white sorghum flour (WSD and WSD+WSE), such as *Megasphaera* and *Romboutsia*. On the other hand, the phenolic extracts show a different clustering and favor other genera.

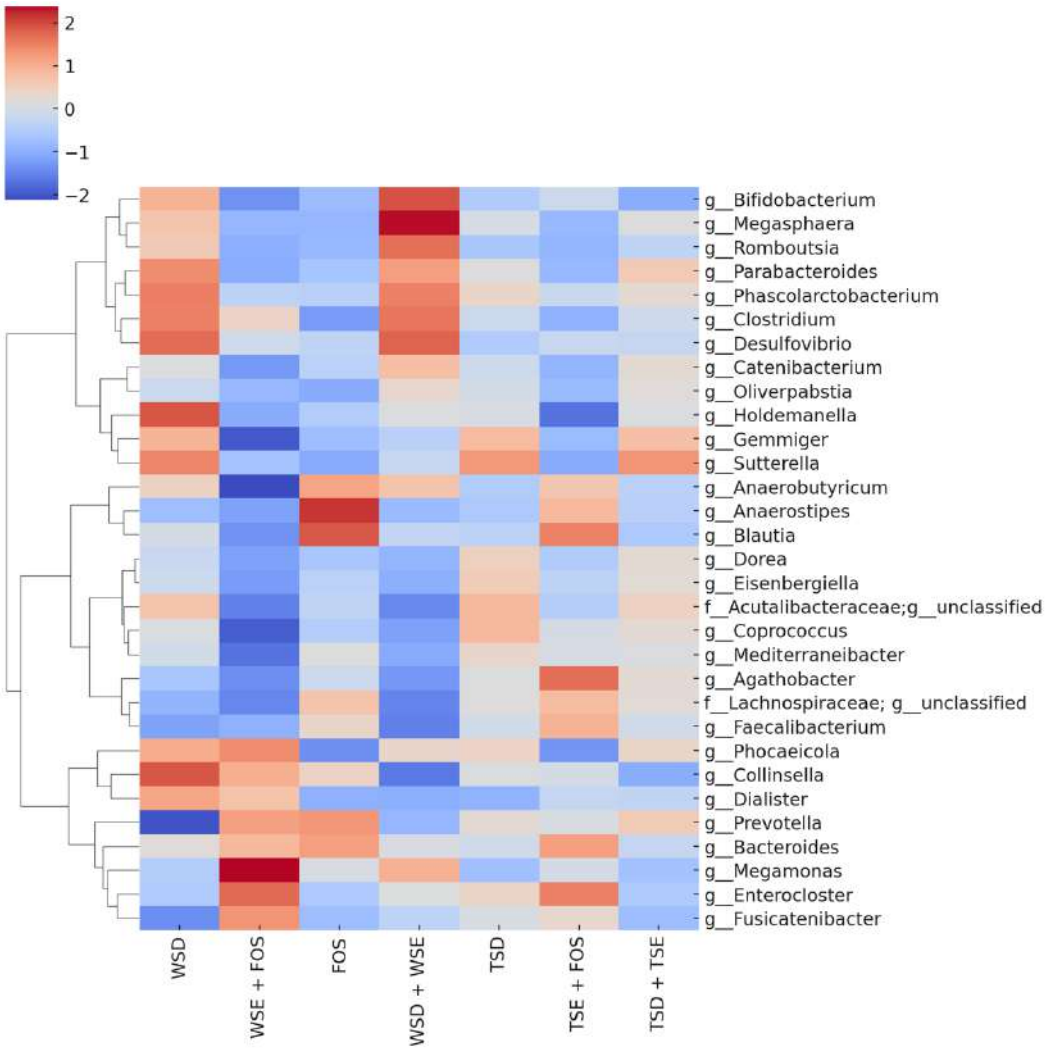

Figure 5. Heatmap analysis with Hierarchical Clustering on Taxa (Ward Method) for each treatment. WSD: white sorghum digested flour; WSE: white sorghum phenolic extract; FOS: fructo-oligosaccharides; TSD: tannin sorghum digested flour; TSE: tannin sorghum phenolic extract.

By analyzing this clustering to identify patterns among the types of fibers (WSD, TSD, or FOS), as well as the effect of phenolics on the growth of bacterial genera, it is possible to observe that some genera, such as *Bifidobacterium* and *Desulfovibrio*, were more abundant in

treatments containing white sorghum flour (WSD and WSD + WSE). It can be observed that the presence of FOS favors the growth of the genus *Bacteroides*, as it was stimulated in all FOS-containing treatments. However, other genera, such as *Enterocloster* and *Agathobacter*, were favored independently of this fiber, having this effect very likely due to sorghum phenolic extracts. Additionally, *Megamonas* appears to be a genus related to white sorghum phenolic extracts (WSE), as it showed greater growth in the WSE+FOS and WSD+WSE treatments, but not in WSD alone or FOS.

8.4 DISCUSSION

There are still significant gaps in our understanding of how various foods interact with the gut microbiota, despite its increasingly recognized importance for overall health. Exploring these interactions is crucial for advancing our knowledge of diet-microbiota relationships and their implications for disease prevention and health promotion. Therefore, this study aimed to understand how toasted flours from white sorghum and tannin sorghum, as well as their phenolic-rich extracts, can alter the gut microbiota and its metabolites, SCFAs.

The alpha diversity of the gut microbiota, measured by the Shannon index, which considers both the richness and evenness of microorganisms, shows an intriguing result, as it would be expected that FOS would benefit a larger number of taxa and maintain an alpha diversity similar to the control due to the lower specificity of this dietary fiber (Cantu-Jungles et al., 2021). However, it was the sorghum flour samples (TSD, WSD) and the tannin sorghum flour and its phenolic extract (TSD+TSE) that maintained a Shannon index similar to the control. Cantu-Jungles & Hamaker (2023), however, point out that gut microbiota diversity should not be the only metric to assess the positive impact of dietary fiber interventions. This may be because its utilization by the microbiota can reduce richness by suppressing the growth of pathogenic bacteria, for example.

Beta-diversity analysis and the Bray-Curtis index assess differences in the microbial community composition between samples. The PCA analysis revealed a clear separation between the white sorghum treatments and their phenolic compounds (Figure 3B), showing that the phenolic profile of the samples influenced the microbiota composition. The distinct separation of fibers indicated that FOS had also a notable impact on the gut microbiota, as did white sorghum, though the latter was closer to tannin sorghum (Figure 3C). The separation observed, based on the Bray-Curtis index, indicates that the treatments significantly altered the

microbiota composition, with marked differences in the relative abundance of key taxa between the groups.

Evaluating SCFA production alongside gut microbiota profiles, it can be observed that the higher acetate production shown in treatments containing FOS is likely related to the promotion of certain genera known for producing this SCFA, such as *Bacteroides* (Shien et al., 2024). As for propionate, the genera *Enterocloster* (Sprague et al., 2024), as well as *Megamonas* and *Blautia* — which produce propionate through pathways involving epimerase, decarboxylase, and methylmalonyl-CoA mutase (Polansky et al., 2016) — are known propionate producers, helping to explain the higher production of this SCFA in the WSE+FOS and TSE+FOS treatments.

The increase in acetate and propionate concentrations caused by the addition of phenolic-rich extract from white sorghum (WSE) to toasted white sorghum flour (WSD) may be explained by the phenolic composition of this sorghum genotype, which differs from tannin sorghum. As seen previously in the characterization of phenolic-rich extracts from both white sorghum and tannin sorghum, most of the abundant phenolic compounds in WSE are also present in TSE, with the latter containing additional compounds not found in white sorghum. Moreover, TSE also contains condensed tannins, which presents a high degree of polymerization (Paes et al., 2024; Hagerman et al., 1998). One hypothesis is that, despite WSE having a lower relative abundance of phenolic compounds, these are easily fermentable, whereas TSE may contain other phenolics that do not impact SCFA production to the same extent as WSE.

Previous studies have shown an increase in SCFA production by daidzein (Chen et al., 2022) and caffeic acid (Parkar et al., 2013) in *in vitro* fecal fermentation, found in both sorghum genotypes. The dicaffeoylquinic acid, which was found only in white sorghum and not in tannin sorghum (Paes et al., 2024), was able to modulate the intestinal microbiota and SCFA *in vitro* by increasing acetate concentration and decreasing propionate and butyrate in the study by Xie et al. (2017).

Bazzoco et al. (2008) investigated the effect of proanthocyanidins (PAs) – also known as condensed tannins – from apples using an *in vitro* colon model with human fecal microbiota. They found that the short-chain PAs were more readily fermented, while long-chain PAs inhibited microbial metabolism of PAs, particularly at early time points. Additionally, the presence of isolated PAs suppressed SCFA formation from carbohydrates compared to other substrates, suggesting a competition between the inhibitory effect of PAs on microbial activity and their conversion by the microbiota. It is not possible to say that there was a suppression of

SCFA production by TSE, since there was an enhancement, which may be attributed to the presence of FOS in some instances, or occurred independently, as in the case of propionate. Although supplementation of TSE in TSD did not cause an increase in SCFA production, studies have shown that profisetinidin, a type of condensed tannin, was capable of increasing SCFA concentration *in vitro* when supplementing other foods (Molino et al., 2021).

Regarding butyrate production, the higher concentration observed in TSE compared to WSE – both with the same FOS dietary fiber – may also have explanations in their phenolic composition. Sorghum with tannins exhibits kaempferol, dihydroquercetin, and naringenin 7-O glucoside among its most abundant compounds, which are not present in white sorghum (Paes et al., 2024). The study by Rha et al. (2019) examined the digestive stability of flavonol aglycones and glycosides from green tea during simulated digestion and anaerobic human fecal fermentation. Kaempferol and its glycosides demonstrated higher stability in simulated gastric and intestinal fluids compared to quercetin and myricetin derivatives. Anaerobic fecal fermentation with flavonol-rich fractions (kaempferol, quercetin, and myricetin) generated organic acids, including acetate, propionate, and butyrate, with butyrate being the most abundant.

The fact that treatments containing FOS show higher production of butyrate, as well as acetate, is not exactly a surprise due to the simpler nature of this dietary fiber, allowing more groups to utilize and ferment it (Cantu-Jungles, et al., 2021). Among the most abundant and expressed bacterial groups in the treatments containing FOS (Figure 5) that are known to be butyrate producers are the genera *Faecalibacterium* (Sprague et al., 2024) and *Anaerostipes* (Schwiertz et al., 2002). The genus *Bifidobacterium* benefits from butyrate for its growth through cross-feeding with other bacterial groups (Rios-Covian et al., 2015). Thus, among the factors that led to the higher abundance of this genus in the WSD and WSD+WSE treatments may be the considerable—but lower than in the FOS treatments—butyrate production, as well as the fiber and phenolics present in the white sorghum flour. On the other hand, *Bifidobacterium* lower expression in the FOS treatments may be due to the high competition for this fiber.

The two digested sorghum flours have significantly different compositions of insoluble dietary fibers, total dietary fibers, and resistant starch, with the toasted tannin sorghum flour having a higher content of these components (Table 1). However, despite the same amount (50 mg) of flour after *in vitro* digestion being used, this difference in dietary fibers did not result in a difference in the production of any type of SCFA between the digested sorghum flours WSD and TSD. Comparing the results with other cereals, the study by Nordlund et al. (2012) showed

that although wheat bran and aleurone exhibited a higher content of total phenolic metabolites generated by the fecal fermentation microbiota *in vitro*, they had lower total SCFA levels. However, considering individual SCFAs, wheat aleurone showed a higher concentration of butyrate. The study by Tuncil et al. (2018) analyzed the *in vitro* fecal fermentation of wheat, sorghum, rice, and corn brans and showed that the total SCFA concentrations from sorghum bran were lower than those from rice and wheat, being higher only than that from corn. In this study, it is possible to observe, as in our results, that FOS produced more acetate and butyrate, but not propionate, than sorghum bran.

The way phenolic compounds impact gut microbiota and, consequently SCFA, is still under investigation to find the mechanisms behind it. According to Mosele et al. (2015), the rise in SCFAs following *in vitro* fermentation of pure phenolic standards may be linked to an accelerated fermentation rate of released glycoside moieties and/or residual carbohydrates in the culture media or feces, while phenolic-rich extracts have shown mixed results. These varied results suggest that the utilization of phenolic compounds by the intestinal microbiota depends on factors such as degree of polymerization, compound type, as well as their bioaccessibility, given that many of them are bound to dietary fibers.

8.5 CONCLUSION

The investigation into gut microbiota modulation and its short-chain fatty acids (SCFAs) production in *in vitro* fecal fermentation provides valuable insights into the potential health benefits of phenolic-rich extracts from white and tannin sorghum. Our findings reveal that these phenolic extracts, when combined with fructo-oligosaccharides (FOS), were able to modulate and change gut microbiota composition, and to increase the concentrations of propionate, but not acetate and butyrate. Both sorghum phenolic extracts and toasted flours showed to favor SCFA-producers bacteria that have been related to good health outcomes.

Furthermore, the treatment of toasted white sorghum flour (WSD) with its phenolic-rich extract (WSE) was able to modulate the production of acetate and butyrate; the same was not observed for tannin sorghum flour (TSD). Our results show that even though the relative abundance of phenolic compounds is higher for tannin sorghum flour, this does not necessarily translate into a substrate for gut microbiota to produce SCFA. This raises questions to be elucidated for a better understanding of the relationship between the phenolic profile of each flour and its effect on the gut microbiota. Thus, both toasted sorghum flours would be potential healthy options for modulating the gut microbiota and its metabolite SCFA production, with

phenolic-rich extracts, especially from white sorghum, being particularly interesting for use as dietary supplements.

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9. OVERALL CONCLUSION

Based on the comprehensive analysis of sorghum health benefits from *in vitro* and *in vivo* studies, it is evident that sorghum phenolic-rich extracts and toasted flours, with an interesting composition of dietary fibers and phenolic compounds, possess a significant functional potential in cancer prevention, antimalarial treatment, SCFA production, and gut microbiota modulation.

Regarding *in vitro* antiproliferative effects, the white sorghum ethanolic extract (WSE) demonstrated superior cytotoxicity against EA.hy926 and A549 cancer cells, as well as notable antimetastatic potential in HCT-8 cells. Moreover, the *in vivo* study revealed that tannin-rich sorghum flour supplementation significantly enhanced oxidative stress defense mechanisms in rats and reduced the incidence of aberrant crypt foci (ACF), which are precursors to colon cancer. Therefore, both WSE and tannin-rich sorghum flour stand out as promising agents for cancer prevention. Regarding gut microbiota modulation, both white and tannin sorghum toasted flours demonstrated significant influence.

In terms of SCFA production, the white sorghum flour showed higher acetate and propionate levels compared to other groups in the *in vivo* study, indicating its efficacy in enhancing SCFA production. This was further corroborated by the *in vitro* fecal fermentation study, where white sorghum phenolic extracts (WSE) combined with fructooligosaccharides or white sorghum flour (WSD) resulted in high concentrations of acetate and propionate. These findings suggest that white sorghum is highly effective in boosting SCFA production through gut microbiota modulation. Despite tannin sorghum flour having a higher dietary fiber content, the supplementation with phenolic extracts proved to be a more critical factor in enhancing SCFA production.

In conclusion, the findings from this thesis underscore the health potential of sorghum and its components. Although more data are needed to understand the mechanisms of action, all these results support the promotion of sorghum consumption, particularly its toasted flours, for health benefits. Furthermore, the various bioactivities of phenolic-rich sorghum extracts highlight their potential application as dietary supplements in capsules, including targeted colon release. Toasted sorghum flours have the potential to be consumed as "farofas," a popular dish in Brazil, serving as a healthy alternative to the widely consumed cassava flour, aiming to combine nutrition and tradition for greater acceptability.

SUPPLEMENTARY MATERIAL

1 – Ethical committee approval

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 13/2022, intitulado **"Avaliação *in vitro* e *in vivo* do potencial funcional de farinhas tostadas de sorgo",** coordenado pelo professor Frederico Augusto Ribeiro de Barros do Departamento de Tecnologia de Alimentos, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC, portanto sendo aprovado por esta Comissão em 07/07/2022, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 13/2022, named **"In vitro and in vivo evaluation of the functional potential of toasted sorghum flours"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008, Normative Resolutions edited by CONCEA/MCTIC, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTIC therefore being approved by the Committee on July 07, 2022 valid for 12 months.

Prof. Fabrício Luciani Valente
Coordenador
Comissão de Ética no Uso de Animais – CEUA/UFV

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Viçosa, 07 de julho de 2022

Prof.
Frederico Augusto Ribeiro de Barros
Coordenador do projeto
DTA/UFV

Sr. Coordenador,

Após avaliação da Metodologia utilizada no Projeto de Pesquisa intitulado **"Avaliação *in vitro* e *in vivo* do potencial funcional de farinhas tostadas de sorgo"**, aqui nomeado Processo 13/2022, a CEUA/UFV emite parecer favorável ao protocolo de utilização de animais proposto, tendo como base para análise a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTIC, bem como a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTIC.

Acresce a esse Parecer a exigência de Relatório Final de Atividades conforme itens a seguir:

*RESUMO DOS RESULTADOS FINAIS OBTIDOS A PARTIR DOS EXPERIMENTOS ENVOLVENDO A **UTILIZAÇÃO DE ANIMAIS** NO PROJETO DE PESQUISA*

1 Número do protocolo de submissão do projeto de pesquisa à CEUA/UFV:
2 Metodologia completa obrigatoriamente com:
- Local (is) Geral (is) e específico (s) oficial (is) onde ocorreu a experimentação;
- O nome científico do animal em questão;
- Número total de animais utilizados na pesquisa.
3 Resultados:
4 Nome do Coordenador do Projeto:
Assinatura:
5 Nome do Responsável Técnico:
Assinatura:
Inscrição em CRMV:

Prof. Fabrício Luciani Valente
Coordenador
Comissão de Ética no Uso de Animais – CEUA/UFV