UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DE ALIMENTOS

Matheus Thomaz Nogueira Silva Lima

EVALUATION OF THE BIOACTIVITY OF HIBISCUS EXTRACTS (*HIBISCUS SABDARIFFA* AND *H. ROSA-SINENSIS*) AND ANTI-INFLAMMATORY POTENTIAL

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Advisor: Prof. Dr. Jacqueline A. Takahashi

CAPES-PrInt colaborator: Prof. Dr. Frédéric J. Tessier (University of Lille, France)

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AVALIAÇÃO DA BIOATIVIDADE DE EXTRATOS DE HIBISCOS (HIBISCUS SABDARIFFA E H. ROSA-SINENSIS) E POTENCIAL ANTIFLAMATÓRIO

MATHEUS THOMAZ NOGUEIRA SILVA LIMA

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RESUMO

O hibisco, comumente conhecido como vinagreira, é uma planta com distribuição mundial e muito utilizada como alimento, no preparo de chás, corantes alimentícios e produtos fermentados. As flores de hibisco vermelho são comumente comercializadas e estudadas em todo o mundo devido aos seus benefícios nutricionais e usos terapêuticos. No entanto, outras variações de cor ocorrem naturalmente e podem apresentar aplicações potenciais na indústria alimentícia. A formulação de alimentos funcionais à base de hibisco seria de grande valia, uma vez que efeitos nutracêuticos foram implicados nesta planta. Primeiro, este trabalho teve como objetivo caracterizar os teores de fenólicos totais (TPC) e flavonoides totais (TFC), os efeitos antioxidante, anti-glicação e neuroprotetor de extratos florais de hibisco. Em segundo lugar, abordar a aplicabilidade da radiação gama como ferramenta na cadeia de produção do hibisco, caracterizando os efeitos dessa radiação ionizante na bioatividade de extratos desta planta. Por fim, de modo a avaliar o estresse pró-inflamatório/oxidativo de compostos dietéticos como produtos finais de glicação avançada, estudar o uso de células endoteliais (HUVECs) como modelo funcional para de inflamação mediada pelo Receptor de Produtos Avançados de Glicação (RAGE). A variedade de hibisco vermelho (*Hibiscus sabdariffa*) é uma fonte bem conhecida de fenólicos e flavonoides. No entanto, aqui foi demonstrado que a variedade rosa de *H. rosa-sinensis* apresentou o maior TPC (31,28±1,24 mg EAG/100 g) e TFC (469,20±3,24 mg EQ/100 g) em extratos aquosos que é o solvente mais comum na preparação do hibisco como alimento. A atividade antioxidante permaneceu acima de 60% e 80% da eliminação de radicais DPPH e ABTS, respectivamente para todas as amostras. Maior distinção

entre o potencial antioxidante foi feito a partir da atividade do poder redutor do férrico, em que apenas os extratos etanólicos das variedades rosa e vermelha ultrapassou 50 %. A variedade vermelha de hibisco também mostrou atividade anti-glicação equivalente (55%), anti-acetilcolinesterásica (> 95%) e efeito neuroprotetor *in vivo* (*D. melanogaster*). O uso de radiação gama não apresentou efeitos negativos consideráveis na bioatividade do hibisco. Doses de 10 kGy a 30 kGy permitiram a descontaminação de altas cargas microbianas, mas 30 kGy tiveram efeitos diversos no TFC. Por fim, a indução da inflamação foi possível em cultura de HUVEC, com aumento da expressão dos genes *TNFα*, *IL*6 e *VCAM1* sob efeitos de TNFα (10 ng/mL). No entanto, HMGB1, um ligante de RAGE de alta afinidade, não induziu inflamação nas condições experimentais aqui apresentadas. Em conclusão, a biodiversidade dentro do gênero hibisco influencia a variação no conteúdo fenólico e bioatividade dos extratos, e novas variedades demonstraram conter antioxidantes, TPC e TFC, comparáveis ao hibisco vermelho comumente consumido e merecem atenção. Além disso, a radiação gama mostrou eficácia na redução da contaminação microbiana, mantendo o potencial bioativo nas diferentes amostras de hibisco. Por fim, os resultados do HUVECS sugeriram que a ativação da inflamação é independente das variações da expressão de RAGE tanto nos níveis transcricionais quanto translacional. No entanto, investigações são necessárias para delinear a participação precisa de RAGE na ativação da inflamação em culturas de HUVEC.

Palavras-chave: *Hibiscus sabdariffa*; *Hibiscus rosa-sinensis*; bioatividade; neuroprotetor; anti-inflamatório; RAGE

ABSTRACT

Hibiscus, commonly known as roselle, is a plant with worldwide distribution and is greatly used as food, in the preparation of teas, food dyes, and fermented products. Red hibiscus flowers are commonly commercialized and studied around the world due to their nutritional benefits and therapeutic uses. However, other color variations occur naturally and may present potential applications in the food industry. The formulation of hibiscus-based functional foods would be of great value once nutraceutical effects have been implied to this plant. At first, this work aimed to characterize the total phenolic (TPC) and total flavonoid contents (TFC), the antioxidant, the anti-glycoxidation, and neuroprotective effects of hibiscus floral extracts. Secondly, we approached the applicability of gamma radiation as a tool in the hibiscus supply chain, by characterizing the effects of this ionizing radiation on hibiscus bioactivity. Lastly, on the way to investigate the pro-inflammatory/oxidative stress of dietary compounds as advanced glycation end-products, we evaluated the use of endothelial cells (HUVECs) as a functional model for RAGE-mediated inflammation screening. The red hibiscus variety (*Hibiscus sabdariffa*) is a well-known source of phenolic and flavonoids. However, here we demonstrated that the pink variety of *H. rosa-sinensis* presented the highest TPC (31.28±1.24 mg GAE/100 g) and TFC (469.20±3.24 mg QE/100 g) in aqueous extracts which is the most common solvent in hibiscus preparation as food. Antioxidant activity remained over 60% and 80% of DPPH and ABTS radical scavenging, respectively for all samples. Grater variation resulted from iron reducing power activity of ethanolic extracts of pink and red varieties (> 50 %). Hibiscus red variety also showed consistent anti-glycation (55 %), anti-acetylcholinesterase (> 95 %), and neuroprotective effect *in vivo* (*D.*

melanogaster). The use of gamma radiation did not present extensive effects on hibiscus bioactivity. Doses of 10 kGy to 30 kGy allowed high microbial loads decontamination but 30 kGy had effects on TFC. Finally, the induction of inflammation was made possible on HUVEC culture, with increased expression of *TNFα*, *IL6*, and *VCAM1* gene under TNFα (10 ng/mL) effects. However, HMGB1, a well-known inflammation activator, did not induce inflammation under the experimental conditions here applied. In a conclusion, the biodiversity within *Hibiscus* influences the variation in the phenolic content and bioactivity of these plants, and new varieties have been demonstrated to hold antioxidants, TPC, and TFC, comparable to the commonly consumed red hibiscus and deserve attention. Moreover, gamma radiation showed efficacy in microbial contamination reduction, while maintaining bioactive potential in the different hibiscus samples. Lastly, results from HUVECS suggested that inflammation activation is independent of variations of RAGE expression both at the transcriptional and translational levels. However, further investigation is required to delineate the precise participation of RAGE in inflammation activation on HUVEC cultures.

Key-words: *Hibiscus sabdariffa*; *Hibiscus rosa-sinensis*; bioactivity; neuroprotector; anti-inflammatory; RAGE

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LIST OF ABBREVIATIONS

ABTS	2,2-Azinobis-[3-etil-benzotiazolin-6-ácido sulfônico]
AChE	Acetylcholinesterase
AGE	Advanced glycation end-products
CDTN	Centro de Desenvolvimento da Tecnologia Nuclear
CFU	Colony forming units
CML	carboxymethyl-lysine
DAD	Diode array detector
dCML	Dietary carboxymethyl-lysine
DIA-1	Diaphanous 1
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
ESI	Electrospray ionization
GAE	Gallic acid equivalents
GSH	Gluthatione
HPLC	High-performance liquid chromatography
IL6	Interleukine 6
JNK	c-Jun N-terminal kinases
kGy	Kilo Gray
LaBB	Laboratório de Biotecnologia e Bioensaios
LIG	Laboratório de Irradiação Gama
MS	Mass spectrometry
NF-κB	Nuclear factor-kappa
NOS	Nitrogen reactive species
QE	Quercetin equivalents
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
ROS	Reactive oxigen species
TFC	Total flavonoid content
TNFa	Tumor necrosis factor
TOF	Time-of-flight
TPC	Total phenolic compounds
U	Enzymatic units
UV	Ultraviolet
VCAM1	Vascular cell adhesion protein 1

SUMMARY

1 INTRODUCTION

The use of edible flowers by humans has been described as part of ancient cultures where flowers were used in food preparations and in the composition of syrups or infusions intended for medicinal treatments (AMROUCHE et al., 2022; TAKAHASHI et al., 2020). In the modern world, the consumption of fresh edible flowers is part of gastronomic niches with Eurasian influences, in salad making, or as garnishes (AMROUCHE et al., 2022). On the other hand, dried flowers such as lavender (*Lavandula* sp.) (BOGDAN et al., 2021), calendula (*Calendula officinalis*) (BRAGUETO ESCHER et al., 2019), and hibiscus (*Hibiscus* sp.) (PARAÍSO et al., 2021) are mainly used in the preparation of infusions. Such use gained worldwide popularity since the Victorian period (19th century, United Kingdom) as an English tradition (FERNANDES et al., 2016).

The *Hibiscus* genus includes annual shrubs from the Malvaceae family, with fleshy petals used as ornaments or in beverage production (ANWAR et al., 2010). The main cultivated species of hibiscus are *H. sabdariffa*, popularly known as *vinagreira* in Brazil, due to the acidic taste, and *H. rosa-sinensis*, *mimo de vênus* (DEY et al., 2024). The extensive commercial uses of hibiscus including the flowers and other derivatives, such as seed oil and leaf fibers, turn these plants into relevant commodities, especially in developing countries due to the easiness of adaptation (DA CASTA ROCHA et al., 2014). The parts intended for human consumption are the floral calyces (the set of petals), rich in vitamins, minerals, and a well-known source of phenolic compounds (DA-COSTA-ROCHA et al., 2014). The color of mature calyces is a trait linked to the composition of flavonoids and anthocyanins. Great petal color variation exists within the *Hibiscus* genus, as well as between varieties of the same species, which may vary from

intense red to pink, white, and orange flowers (Figure 1) (HUSSEIN et al., 2010; WARNER; ERWIN, 2001).

Figure 1. Some examples of floral color variations within the *Hibiscus* genus.

The phytochemical profile of hibiscus extracts is predominantly composed of organic acids, malates, oxalates, citric acid, and hibiscus acid; phenolic acids, such as caffeic acid and chlorogenic compounds, and other phenolic compounds such as flavonoids and anthocyanins have also been reported as part of the biochemical repertoire of these plants. These natural products have been associated with different therapeutical properties like antioxidant, antihypertensive, and hepatoprotective compounds (Table 1) (EZZAT et al., 2016; SERBAN et al., 2015).

Table 1. Attributed therapeutical features to *Hibiscus* sp.

Species	Application
Hibiscus sabdariffa (Roselle)	Antioxidants (ZANNOU et al., 2020) Anti-anxiety (JYOTISHI; CHANDEL; GUPTA, 2021) Anti-pyretic (AZIZ et al., 2021) Angiogenic (SHARMA; KOTA, KARUNAKAR; PANDYA, NİLAY, 2021)
Hibiscus rosa-sinensis (Chinese rose mallow)	Neuroprotective (SHEN et al., 2021) Diuretic (NJINGA et al., 2020) Cardioprotective and nephroprotective (AL-QAHTANI; SHAIKH; HABEEB, 2017) Anti-cancer (NGUYEN et al., 2019)

Although great phytochemical diversity has been reported in hibiscus extracts, its polyphenolic content is marked by the predominance of hibiscin and its glycosylated derivatives (Figure 2). Some studies have indicated that approximately 7 mg/g of dried flowers correspond to the content of these anthocyanins (RIAZ; CHOPRA, 2018). As a consequence of the abundance of hibiscin, at least 50% of the total antioxidant activity attributed to *H. sabdariffa* is exclusively associated with the occurrence of these compounds (TSAI et al., 2002). Gossypitrin, hibiscitrin, quercetin, sabdaritrin, and ergosterol have also been identified in hibiscus flowers and levels may vary in accordance to genetic and environmental factors such as soil composition and/or seasonality, which demands extensive investigation of locally available cultivars (VARGAS-LEÓN et al., 2018; SANOU et al., 2022).

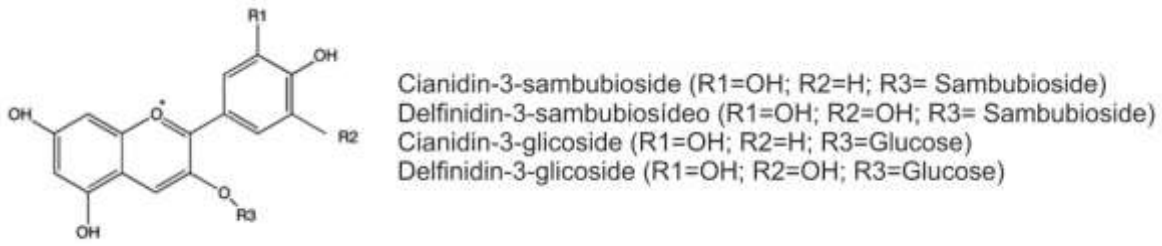

Figure 2. Hibiscin is the main anthocyanin derived from hibiscus extracts. Adapted from DA COSTA ROCHA et al. (2014).

Despite the extensive bibliography cementing the bioactive properties of hibiscus, there is a gap in exploring the different hibiscus varieties and the understanding of the correlation between floral pigmentation and the implications on bioactivity variations. Exploring these traits would pave the way to possible biotechnological applications of this plant as food additives, natural dyes, or nutraceuticals in the food industry.

2 GOALS

Considering the market acceptance of hibiscus, the biodiversity within the *Hibiscus* genus, the food security associated with this product, and the scientific support for the positive health effects of *Hibiscus sabdariffa*. We hypothesized that the possible nutraceutical effects such as neuroprotection, as well as biotechnological applications such as antioxidants or anti-glycoxidation, may be related to the biodiversity of hibiscus, a fact that could be related to the reduction of the effects of the aging process, oxidative stress and neurodegeneration. Therefore, the goal of this work was to explore the different hibiscus varieties and understand the correlation between floral pigmentation and the implications on bioactivity variations. In addition, to evaluate the effects of gamma radiation treatment on hibiscus bioactivity and the elimination of microorganisms. In complement, a cellular model, HUVECs, on inflammation was explored as a potential method for anti-inflammatory effect validation.

3 BIBLIOGRAPHICAL REVIEW

3.1 Phenolic compounds and their beneficial effects on human health

Oxidative stress has been implicated in the genesis of several chronic diseases and associated mechanisms such as glycoxidation. The use of dietary natural antioxidants has been prospected as an important therapeutic strategy for the re-establishment of the redox homeostasis and control of the progression of the above-mentioned pathologies (TELEANU et al., 2019; MONTANÉ et al., 2020). There is a well-established positive correlation between the consumption of diets rich in vegetables and minimally processed ingredients and human well-being promotion, which is driving great scientific interest in this field (TSIGALOU et al., 2020; MCMACKEN; SHAH, 2017).

Natural bioactive compounds are the result of the secondary metabolism of plants. The functional and structural diversity of these molecules is directly related to their biotechnological versatility and commercial relevance due to the vast functional roles they can play (ALAMGIR, 2018; CHANDRAN et al., 2020). Among the five main classes of plant secondary metabolites - terpenoids, phenolic compounds, cyanogenic glycosides, glucosinolates, and alkaloids - phenolic compounds correspond to an important group of molecules that can attenuate pathological processes when acquired through the diet, especially related to oxidative stress, aging, and neurodegeneration (MARTINEZ POMIER; AHMED; MELACINI, 2020; ROJAS; BUITRAGO, 2019). The nutritional importance of these molecules remains associated with the remarkable antioxidant activity - in the scavenging of free radicals, blocking enzymes related to metabolic pathways for the production of reactive oxygen species (ROS), or by metal chelation (YAN et al., 2020).

Phenolic compounds comprise a group of molecules structurally characterized by aromatic rings with hydroxyl group substitutions. These compounds are classified as flavonoids, phenolic acids, stilbenes, xanthones, or lignins. Flavonoids are the largest class of phenolics, with more than 6000 structures identified, coming solely from the diet, as they are not biosynthesized by humans (VUOLO; LIMA; MARÓSTICA JUNIOR, 2019). Phenolic compounds are present in foods such as cocoa, citrus fruits, coffee, and teas. When it comes to consumption levels, studies carried out in developed and developing countries indicate that there is great variability in the daily intake of these compounds. In Denmark, for instance, the average daily consumption is estimated at 1786 mg/day (ZAMORA-ROS et al., 2016). In Brazil, the average consumption was

estimated at 364 mg/day, essentially acquired through fruits and beverages (CARNAUBA; HASSIMOTTO; LAJOLO, 2021).

From the bioactivity perspective, the chemical structure of polyphenolic compounds has a great influence on bioavailability. Quercetin (Figure 3), for instance, is a flavonoid widely found in different plant groups. Quercetin has considerable therapeutic importance, including the modulation of inflammatory processes, the prevention of platelet aggregation, and cardioprotective effects (FERENCZYOVA; KALOCAYOVA; BARTEKOVA, 2020). However, quercetin occurs as a heteroside, or as a glycosylated compound also known as rutin (Figure 3). As the aglycone form of quercetin is more bioavailable, its antioxidant capacity, both concerning the DPPH radical scavenging role and the reduction capacity of ferric ions, is significantly higher than the glycosylated form (LESJAK et al., 2018).

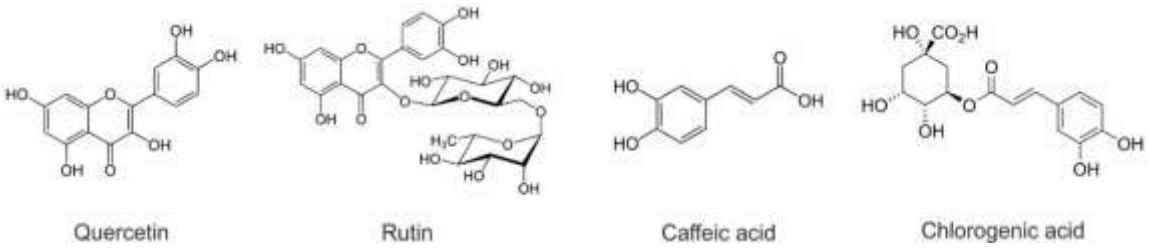

Figure 3. Chemical structures of the aglycone quercetin, its glycosylated derivative rutin, caffeic acid, and its chlorogenic acid ester.

3.2 Oxidative stress and its relationship with neurodegenerative diseases: the physiopathology of parkinson’s disease

The pathophysiology of Parkinson's disease has common mechanisms to Alzheimer's disease. Parkinson’s disease is the second most prevalent degenerative disease worldwide (AARSLAND et al., 2021). A hallmark of the pathogenesis of Parkinson's disease is the formation of α-synuclein protein

aggregates, which compose the so-called Lewy bodies (WALKER; STEFANIS; ATTEMS, 2019). The occurrence of these aggregates in dopaminergic neurons, especially in the substantia nigra, is involved in the rise of motor dysfunctions associated with dopamine loss (TRIST; HARE; DOUBLE, 2019). The neuronal vulnerability in Parkinson's patients is, therefore, related to the accumulation of Lewy bodies, leading to mitochondrial dysfunction, increasing ROS levels, and the establishment of an oxidative environment, which compromises synaptic communications in dopaminergic neurons (Figure 4a). Such cellular dysfunction is followed by the aggravation of neuroinflammatory activation (MACMAHON COPAS et al., 2021).

Oxidative stress on Parkinson's progression is, therefore, established from the selective degeneration of dopaminergic neurons and the accumulation of cellular debris, α-synuclein proteins (a ligand of inflammatory TLR-4 membrane receptors), and microglia activation (GAN et al., 2020) (Figure 4b). Moreover, the inefficiency at storing dopamine inside vesicles is also a source of cytosolic free radicals (*i.e.* via dopamine autoxidation) which implicates in the perpetuation of the cycle of ROS generation and mitochondrial dysfunction. Part of this cycle is continued by the constant activation of inflammation, which is intimately linked to ROS formation (DIAS; JUNN; MOURADIAN, 2013; TRIST; HARE; DOUBLE, 2019).

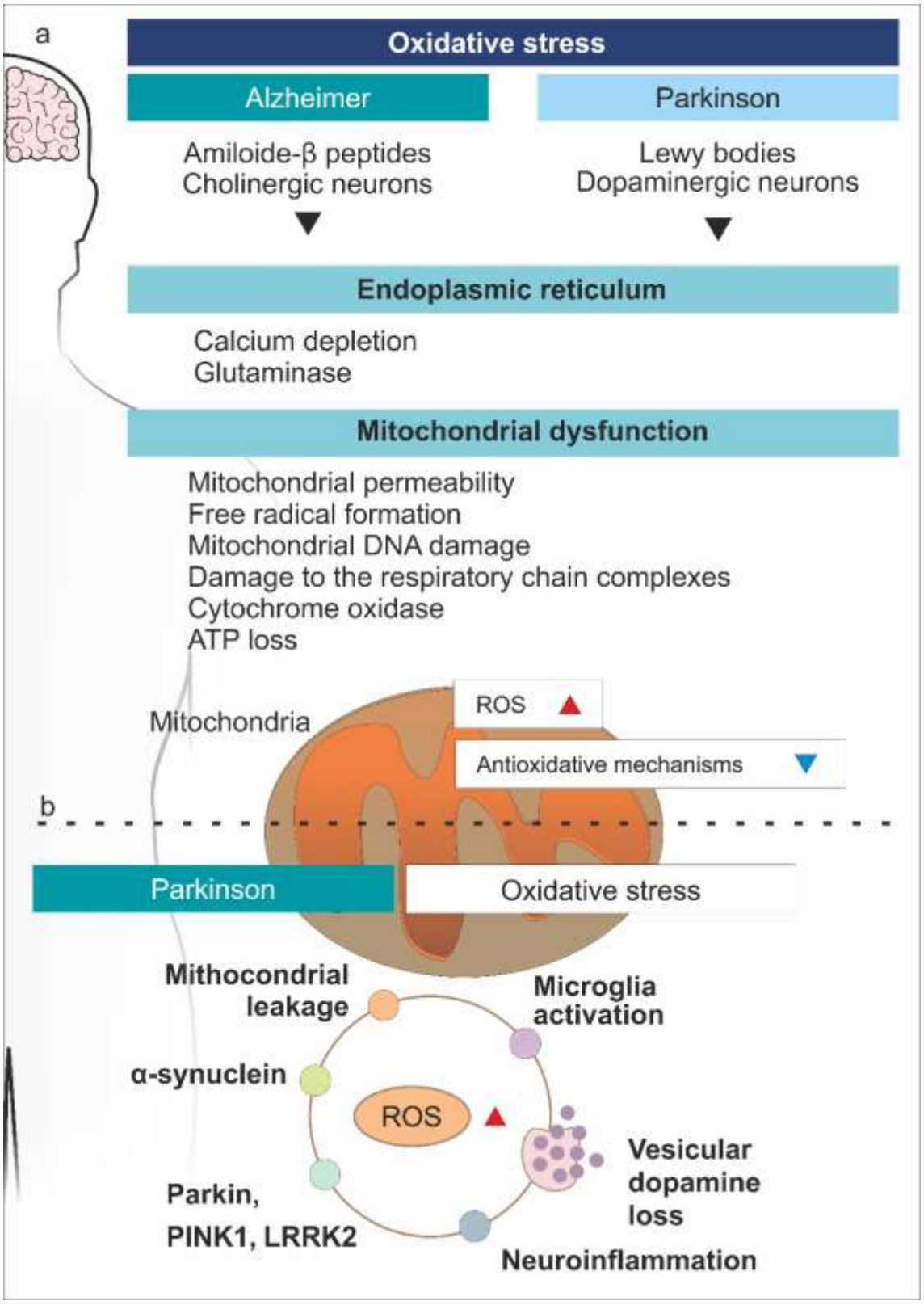

Figure 4. Pathophysiological mechanisms related to the genesis of Alzheimer's and Parkinson's are associated with oxidative stress and mitochondrial dysfunction. (a) consequences of ROS formation, reduced capacity antioxidants, and damage caused by mitochondrial membrane permeability; (b) Factors

contributing to oxidative stress progression in Parkinson’s disease. Decreased mitochondrial energy efficiency and consequent reduction in dopamine levels associated with vesicle loss and dopamine autoxidation.

Drosophila melanogaster has been used as an *in vivo* model of Parkinson’s disease once several genes related to the pathology have homologs in the fly genome. Parkin, PINK1, and LRRK2 for instance are related to energetic metabolism affected during the progression of Parkinson's, added to the conservation of a dopaminergic system (AYAJUDDIN et al., 2018). Moreover, added to the qualities of a short-life spam model, with the easiness of reproduction in lab conditions, the promotion of dopaminergic system modulation is easily achieved by the administration of paraquat or rotenone (HARTENSTEIN et al., 2017).

The control of oxidative processes and inhibition of mechanisms associated with degeneration present in Alzheimer's, such as the inhibition of the enzyme acetylcholinesterase, and in Parkinson's, such as the protection of dopaminergic neurons, is of fundamental importance (TRIST; HARE; DOUBLE, 2019). The relationship between the consumption of antioxidants such as polyphenols and carotenoids is still not clear or consistent, but some evidence has pointed to the therapeutic effectiveness of such compounds on neurodegenerative diseases (CAMILLERI et al, 2013). The lower incidence of Parkinson’s disease in Asia has been for so long an intriguing fact that has been associated with the dietary habits of Japanese and Chinese populations toward higher consumption of tea polyphenols. Epidemiological studies have repeatedly demonstrated an inverse relationship between the consumption of teas and the prevalence of Parkinson's (CARUANA; VASSALLO, 2015). However, more

recent evidence based on powerful statistical analysis, shows that modern lifestyle factors such as the overconsumption of caffeine or tobacco may act as confounding factors, reducing the beneficial effects of polyphenols on health (TAN et al., 2008).

3.3 Tea manufacture and the use of gamma radiation for microbial decontamination

In the manufacture of teas, leaves, flowers, and/or stems from herbal plants are hand or machine collected; however, no deep washing or cleaning occurs to preserve product integrity, color, flavor, and aroma (LANGFORD, 2021). Different origins may account for the microbial content in this class of products, including phylloplane flora, human handling, and storage conditions (MISHRA; GAUTAM; SHARMA, 2006). Gamma irradiation treatment is used by the food industry to increase the shelf life while maintaining the nutritional value of foods (VAN DOREN et al., 2013). Gamma irradiation stands out in substitution for conventional thermal methods, which promote up to 30% loss of water-soluble and thermally unstable molecules, such as phenolic compounds (VOLF et al., 2014). Artificial (80 °C) or sun-drying processes reduce water and, consequently, microbial content. However, the occurrence of spore-forming microorganisms may remain, including pathogenic bacteria or fungal species (MISHRA; GAUTAM; SHARMA, 2006). Gamma irradiation is considered a safe technology and has been successfully used in the microbial control of dried products such as spices and teas. The United States Food and Drug Administration (FDA) limits the use up to a dose of 30 kGy (FDA 2021). However, the extension of the effects of ionizing radiation over hibiscus bioactivity is not yet known. The resulting free

radicals, for instance, promote the degradation or oxidation of polyphenols with antioxidant effects (FANARO et al., 2014).

3.4 The application of gamma radiation to the hibiscus supply chain

Technologies such as gamma irradiation or the use of electrical pulses are capable of increasing food processing efficiency and reducing organoleptic losses, maintaining nutritional characteristics, including the content of antioxidant compounds, such as polyphenols, in a less invasive and more efficient and cost-effective fashion (DIEHL, 2002; MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005).

A common irradiation source used in food processing is gamma radiation derived from cobalt (60Co) or cesium (137Cs) (GAUTAM; TRIPATHI, 2016). The penetration capacity of these forms of ionization is high and can be applied in different food matrices, including already packaged foods, making the chain faster and more fluid production (FARKAS, 2006). Gamma irradiation would take place in different blocks, at local or international levels, especially during packing or repacking steps in product retailers along the hibiscus supply chain (briefly summarized in Figure 5 (PAUL et al., 2021).

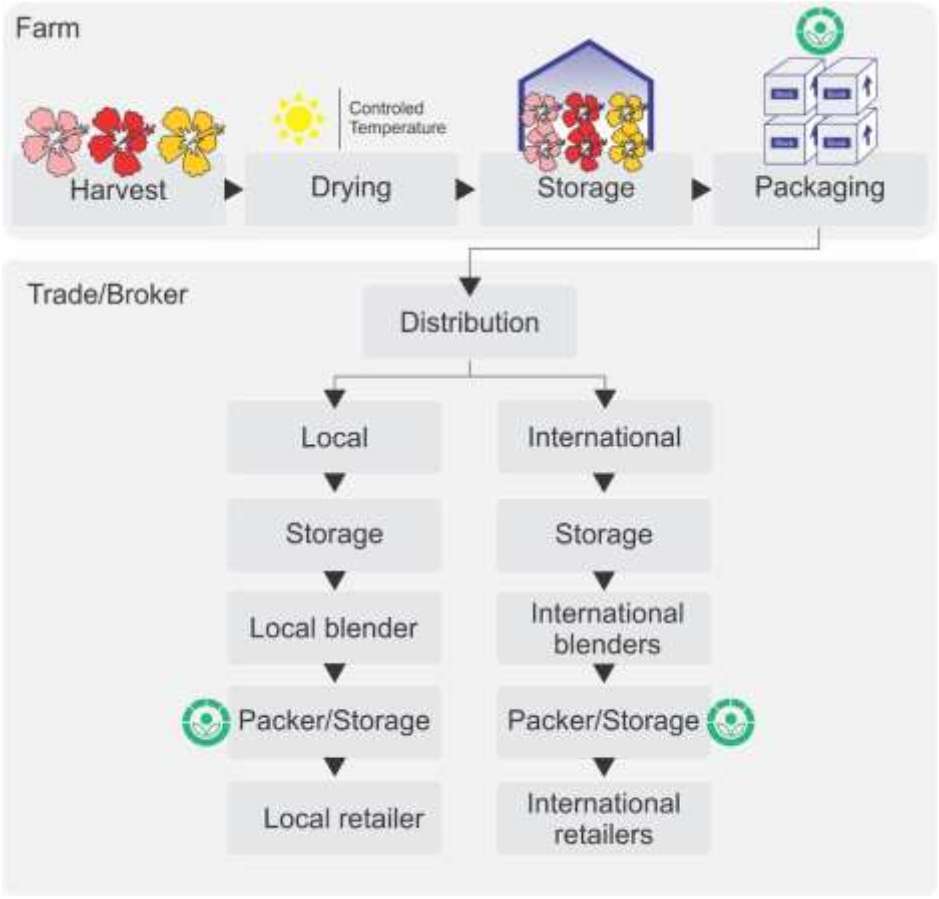

Figure 5. General tea supply chain representation adapted to hibiscus production, distribution, and sales scheme.

Gamma irradiation is a form of ionizing radiation that acts primarily to break the DNA of microorganisms, insects, or protozoa present in the food. In this way, microbial contamination and possible food spoilage might be blocked as demonstrated by the application of gamma radiation to coffee beans to eliminate *Aspergillus ochraceuse* and inactivation of ochratoxin (KUMAR et al., 2012). The use of gamma radiation may also promote the elimination of anti-nutritional compounds such as phytates (FANARO et al., 2014; KHAN et al., 2018).

The application of gamma radiation in foods rich in phenolic compounds has been reported to improve nutritional quality by increasing the bioavailability of phenolic compounds as a result of the glycosidic bond breakage (KHAN et al.,

2018). The breakdown of the glycosidic bonds, as previously described for the Quercetin in Chapter 2, releases the aglycone forms that are more bioavailable and more active than the glycosylated forms. In soybeans, for example, increasing the irradiation dose gamma to 5 kGy and the consequent release of aglycones from polyphenols present in vegetable seeds, potentiate the antioxidant capacity (VARIYAR; LIMAYE; SHARMA, 2004). The same effect was reported for almond extracts submitted to doses of irradiation greater than 4 kGy (HARRISON; WERE, 2007).

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CHAPTER 1

Hibiscus, Rooibos, and Yerba Mate for Healthy Aging: A Review on the Attenuation of *in Vitro* and *in Vivo* Markers Related to Oxidative Stress, Glycoxidation, and Neurodegeneration

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ABSTRACT

The world is currently undergoing a demographic change towards an increasing number of elderly citizens. Aging is characterized by a temporal decline in physiological capacity and oxidative stress is a hallmark of aging and age-related disorders. Such an oxidative state is linked to a decrease in the effective mechanisms of cellular repair, the incidence of post-translational protein glycation, mitochondrial dysfunction, and neurodegeneration, just to name some of the markers contributing to the establishment of age-related redox imbalance. Currently, there are no prescribed therapies to control oxidative stress; however, there are strategies to elevate antioxidant defenses and overcome related health challenges based on the adoption of nutritional therapies. It is well known that herbal teas such as hibiscus, rooibos, and yerba mate are important sources of antioxidants, able to prevent some oxidation-related stresses. These plants produce several bioactive metabolites, have a pleasant taste, and a long-lasting history as safe foods. This paper reviews the literature on hibiscus, rooibos, and yerba mate teas in the context of nutritional strategies for the attenuation of oxidative stress-related to glycoxidation, and neurodegeneration and here Alzheimer's Disease is approached as an example. The focus is given to mechanisms of glycation inhibition as well as the neuroprotective *in vitro* effects and in animal studies to frame the interest in these plants as nutraceutical agents related to current health concerns.

Keywords: herbal teas; oxidative stress; glycoxidation; neurodegeneration; therapeutics.

1 INTRODUCTION

People are living longer. Currently, emergent and developed countries are undergoing a demographic transition with an increasing number of elderly citizens. According to the World Health Organization (WHO), the total number of people over 60 years of age will account for 22% (2.1 billion) of the global population by 2050 (WHO, 2021). The demographic aging process imposes a series of socioeconomic challenges owing to the elevated number of geriatric individuals affected by age-related diseases (*e.g.* metabolic dysfunction, neurodegenerative diseases, and cardiovascular diseases) (LU; PIKHART; SACKER, 2018). These changes are linked to the temporal decline of physiological and cognitive capacities resulting from inefficient mechanisms of cellular repair (*e.g.* polymerase read-proofing activity), accumulation of non-functional cellular proteins (*e.g.* glycated proteins), genetic degradation (*e.g.* mutations), and mitochondrial dysfunction, which merge to define the molecular aging process. In addition to environmental factors such as lifestyle (*e.g.* smoking, retirement, housing), exercise practice, and/or diet also play a role in aging acceleration (KIM et al., 2022; XIA et al., 2021).

An aging hallmark is the establishment of an oxidative stress state, which led to the formulation of the Oxidative Stress Theory of Aging (HARMAN, 1956). This oxidative status coincides with the incidence of several chronic diseases, accounting for the accumulation of defective cellular apparatus (IONESCU-TUCKER; COTMAN, 2021). At the biological level, distinct mechanisms create a fruitful environment for the aggravation of oxidative stress, including the accumulation of misfolded proteins (*e.g.* amyloid-beta peptides) (IONESCU-TUCKER; COTMAN, 2021), glycation (RUNGRATANAWANICH et al., 2021),

shifts in calcium homeostasis (LUDHIADCH et al., 2022), dysfunction of the mitochondrial electron chain (OBRADOR et al., 2021), and cellular signaling cascades disruptions (KASHYAP et al., 2019). Additionally, as cells undergo senescence, repair mechanisms become less efficient, including anti-oxidative (*e.g.* reduction in glutathione levels) (MINICH; BROWN, 2019), as well as anti-glycation barriers (*e.g.* glyoxalase system) (HE et al., 2020).

A therapeutic strategy to elevate antioxidant defenses and overcome health challenges is the use of nutritional therapies (XIA et al., 2021). Recently, there has been keen interest in healthier lifestyles, leading to the search for functional foods capable of reducing the deleterious effects of molecular aging (DOMINGUEZ et al., 2022). This interest has been driven by many studies on natural products, mainly medicinal plants, which have provided insights into the protective effects of diverse phytochemicals (BRIMSON et al., 2021; LUTHRA; ROY, 2022). Polyphenols are the most abundant bioactive compounds present in a variety of plant species (ADNAN et al., 2022). When acquired through diet, polyphenols attenuate pathological processes related to oxidative stress, aging, and neurodegeneration, turning these metabolites into a commercial target for the food and pharmaceutical industries (KESAVAN et al., 2018). Other plant metabolites, such as alkaloids, have also been demonstrated to have a protective effect on the central nervous system as emerging alternative treatments for anxiety reduction, and as antidepressant drugs (AHMED et al., 2021; AKKOL et al., 2021).

The data presented in this review highlight the bioactivity of herbal plants such as hibiscus, rooibos, and yerba mate in the attenuation of oxidative stress markers, glycoxidation, or neurodegeneration, both *in vitro* and *in vivo* (animal

studies), which may contribute to the aging slowdown (LUNCEFORD; GUGLIUCCI, 2005; CHEN et al., 2013; SANTOS et al., 2018; CITTADINI et al., 2019; SARIMA; ASTUTI; MERYANDINI, 2019; EL-SHIEKH et al., 2020). Hibiscus, rooibos, and yerba mate teas are herein contextualized as alternative strategies for the attenuation of oxidative stress, to frame the current interest in these plants as nutraceutical agents. The essential mechanisms of age-related oxidative stress, particularly those associated with protein glycation and neuropathologies are presented. The following sections describe the recent discoveries regarding the use of these plants in such pathological contexts. Finally, we address the perspectives and research gaps that remain to be filled.

2 OXIDATIVE STRESS PROMOTING MECHANISM RELATED TO GLYCATION AND NEURODEGENERATION

2.1. Protein glycation and oxidative stress

Post-translational protein modifications have a potential effect on molecular aging, cell dysfunction, and chronic disease development (GORISSE et al., 2016). First introduced by the French biochemist Louis Camille Maillard in 1912, the Maillard Reaction characterizes a series of non-enzymatic reactions between free amino acids or protein-amino residues and reducing carbohydrates resulting in a group of heterogeneous and chemically stable neoformed compounds called Advanced Glycation End-Products (AGEs) (TESSIER, 2010). Clinical consequences were later discovered in the association between glycation and diabetes progression, cardiovascular implications, and vascular stiffness promotion (TEISSIER et al., 2019; TEISSIER; BOULANGER, 2019). Besides the spontaneous occurrence of glycation *in vivo*, thermal processing of foods also promotes the formation of AGEs (NAIK; WANG; SELOMULYA, 2021). Some

dietary AGEs are of great interest to the food industry because of the appealing and sensorial aspects of food such as flavor, aroma, and color, in addition, to increasing the pool of glycation products *in vivo* (MURATA, 2021; XIANG et al., 2021). In contrast, several AGEs (*e.g.* acrylamide, and carboxymethyl-lysine - CML) act as potential activators of inflammation, oxidative stress, or even disturbing gut epithelial homeostasis (GUIBOURDENCHE et al., 2021).

Some glycation pathways are described as aging-promoting mechanisms such as the modifications in extracellular matrix proteins, the reduction of cellular connectivity, elasticity and tissue flexibility, the promotion of tissue loss of function, and oxidative stress/inflammation activation mediated by the specific interaction with the Receptor for Advanced Glycation End-products (RAGE) (KIM; PARK; KIM, 2017). RAGE is a multi-ligand receptor part of the immunoglobulin superfamily that is involved in the initiation of innate pro-inflammatory responses and oxidative stress trigger (ALEXIOU et al., 2010; BOULANGER et al., 2007). Studies on AGE-RAGE axis activation have linked the increased expression of intracellular oxidative and pro-inflammatory signals such as NADPH oxidase, and cytokines in renal tissue, respectively (SERBAN et al., 2015). Another important implication of glycation in the cellular redox control is related to the loss of function of proteins related to redox control. Macromolecular changes have been reported in catalases (BAKALA et al., 2012), and glutathione (SURAVAJJALA et al., 2013) which are essential for redox homeostasis.

Together, glycation and oxidative stress lead to glycoxidation and a downstream oxidative stress activation (Figure 1A) (AHMAD et al., 2018). The early stage of the Maillard Reaction involves a series of reversible sugar-amino

group rearrangements. The oxidative degradation of Schiff bases and Amadori Products, together with glucose autoxidation, and the cellular metabolism (*e.g.* glycolysis), result in the generation of α-oxaldehydes (*e.g.* glyoxal, methylglyoxal) (Figure 1A). The formation of CML, one of the most discussed AGE in the pertinent literature, has been consistently demonstrated to increase under aerated conditions with different α-carbonyl precursors (glycolaldehyde and glyceraldehyde) (GLOMB; MONNIER, 1995; RAU; GLOMB, 2022).

Over the last decades, several compounds have been demonstrated to inhibit AGE formation as thiamine, and pyridoxamine (BOOTH; KHALIFAH; HUDSON, 1996), to inhibit the related redox imbalance during the early stages of the reactions as Epalrestat (HAMADA et al., 2000), or by breaking AGE cross-links as alagebrium (HARTOG et al., 2011) (Figure 1A). Aminoguanidine, a hydrazine derivative, was shown to be the most promising synthetic drug for AGE formation inhibition but severe countereffects associated with kidney damage diminished its clinical potential (Phase III) (NEPHROGENEX, INC., 2016). On the other side, natural products have been used as a strategy to mitigate AGEs formation (SONG et al., 2021). Currently, the screening of natural compounds (*e.g.* polyphenols, polysaccharides, terpenoids, vitamins, and alkaloids) for new glycation inhibitors has gained attention because of the historically safe consumption profile of these molecules (SONG et al., 2021). Natural products can act on any phase of the series of reactions on the AGEs formation pathways, from sugar-protein interaction blocking, to attenuating glycoxidation through trapping intermediates including reactive dicarbonyl, or free radicals, and/or by breaking down formed AGE crosslinks (ANWAR et al., 2021; VELICHKOVA; FOUBERT; PIETERS, 2021).

Two main research fronts have been explored in the literature investigating natural products as inhibitors of protein glycation. First, is the use of natural products as nutraceuticals. Quercetin, for instance, has been demonstrated to trap methylglyoxal and glyoxal, thereby reducing subsequent AGE formation *in vitro* (LI et al., 2014). A similar effect was observed in mice that received oral quercetin supplementation. After 6 weeks of feeding, the group of animals in which quercetin was provided together with methylglyoxal had lower circulating levels of methylglyoxal and AGEs (ZHAO; TANG; SANG, 2021). Second, food additives can inhibit glycation during food processing. In this second strategy, natural products are less explored because of the significant sensory changes in food (*e.g.* texture, color). Quercetin has been investigated in both cookies and bread models. In both foods, quercetin addition was able to consistently mitigate total AGE formation, but sensorial changes in dough elasticity were observed (LIN; ZHOU, 2018; ZHANG; CHEN; WANG, 2014).

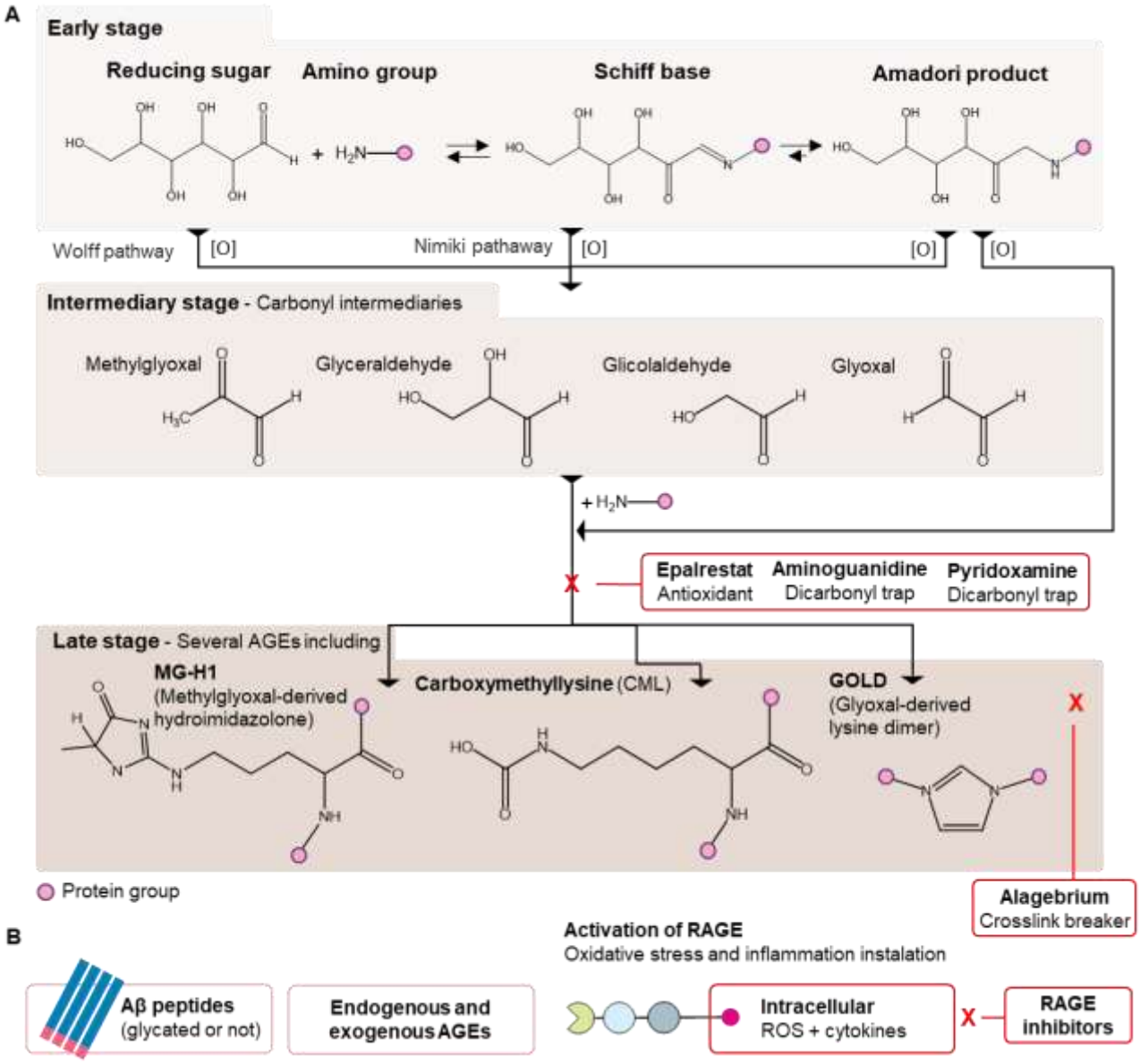


Figure 1. (A) Schematic representation of early stage of the Maillard Reaction and related oxidative pathways. The oxidative cleavage of Schiff base and Amadori Products results in the formation of reactive dicarbonyls. Dicarbonyls are also by-products of glucose autoxidation and glycolysis. Reactive dicarbonylic intermediaries are further involved in glycoxidation leading to the formation of AGEs (SERGI et al., 2020). Epalrestat, aminoguanidine, pyridoxamine, and alagebrium have been identified as mitigators of the Maillard Reaction, taking part in different steps. (B) The resulting AGEs in the latest phase of the Maillard reaction, both from endogenous and exogenous sources, have been identified as ligands and potential activators of RAGE. Such interaction leads to downstream activation of oxidative stress and inflammation. From the clinical perspective, a therapeutic strategy to mitigate the activation of RAGE, as

well as the progression of Alzheimer's Disease, has been focused on the development of RAGE inhibitors *(KONG et al., 2020)*.

2.2. Oxidative stress and neurodegeneration: a case of alzheimer's disease

The occurrence of exacerbated oxidative process is common in Alzheimer's Disease and other chronic neurodegenerative disorders (HASSAN et al., 2021). Many mechanisms are involved in the progression of oxidative stress in the brain (Figure 2). The gradual loss of neurons, motor impairment, and aggregation of proteins (*e.g.* TAU proteins, Aβ-peptides) characterize these diseases (JAGUST, 2018). From the oxidative perspective, mitochondrial dysfunction is a common factor since neurons are post-mitotic cells that demand large amounts of energy, use high levels of oxygen, and are inserted into an environment with lower antioxidant capacity, therefore, more susceptible to damage, composing the Mitochondrial Hypothesis of aging (KOWALSKA et al., 2020). This hypothesis identifies mitochondria, the cellular organelle responsible for energy production, as the main source of ROS (reactive-oxygen species), especially in energy-demanding organs such as the brain (LIN; BEAL, 2006). In this context, excessive production of oxygen and reactive nitrogen species (NOS), promotes the oxidation of proteins, lipids, and nucleic acids, providing the emergence of pathologies such as Alzheimer's Disease (IONESCU-TUCKER; COTMAN, 2021).

Alzheimer's Disease is a complex neurological dysfunction and the most prevalent form of dementia worldwide (CAO et al., 2020). The origin of the disease can be either genetically determined, developed in early adulthood, or

associated with aging (ZHANG et al., 2021a). The main pathophysiological feature of Alzheimer's Disease is the deposition of extracellular fibrillar protein aggregates called amyloid-β peptides (Aβ) (a well-known RAGE ligand) and hyperphosphorylation of the TAU protein. These protein aggregates initiate neuroinflammatory processes, leading to neuronal death and synaptic communication failures (BUSCHE; HYMAN, 2020). They lead to a depletion of calcium in the endoplasmic reticulum, reducing the activity of GSH, and leading to the accumulation of ROS (CENINI; LLORET; CASCELLA, 2019). A clear association between the deposition of amyloid aggregates is related to the impairment of complexes IV and V of the mitochondrial electron transport chain, and increased permeability of mitochondrial membranes (PICONE et al., 2020). Mitochondrial impairment leads to a second molecular marker identified in Alzheimer’s Disease patients: the reduction in the synthesis of acetylcholine. Acetyl-CoA, an acetylcholine precursor, is synthesized in the mitochondrial matrix (WONG et al., 2020). This neurophysiological landmark, described by the Cholinergic Hypothesis, is one of the main characteristics of the disease, and it may occur up to 30 years before the appearance of the first clear signs of the pathology (HAMPEL et al., 2019). Based on this physiological framework, the only commercially available drugs for the treatment of Alzheimer’s disease are inhibitors of the acetylcholinesterase enzyme that promotes the hydrolysis of acetylcholine, attenuating the effects of low levels of the neurotransmitter (TAKAHASHI et al., 2019).

Glycation-related pathways are also involved in Alzheimer’s disease pathophysiology. Aβ-peptides have been identified as RAGE ligands (JANGDE; RAY; RAI, 2020) (Figure 1B). Aβ peptide crosslinks have been demonstrated to

be associated with glycation, which was shown to modulate the interaction Aβ-RAGE, increasing the affinity of the glycated clusters to the receptor (BATKULWAR et al., 2018). Furthermore, based on the in-situ analysis of neurons, Rudy et al. (2001) demonstrated the presence of CML in neurofibrillary tangles of patients with Alzheimer's Disease (CASTELLANI et al., 2001).

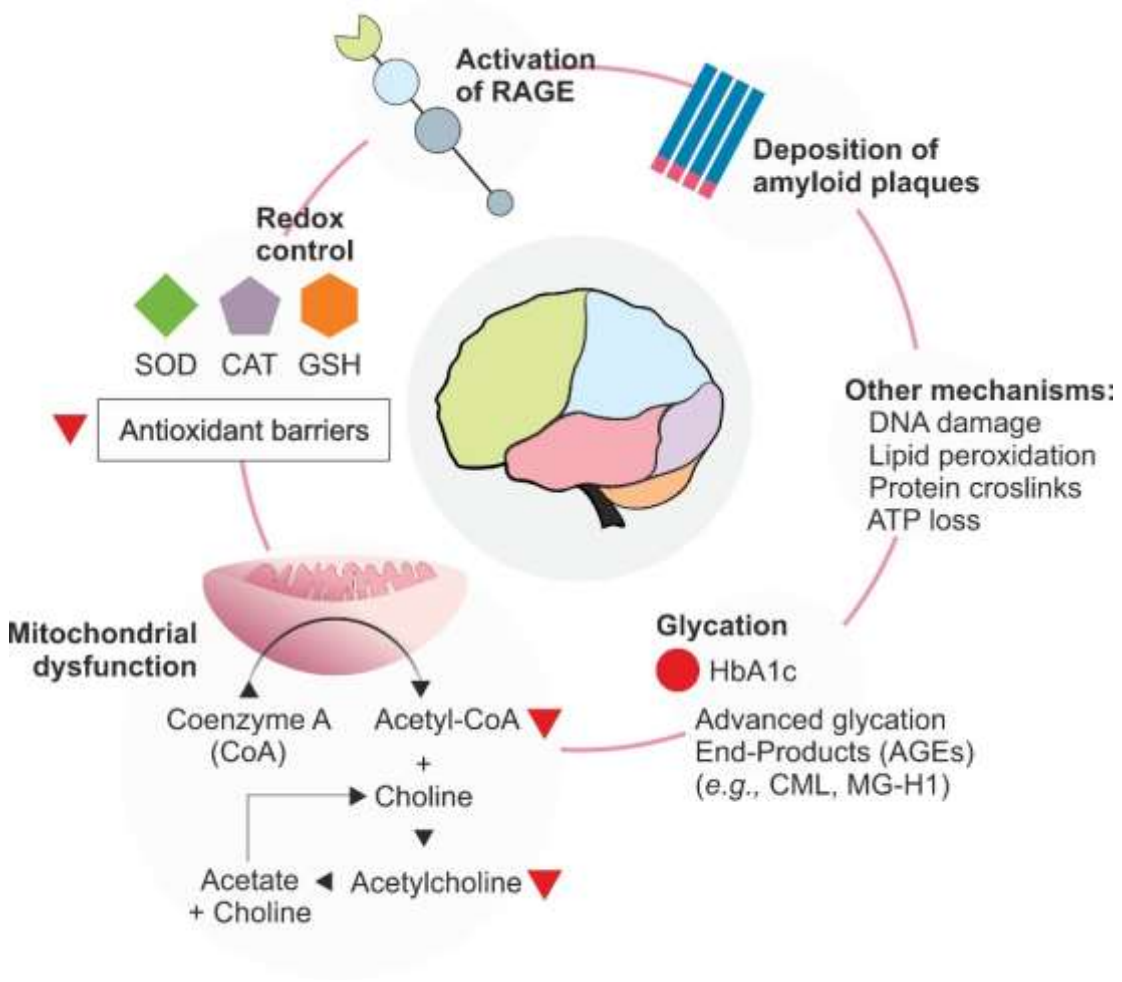


Figure 2. Schematic representation of some biological mechanisms on the interplay of glycation, neurodegeneration, and the progression of oxidative stress. Mechanisms in brain degeneration are highlighted. Both glycation as well as cellular degeneration, are involved in the activation of local (i.e. brain) and systemic oxidative stress. The accumulation of dysfunctional mitochondria, DNA, damage, lipid peroxidation, and/or energetic imbalance is able of inducing severe damage to cells. AGEs as well as Aβ-peptides have a related stress activation on membrane-RAGE, which is a promiscuous receptor interacting with both

ligands. CML: carboxymethyl-lysine; MG-H1: Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine.

3 HIBISCUS, ROOIBOS, MATE AS SOURCES OF NATURAL BIOACTIVE COMPOUNDS

As several pathological mechanisms hold an oxidative stress factor, some challenges have been limiting the real perspectives on antioxidant therapy application. There is a lack of consensus in the scientific literature regarding the effectiveness of antioxidant therapies. Some reasons have been recently pointed out concerning the definition of the real extension of oxidative stress as a disease-promoting factor added to the underestimation on the related pathways these therapies could engage. Yet, there is no definition of effective doses for *in vivo* effect (FORMAN; ZHANG, 2021). The simple scavenging of radicals would be a simplistic use to fulfill the gaps in the potential of plant bioactive compounds as potential antioxidant molecules, and an outlook should be done on the related mechanism as the activation of physiological pathways as the SOD (Superoxide dismutase) system, CAT (Catalase), or the downregulation of pro-inflammatory cytokines as TNF-α related to the downstream activation of oxidative stress. Flavonoids from Chinese medicinal herbs have been demonstrated to modulate such pathways, contributing to the regulation of oxidative stress in mice brains (MAI et al., 2021).

The *in vitro* antioxidant effect of plant bioactive compounds has been largely demonstrated. When it comes to the epidemiological approach, studies on the consumption of plant-based diets rich in polyphenols have been published since early 1990. A follow-up study (5 years) with 805 individuals in the

Netherlands indicated the daily consumption of 259 mg of flavonoids mainly from teas (61%) was inversely proportional to the risk of coronary heart diseases. Such a relationship was later demonstrated in other populations, but a limiting factor in this issue has recently been raised from metadata analysis, which demonstrated that the great variation in terms of bioactive compound intake across different studies limits the effects of translation from *in vitro* to *in vivo* trials (CONDEZO-HOYOS; GAZI; PÉREZ-JIMÉNEZ, 2021).

Many plant species exhibit significant neuroprotective *in vitro* and *in vivo* activities (GREGORY et al., 2021). These plants contain multiple molecules, capable of working through different mechanisms of action, which could benefit the search for therapeutics for complex neurodegenerative disorders. Flavonoids, for instance, are ubiquitously distributed plant constituents, with more than 6000 structures already identified. These polyphenolic compounds are notorious antioxidants that must be acquired from the diet, as they are not biosynthesized by the human body (VUOLO; LIMA; MARÓSTICA JUNIOR, 2019). Flavonoids have been reported to be effective neuroprotective agents with several advantages, such as safety, good pharmacokinetic flow, capacity to penetrate the blood-brain barrier, and cost-effectiveness (HAMSALAKSHMI et al., 2022). Alkaloids are another class of ubiquitous bioactive natural products. These nitrogen-containing natural products have a wide array of chemical structures, that have long been known for their therapeutic activities, especially by stimulating the central nervous system in humans (DEBNATH et al., 2018). Alkaloids also act as anti-cancer and neuroprotective agents (LI et al., 2021; PLAZAS et al., 2022) as well as in the cardiovascular system (ZHANG et al.,

2022). The main alkaloid present in food sources is caffeine, a dimethylxanthine present in coffee and cocoa (Table 1) (GUTIÉRREZ-GRIJALVA et al., 2020).

Flavonoids and alkaloids are present in a wide variety of foods, including herbs that have remarkable therapeutic properties and have long been used in tea preparations. Teas are the most consumed plant-based beverages with a high-value market around the world, expected to reach $318 billion by 2025 (KHAN; MUKHTAR, 2018; PATIL; BACHUTE; KOTECHA, 2021). The tea market is mainly driven by black, oolong, and green tea products derived from *Camellia sinensis* (TEA ASSOCIATION OF THE USA, 2021). Comprehensive reviews have been published on the phytochemical composition of fermented and non-fermented *C. sinensis* leaves, which are composed of phenolic compounds (flavan-3-ols, epicatechins, catechins), and alkaloids such as theobromine (BORTOLINI et al., 2021). These plant metabolites have been associated with positive health effects including antioxidant, anti-inflammatory, cardioprotective, and neuroprotective (SAMANTA, 2020). In response to market demand, the food industry is urging the development of new products with functional and health claims, leading other herbal infusions to gain public attention in European, American, and Asian markets (SILVA et al., 2016). Hibiscus (*Hibiscus* sp.), rooibos (*Aspalathus linearis*), and yerba mate (*Ilex paraguariensis*) are important examples of herbal plants (Figure 3) that are mostly found in African and South American local markets and have been traditionally used in the production of flavorful non-alcoholic beverages (DA-COSTA-ROCHA et al., 2014; BIÉNABE; MARIE-VIVIEN, 2017; GREGIANINI; WINGE, 2019). These plants have been demonstrated to have important health effects as anti-cancer (*in vitro*) (HUANG et al., 2020), anti-diabetes (*in vivo*) (ORLANDO et al., 2019), or anti-inflammatory

(*in vivo*) (GEBARA et al., 2021). These health benefits play a role as commercial boosters for increasing market demands for new plant-based products with functional properties. The hibiscus market, for instance, is projected to grow 7% by 2027. During 2021-2027, the yerba mate market is estimated to increase by almost 5%, while the rooibos market is mainly held by South African farmers with 7000 hectares and 15000 tones yearly produced (ELISHA; VILJOEN, 2021; HEIBERG; HUTCHINGS, 2021; YERBA MATE MARKET SIZE 2022-2027 | KEY PLAYERS, REGIONAL ANALYSIS, SEGMENTATION BY TYPES AND APPLICATIONS | OPPORTUNITIES, CHALLENGES, TRENDS, DRIVERS | SHARES, REVENUE, AND SALES, [s.d.]). These crops are alternatives to *C. sinensis* tea, which market has shrunk over the last years due to adverse climate conditions (FAO INTERGOVERNMENTAL GROUP ON TEA | FAO | FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, [s.d.]). Therefore, investing in hibiscus, rooibos, and mate markets may contribute to the local crop expansion, more sustainable and biodiverse agricultural development, and the reduction of local inequalities among small-scale farmers.

Figure 3. Commercial **(A)** rooibos, **(B)** hibiscus, and **(C)** green yerba mate dry herbal teas.

The *Hibiscus* genus is native to Africa, with distribution between the Middle East, Asia, and Latin America, and has great taxonomic diversity with more than

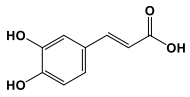
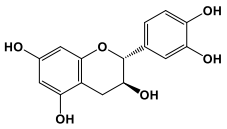
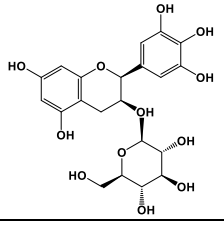
300 species cataloged between the tropics and subtropics (DA-COSTA-ROCHA et al., 2014). The main species of hibiscus intended for human consumption is *H. sabdariffa* (Figure 3), popularly known as roselle, and *H. rosa-sinensis*, the Chinese mellow. In African countries such as Egypt and Nigeria, extracts of *H. sabdariffa* are added to gelatin and fruit juices to improve the nutritional value and attractiveness of foods (ISLAM, 2019). The nutritional value of hibiscus petals is corroborated by the presence of vitamins A and E, ascorbic acid (vitamin C), calcium, and iron (DA-COSTA-ROCHA et al., 2014). The phytochemical profile of hibiscus extracts also comprises tocopherol and linoleic acid, organic acids (malate, oxalate, citric, and hibiscus acid); phenolic acids (caffeic and chlorogenic acids), and other phenolic compounds such as flavonoids and anthocyanins (JABEUR et al., 2017). The first anthocyanin isolated from aqueous extracts of hibiscus was hibiscin (delphinidin-3-sambubioside) which corresponds to 71% of the anthocyanin content in hibiscus extracts (TSAI et al., 2002). This metabolite has long been known to participate in the modulation of mitochondrial ROS production and the induction of apoptosis in human leukemic cells (HOU et al., 2005). Furthermore, it can mediate inflammatory processes by reducing intracellular inflammatory signals such as IL-6 and TNF-α (SOGO et al., 2015). The related effects of hibiscin and other frequently investigated phytochemicals on hibiscus, rooibos, and mate (following discussion) are presented in Table 1.

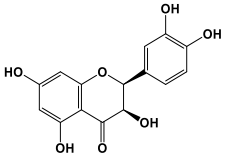
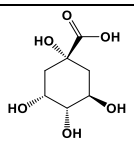
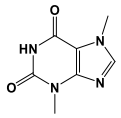
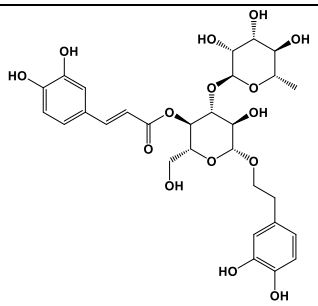
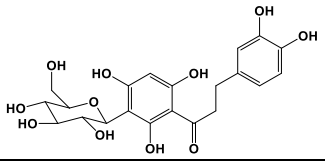
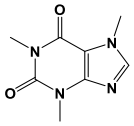
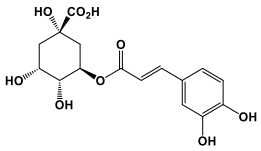
Rooibos (Figure 3) is a common name for the shrubby legume from the South African species *A. linearis*. Rooibos is prepared from either leaves or stems, and consumers have been increasing because of its health benefits and caffeine-free composition compared to other teas (STANDER; JOUBERT; DE BEER, 2019). In addition, this plant has a unique flavonoid profile including

dihydrochalcone aspalathin in both fermented and non-fermented rooibos which has been demonstrated to help with type-2 diabetes slowdown (MULLER et al., 2022). Non-exclusive polyphenols such as orientin, isoorientin, isoquercitrin, rutin, and quercetin, have also been described in rooibos (JOUBERT et al., 2012).

Mate (Figure 3) tea results from *I. paraguariensis* leaf infusions, a plant originally from South America, that is endemic to Brazil, Argentina, Paraguay, and Uruguay where the beverage is traditionally consumed (CARDOZO et al., 2021). Yerba mate infusions are either consumed from green or toasted leaves, to which scientific support has been demonstrated to be predominantly composed of chlorogenic acids and xanthine derivatives, such as caffeine and theobromine, which have antioxidant (*in vitro*) (TESELKIN et al., 2021), as well as hepatoprotective (*in vivo*) (JANG et al., 2018), antimicrobial (PALUCH et al., 2021), and anti-cancer (*in vitro*) effects (SALEH et al., 2021).

Table 1. Reported bioactive natural products in rooibos, hibiscus, and/or mate extracts.

Experimental condition	Compound [Class]	Chemical structure	Associated bioactivity [model]	Effect
In vitro	Caffeic acid [Phenolic compound]		Anti-glycation [Fluorescence 370/440 nm]	AGE formation Control: 180 % Caffeic acid (0.2 mM): 80 % (CAO et al., 2019)
	Epicatechin [Polyphenol]		Neuroprotective [SHSY5Y cells]	Parkin expression Rotenone (1 µM): 110 (a.u) Rotenone + Epicatechin (10 µM): 60 (a.u.) (LUO et al., 2021)
	Hibiscin [Polyphenol]		Anti-inflammatory [RAW264.7 macrophage cells]	IL-6 expression Control (LPS 1 mg/kg): 1750 pg/mL LPS+ Hibiscin (15 µM/kg): 750 pg/mL (SOGO et al., 2015)

Experimental condition	Compound [Class]	Chemical structure	Associated bioactivity [model]	Effect
	Quercetin [Polyphenol]		Attenuation of mitophagy [Primary microglia]	MitoSox Control (LPS (100 ng/mL): 28 (a.u.) LPS+Quercetin (30 µM): 8 (a.u.) (HAN et al., 2021)
	Quinic acid [Polyol]		Photoprotective [HaCaT keratinocytes]	UVB irradiation-induced ROS generation Control: 2000 (a.u.) Quinic acid: (10 uM): 1500 (a.u.)
	Theobromine [Methylxanthine]		Adipogenesis attenuation [SGBS cells]	Adipogenic differentiation Control: 100% Teobromine (100 µg/mL): 60% (FUGGETTA et al., 2019)
In vivo	Acteoside [Polyphenol]		Glucose metabolism [Male Wistar albino rats]	Blood glucose Control: 318 mg/dL Acteoside (40 mg/kg): 75 mg/dL (Oral) (EL-MARASY et al., 2020)
	Aspalathin [Polyphenol]		Nephroprotective [Male C57BL/6 mice]	Creatinine levels Control: 0.47 mg/dL Aspalathin (1.00 mg/kg): 0.25 mg/dL (Intravenous injection) (YANG et al., 2018)
	Caffeine [Methylxanthine]		Anti-AChE [Male albino rats]	AChE activity Control: 7 µmol/min/g Caffeine (20 mg/kg/day): 2 µmol/min/g (Oral) (HOSNY et al., 2019)
	Chlorogenic acid [Polyphenol]		Neuroprotective [Swiss albino mice]	Mitochondrial damage Control (MPTP): 3 nM Chlorogenic acid + MPTP: 5 nM (Oral) (SINGH et al., 2020)

SGBS: human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell; SHSY5y: human neuroblastoma cell line; RAW262.7: murine monocyte/macrophage-like cells.

4 THE POTENTIAL OF HIBISCUS, ROOIBOS, AND MATE IN GLYCOXIDATION AND NEURODEGENERATION ATTENUATION

The well-known food safety associated with the consumption of hibiscus, rooibos, or yerba mate and the acceptance of these herbs linked to pleasant taste, have elicited great interest in defining their nutraceutical potential. So far, no acute toxic effects have been inferred from the consumption of hibiscus or yerba mate extracts based on short or long-term experiments in rodents, neither from histochemical tissue essays nor biochemical serum analysis (ANDRADE et al., 2012; SIREERATAWONG et al., 2013). On the other hand, cases of acute hepatitis in South Africa and France have been recently described to be potentially associated with rooibos consumption but no clear link has been stated in both case reports (CARRIER et al., 2021; ENGELS et al., 2013). Recent discoveries have been made both *in vitro* and *in vivo* (animal models) on the effects of these plants as antioxidants, for the prevention of AGE formation, and neuroprotective effects. The major bibliography addressing these plants relies on the effects of crude aqueous extracts in a way that mimics the traditional methods of preparation. With the increasing understanding of the diverse mechanisms leading to elevated ROS levels, more significant biomarkers have been explored in the literature to draw conclusions on the effects of phytochemicals in this context. Table 2 summarizes some major targets investigated on hibiscus, rooibos, and mate-related literature associated with oxidative stress, extrapolating ROS production measures. Estimating oxidative constraints may range from the direct detection of oxidative species to their impacts as oxidized lipids. However, while the expression of CAT, SOD, or MAO (Monoamine oxidase) is well explored in the context of the consumption of these herbal drinks,

other markers of oxidative stress remain to be investigated as glycation products (*e.g.* pentosidine, carboxyethyl-lysine, CML) (SHAW; BAYNES; THORPE, 2002; TSUKAHARA et al., 2003). Due to increasing clinical interest and the significance of the causal role of redox modifications related to glycoxidation and neuro-oxidation, the use of herbal teas in recent years is highlighted as an attenuation strategy.

Table 2. Biomarkers related to oxidative stress, glycoxidation, and neurodegeneration.

Physiological target	Biomarker	Pathological implication	Reference
Neurodegeneration	Aβ-peptides (Amyloid beta-peptide)	Aβ cerebral deposition increases with AD progression	(BLENNOW; ZETTERBERG, 2018)
	AChE (Acetylcholinesterase)	Participates in acetylcholine level decline in the genesis of AD	
	γ-secretase (Gamma secretase)	γ-secretase participates in Aβ-protein processing	
Glycoxidation	Fluorescence (355/460 nm)	Marker of AGE occurrence (*e.g.* skin)	(PAOLILLO et al., 2019)
Oxidative stress	CAT (Catalase)	Takes part in cellular oxidative stress mitigation	(NANDI et al., 2019)
	COX-2 (Cyclooxygenase-2)	Inflammation and inflammation mediator	(ONODERA et al., 2015)
	GSH/GSSG (Reduced glutathione/oxidized glutathione ratio)	Redox balance indicator	(SENTELLAS et al., 2014)
	H2O2 (Hydrogen peroxide)	Mitochondrial dysfunction	(GIORGIO et al., 2007)
	LDH (Lactate dehydrogenase)	Energy metabolism and cell senescence control	(SABBATINELLI et al., 2019)
	Lipid peroxidation	Cellular lipid integrity biomarker	(NIKI, 2014)
	MAO-A (Monoamine oxidase A)	Regulates amine metabolism, especially important for neurophysiology, associated with anxiety or depression studies	(UZBEKOV, 2021)
	MPO (Myeloperoxidase)	MPO is mostly produced by immune cells, especially neutrophils, being involved with both inflammation and oxidative stress	(LASOLA et al., 2020)
	SOD (Superoxide dismutase)	Plays a role in oxidative stress and cell injury indication	(COSTA et al., 2016)
	HIAA (5-Hydroxyindoleacetic acid)	Product of serotonin metabolism pathway used as a biomarker of neurological injury	(JAYAMOHANANAN; MANOJ KUMAR; T P, 2019)

4.1. Antioxidant and anti-glycation effects of hibiscus, rooibos, and yerba mate

The brain appears to be especially sensitive to oxidative stress due to its high oxygen demand (SALIM, 2017). Some body of work has addressed the potential of hibiscus, rooibos, and mate crude extracts in the mitigation of ROS production as well as anti-glycation both *in vitro* (Table 3) and *in vivo* (Table 4) approaching major biomarkers as glutathione, SOD, CAT, and the formation of autofluorescent AGEs. *In vitro* studies on neuroblastoma cell culture (SH-SY5Y) demonstrated that hibiscus ethanolic extracts (100 µg/mL) reduced ROS production, and more significantly lipid peroxidation when compared to cells exposed to H2O2 stress, which is supposed to contribute to cell membrane lipid layer maintenance (SHALGUM et al., 2019). Under *in vivo* conditions, such antioxidant potential was translated as increased engagement of CAT and SOD enzyme in the brain of diabetic male Sprague-Dawley who received orally 25 mg/kg body weight of hibiscus aqueous extract (PILLAI; MINI, 2016).

The effect of rooibos was similar over SOD and CAT as observed in immobilization-induced oxidative stress Sprague-Dawley animals received a supplement of rooibos along with a 4-week study. The intake of rooibos aqueous extract (10 mg/mL) was demonstrated to result in greater activity of both enzymes in comparison to animals under stress but not receiving rooibos supplementation (HONG; LEE; KIM, 2014). In consequence, in this same study, rooibos was associated with lower brain lipid oxidation. Rooibos is considered to act over DAF-16/FOXO signaling pathway, which mediates SOD, CAT, and GST levels, modulating life spam (CHEN et al., 2013).

Table 3. *In vitro* antioxidant and anti-glycation effects of rooibos, hibiscus, mate extracts.

Assay	Species [extract]	Measure	Dose or EC50	Reference
Antioxidant	*H. sabdariffa* [Ethanolic]	Lipid peroxidation (SH-SY5Y cells)	Control: 800 % Extract (100 µg/mL): 300 %	(SHALGUM et al., 2019)
		ROS production (SH-SY5Y cells)	Control: 130 % Extract (100 µg/mL): 100 %	
	H. sabdariffa [Methanolic]	Malondialdehyde	EC50 22 µg/mL	(OBOH et al., 2018)
		Monoamine Oxidase	EC50 44 µg/mL	
		ATPase activity	EC50 22 µg/mL	
Anti-glycoxidation	*A. linearis* [Aqueous]	AGE formation inhibition (Fluorescence 340/420 nm) Glucose in BSA system	Control (aminoguanidine): 45 % Green extract (200 µg/mL): 45 % Fermented extract (200 µg/mL): 55 %	(PRINGLE et al., 2018)
	H. rosa-sinensis [Aqueous]	AGE formation inhibition (Fluorescence 340/420 nm) Fructose in BSA system	Control (Aminoguanidine): IC_{50} 6 µg/mL Extract: IC_{50} 67 µg/mL	(SANTHOSH; VEERESHAM; RAMA RAO, 2017)
	I. paraguariensis [Aqueous]	AGE formation inhibition (Fluorescence 340/420 nm) Fructose in BSA system	Control (Fructose): 4000 a.u. Extract (2.5 µg/mL): 3000 a.u.	(PEREIRA et al., 2012)
		AGE formation inhibition (Fluorescence 340/420 nm) Methylglyoxal in BSA system	Control (green tea): 65 a.u. Extract (20 µg/mL): 42 a.u.	(LUNCEFORD; GUGLIUCCI, 2005)

BSA: bovine serum albumin.

Oxidative stress and inflammation are interconnected mechanisms that play roles in chronic disease progression (HUSSAIN et al., 2016). Hibiscus was also demonstrated to attenuate the effect of markers on the interface between oxidative stress and inflammation. COX-2 is a mediator in inflammatory action while monoamine oxidase (MAO) plays a major role in the outer mitochondrial membrane regulating the metabolism of monoaminergic neurotransmitters (ONODERA et al., 2015; UZBEKOV, 2021). Compelling evidence involves both biomarkers in the progression of ROS-related inflammation in major metabolic disorders (MUÑOZ et al., 2015; STURZA et al., 2019). Oboh et al. (2018) reported that roselle methanolic extract reduced MAO expression *in vitro* (EC50 = 43.69 µg/mL), while diabetic Wistar albino mice had decreased COX-2 activity toward the inversion of oxidative stress (EL-SHIEKH et al., 2020).

Glutathione (GSH) is a powerful mechanism in animal cell redox control (BAJIC et al., 2019). It has been demonstrated that aging neurons have lower levels of the reduced form (GSH) which is converted into the oxidized version (GSSG) (CASTELLI et al., 2019). Oral supplementation of rooibos (10 mg/mL) and mate (200 mg/mL) extracts showed effects on the increase of the GSH/GSSH ratio. Such behavior attributed to yerba mate was also observed in synaptosomal/mitochondrial P2 fractions (LIMA et al., 2017) as well as in brain homogenates of chronic immobilized rats (COLPO et al., 2017) which suggests that synaptosomal cells are key in GSH control in rats.

Rooibos, hibiscus, and mate provide an important phytochemical repertoire with anti-glycoxidation activity. Reactive saccharides such as glucose, fructose, and ribose, as well as carbonyl compounds, such as glyoxal, and methylglyoxal have been described as important precursors of AGEs (GLOMB; MONNIER, 1995). Therefore, in the search for anti-glycation molecules, different glycation precursors are investigated. Several glycation derivatives, including protein cross-links, are autofluorescent and can be detected at excitation/emission wavelengths of 335/385 nm, for total AGE estimation, and 485/520 nm for cross-link estimation (BEISSWENGER et al., 2012, p. 440; RAPOSEIRAS-ROUBÍN et al., 2013). This characteristic is explored *in vitro* for bioassays on the inhibition of AGE formation. Caffeic and chlorogenic acid were found to be major components in *I. paraguariensis* extracts. Along with the study of the inhibition of AGEs based on fluorescence measures, caffeic acid showed the most significant effect (90%) in a methylglyoxal-BSA system compared to aminoguanidine (60%) control (GUGLIUCCI et al., 2009). Chlorogenic acid, on the other hand, showed similar EC50 to aminoguanidine, 10 mM and 8 mM

respectively, in fructose/inhibition in the ovalbumin system (BAINS; GUGLIUCCI, 2017). When it comes to the crude extracts of mate (2.5 µg/mL), it was shown a reduction of 25% in the formation of fluorescent AGEs (PEREIRA et al., 2012) while rooibos non-fermented extract (200 µg/mL) was shown to limit fluorescence up to 45%, equivalent to aminoguanidine control (SANTHOSH; VEERESHAM; RAMA RAO, 2017). *In vivo*, elevated glucose levels in diabetic patients have been correlated to the occurrence of glycated hemoglobin (INCANI et al., 2015). These polyphenols as well as rutin and quercetin (also part of the phytochemical composition of these plants) acted mainly by the inhibition of Amadori product formation in the early stage of the Maillard Reaction (CHINCHANSURE et al., 2015; CAI; CHEN; JIANG, 2018). In addition, it may also contribute to glucose homeostasis by insulin resistance reduction, decreasing circulating AGEs, and lipid peroxidation in diabetic rats, hibiscus tisane was demonstrated to play a role in circulating glucose and AGE reduction while reducing the incidence of glycated hemoglobin (PENG et al., 2011).

Table 4. *In vivo* antioxidant and anti-glycation effects of rooibos, hibiscus, mate extracts.

Target effect/Organ	Species [Extract]	Concentration	Animal model	Measure	Effect	Tendency	Reference
Antioxidant/Brain	*A. linearis* [Aqueous]	1 g/100 mL	Immobilization-induced oxidative stress Sprague Dawley rats	CAT	Control (Stress): 2 unit/mg Extract: 3 unit/mg	↑	(HONG; LEE; KIM, 2014)
				FFA	Control (Stress): 700 µg/mL Extract: 650 µg/mL	↓	
				GSH/GSSG	Control (Stress): 7.5 Extract: 9	↑	
				HIAA	Control (Stress): 400 mg/g tissue Extract: 350 mg/g tissue	↓	
				Lipid peroxidation	Control (Stress): 50 nmol/g tissue Extract: 40 nmol/g tissue	↓	
				SOD	Control (Stress): 1 unit/mg Extract: 1.7 unit/mg	↑	
	H. rosa-sinensis [Aqueous]	25 mg/kg body weight	STZ induced diabetic Male Sprague-Dawley	CAT	Control (Diabetic): 5 U/mg Extract: 10 U/mg	↑	(PILLAI; MINI, 2016)
				SOD	Control (Diabetic): 7 U/mg Extract: 15 U/mg	↑	
	H. sabdariffa [Aqueous]	200 mg/kg body weight	Male Swiss albino mice	MDA	Control (STZ): 3 nmol/g White hibiscus extract: 0.5 nmol/g Red hibiscus extract: 0.5 nmol/g	↓	(EL-SHIEKH et al., 2020)
				MPO	Control (STZ): 75 µg/mg tissue White hibiscus extract: 20 µg/mg tissue Red hibiscus extract: 20 µg/mg tissue	↓	
				Cox-2	Control (STZ): 4 (fold change) White hibiscus extract: 1 (fold change) Red hibiscus extract: 1 (fold change)	↓	
	H. sabdariffa [Ethanolic]	500 mg/kg body weight	Cypermethrin oxidative stress male mice (Mus musculus)	AChE	Control (Cypermethrin): 0.5 µmol/min/mg Extract: 2.5 µmol/min/mg	↓	(MEZNI et al., 2020)

Target effect/Organ	Species [Extract]	Concentration	Animal model	Measure	Effect	Tendency	Reference
				CAT	Control (Cypermethrin): 0.04 µmol/min/mg Extract: 0.06 µmol/min/mg	↓	
				H2O2	Control (Cypermethrin): 1.2 µmol/mg Extract: 0.3 µmol/mg	↓	
				MDA	Control (Cypermethrin): 2 µmol/mg Extract: 0.5 µmol/mg	↓	
	I. paraguariensis [Aqueous]	200 mg/mL	Chronic immobilization stress male Wistar rats	GSH/GSSG	Control: 0.48 Extract: 0.50	→	(COLPO et al., 2017)
				Lipid peroxidation	Control: 2.1 TBA/mg Extract: 1.3 TBA/mg	↓	
		200 mg/mL	Male Wistar rats	GSH/GSSG	Control: 4.7 Extract: 16.6	↑	(LIMA et al., 2017)
				Lipid peroxidation	Control: 1.3 MDA eq/mg Extract: 0.3 MDA eq/mg	↓	
		50 mg/kg BW	PTZ-induced seizure male Wistar rats	CAT	Control (PTZ): 5 mmol/min/mg Extract: 9 mmol/min/mg	↑	(BRANCO et al., 2013)
				SOD	Control (PTZ): 15.50 U/mg Extract: 23 U/mg	↑	
				Sulfhydryl protein	Control (PTZ): 0.09 nmol DTNB/mg Extract: 0.31 nmol DTNB/mg	↑	
Anti-glycoxidation	*H. rosa-sinensis* [Ethanolic]	25 mg/kg BW	STZ induced diabetic Male Sprague-Dawley	Glycated hemoglobin	Control: 13% Extract: 6%	↓	(PILLAI; MINI, 2016)
	H. sabdariffa [Methanolic]	200 mg/kg BW	STZ induced diabetic Male Sprague-Dawley	Serum glucose	Diabetic control: 400 mg/dL Extract: 100 mg/dL	↓	(PENG et al., 2011)
				AGE levels	Diabetic control: 4.5 mg/mL Extract: 3 mg/dL	↓	

4.2. Neuroprotective effects of hibiscus, rooibos, and yerba mate

Several studies have shown that plant metabolites such as flavonoids, anthocyanins, and phenolic acids are active components with neuroprotective properties (ZHANG et al., 2021b). Complementary *in vitro* and *in vivo* assays demonstrated that *H. sabdariffa* led to the inhibition of AChE and butyrylcholinesterase (BChE), both related to the hydrolysis of acetylcholine (EL-SHIEKH et al., 2020; OBOH et al., 2018) (Table 5). So far, more prolific research on this issue is found over hibiscus tisane. Table 5 exemplifies the investigation of different organic extractions of *H. sabdariffa*. Data from PC12 cells, a cell model for neural crest neuroblastic cells, demonstrated that hibiscus ethanolic extract (60 µg/mL) allowed the reduction of apoptotic cell counts (BAKHTIARI; HOSSEINI; MOUSAVI, 2015).

Table 5. *In vitro* neuroprotective effect of aqueous, ethanolic, and methanolic *H. sabdariffa* extracts.

Extract	Measure	Dose or EC50	Reference
Aqueous	AChE inhibition	Control (galantamine): IC_{50} 7 µg/mL White hibiscus extract: IC_{50} 123 µg/mL Red hibiscus extract: IC_{50} 106 µg/mL	(EL-SHIEKH et al., 2020)
Ethanolic	PC12 cells Inhibition of cell apoptosis	Control (SGD): 65 apoptotic cells Extract (60 µg/mL): 30 apoptotic cells	(BAKHTIARI; HOSSEINI; MOUSAVI, 2015)
Methanolic	AChE inhibition	IC_{50} 46.96 µg/mL	(OBOH et al., 2018)
	BChE inhibition	EC50 40.38 µg/mL	

When it comes to *in vivo* assays (Table 6), a diet enriched with hibiscus anthocyanins was able to downregulate several aspects of Alzheimer's Disease as neuroinflammation. The aggregation of Aβ-peptides in the brain is a source of oxidative stress and was demonstrated to lead to lipid peroxidation (BUTTERFIELD; BOYD-KIMBALL, 2018). In addition, Aβ-peptides play a role as

a RAGE ligand, which accounts for a factor in oxidative stress in astrocytes and cerebral endothelial cells as reported by (ASKAROVA et al., 2011). In non-transgenic Alzheimer's Disease model mice, Aβ-42 accumulation was reduced following γ-secretase, APH1a, and BACE1 activity (EL-SHIEKH et al., 2020). *C. elegans* is a simple nematode with an approximately 83% genome similar to humans, which turns it extremely useful in human physiology studies (LAI et al., 2000). Yerba mate extract was able to downgrade neuro-oxidative biomarkers as Aβ-42 expression and ROS levels in *C. elegans*. Most important, such effects were correlated to increased worm lifespan, suggesting that can help to slow down aging (MACHADO et al., 2021).

In addition to these findings, some data on animal behavior shed light on the neuroprotective effect of hibiscus and mate teas. Some strategies are used for neuronal damage perception as behavioral assay associated with anxiety-related, cognitive and spatial learning, and aversive memory. Respectively, elevated plus maze, Morris water test, and step-down avoidance task are behavioral tests able to estimate such cognitive impacts (BORBA FILHO et al., 2015; VORHEES; WILLIAMS, 2006; WALF; FRYE, 2007). The Morris water maze test evaluates animal mice spatial reference. In this issue, El-Shiekh et al. (2020) demonstrated that hibiscus flower extracts (both red and white flowers) (200 mg/kg) were able to restore mice spatial capacities compared to STZ-induced Alzheimer's Disease model mice, hibiscus was suggested to attenuate neuroinflammation and amyloidogenesis in treated animals. In anxiety and memory assessment, it has been demonstrated that mate hydroethanolic extract (300 mg/kg body weight) increased anxiolytic-like behavior in mice, which was suggested by the bioactivity of mate extracts over the cholinergic system together

with the levels of caffeine in this plant. On the other hand, the scopolamine-induced deficit was prevented by ilex extract (SANTOS et al., 2018).

Table 6. *In vivo* neuroprotective effects of rooibos, hibiscus, mate extracts.

Species [Extract]	Concentration	Animal model	Measure	Effect	Tendency	Reference
A. linearis [Aqueous]	100 mg/mL	Zebrafish larvae	Monoamine oxidase	Control (Clorgyline): 100% Extract: 60 %	↓	(LÓPEZ et al., 2022)
			Cell viability	Control: 100 % Extract: 40 %	↓	
	12.5 µg/mL	Zebrafish larvae	ROS production	Control: 600 % (120 min) Extract: 200 % (120 min)	↓	
H. sabdariffa [Aqueous]	200 mg/kg BW	Male Swiss albino mice	Moris water test	Control (STZ): 20 s Extract: 30 s	↑	(EL-SHIEKH et al., 2020)
			BACE1	Control (STZ): 5 (fold change) White hibiscus extract: 2 (fold change) Red hibiscus extract: 2 (fold change)	↓	
			Aβ-42	Control (STZ): 250 mg/mg tissue White hibiscus extract: 100 mg/mg tissue Red hibiscus extract: 100 mg/mg tissue	↓	
			γ-secretase	Control (STZ): 3.5 (fold change) White hibiscus extract: 1 (fold change) Red hibiscus extract: 1 (fold change)	↓	
H. sabdariffa [Ethanolic]	500 mg/kg BW	Swiss albino mice	AChE activity	Control (Scopolamin): 44 nM/min/g tissue Extract: 33 nM/min/g tissue	↓	(ALSHABI; SHAIKH; HABEEB, 2021)
I. paraguariensis [Aqueous]	10.5 mg/L	*Caenorhabditis elegans*	Aluminum induced oxidative stress	Control: 0.6 µM/h/mg Extract: 0.4 µM/h/mg	↓	(BORTOLI et al., 2018)
I. paraguariensis	4 mg/mL	*C. elegans*	Aβ-42 expression	Control: 1 a.u. Extract: 0.6 a.u.	↓	(MACHADO et al., 2021)

Species [Extract]	Concentration	Animal model	Measure	Effect	Tendency	Reference
[Ethanolic]			AChE activity	Control: 100 % Extract: 50 %	↓	
			Lifespan	Control: 15 days Extract: 17 days	↑	
			ROS production	Control: 100 % Extract: 50 %	↓	
	500 mg/kg	Male C57Bl/6 mice	Catalepsy	Control (reserpine): 120 s Extract: 60 s	↓	(MILIOLI et al., 2007)
	300 mg/kg BW	Male Swiss mice	Elevated Plus Maze	Control: 17 % Extract:40 %	↑	(SANTOS et al., 2015)
			AChE	Control: 4.5 mmol/min/mg Extract: 8.0 mmol/min/mg	↑	
			Step-down avoidance task	Control: 170 s Extract: 70 s	↓	

5 CONCLUSIONS

Over the past few years, the mitigation of oxidative stress has gained much importance due to the implications on several modern diseases related to age progression, glycoxidation, and aging. The lack of effective treatments for neurodegenerative diseases or strategies that could prevent the onset of age-related diseases, for instance, encourages and may answer to the keen interest of pharmaceutical and food industries in the search for products with improved bioactivity. Yet, attenuating the implications of glycation on aging has catch attention as an anti-aging strategy in potential.

Plants have important applications in the food industry and as nutraceuticals potentially minimizing the negative consequences of oxidative stress. Plants have a repertoire of phytochemicals that can be explored as food supplements, attenuating the progression of diseases, or as food additives, preventing the formation of neoformed compounds with deleterious effects on human health. Rooibos, hibiscus, and mate tisanes have been demonstrated to contribute positively to the reduction of oxidative stress, inhibit glycoxidation, and promote neuronal oxidative protection both *in vitro* and *in vivo*. Investigating these plants has important market impacts. The associated health claims and industries may be encouraged by the appealing taste that these plants present, great market acceptance, and the lower health risks due to long use as foods. Further characterization of the mechanisms of action on glycation inhibition or neuroprotection remains to be investigated together with the identification of target compounds contributing to such effects. In addition, further investigation may benefit both the pharmaceutical and the food industries.

From the clinical point of view, it would be of great importance to approach the translation of described *in vitro* and pre-clinal results into human physiology. Such investigation may answer questions on the real effectiveness of these plants as nutraceuticals, as well as on the definition of optimal dose, and digestibility. From the food industry perspective, it would be of considerable importance the investigation of the stability of key-bioactive compounds during food processing. Therefore, new insights are required to expand the biotechnological uses of these plants to help improve the human aging process. Lastly, besides the scope of this article, it is important to highlight that investing and boosting hibiscus, rooibos, and mate markets may contribute to the local crop expansion, towards more sustainable development, with technological methods of production, and the reduction of local inequalities.

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CHAPTER 2

Characterization of hibiscus extracts and the effects of gamma radiation on microbial decontamination, phenolic content, and bioactivity of hibiscus (*Hibiscus* sp.) floral varieties

ABSTRACT

This work section aimed to explore the different hibiscus varieties and understand the correlation between floral pigmentation and the implications on the total phenolic and flavonoid content, the antioxidant, the anti-glycation, the *in vivo* neuroprotective effect, and the antiacetylcholinesterase potential of aqueous and ethanolic extracts of different varieties of hibiscus flowers with distinct floral pigmentation; in addition, a series of experiments were undertaken to assess the effects of γ-radiation (5, 10, and 30 kGy doses) on microbial decontamination, antioxidant, and neuroprotective capacities of *Hibiscus sabdariffa* and *H. rosa-sinensis*. Initially, the total phenolic and flavonoid contents were determined by spectrophotometric techniques. Aqueous extracts showed higher levels of phenolic and flavonoids than ethanolic ones. The aqueous extract derived from pink hibiscus (extract HR5) presented the highest total phenolic content, 31.28±1.24 mg GAE/100 g, and total flavonoids, 469.20±3.24 mg QE/100 g. The high phenolic content may be related to the greater diversity of peaks under 280 nm, only presented by the chromatographic profile of this extract. The aqueous extract of the orange variety (HL10), showed the lowest levels of phenolics, 4.69±0.22 mg QE/100 g, and total flavonoids, 66.53±11.55 mg QE/100 g. The antioxidant activity was evaluated by DPPH and ABTS radical scavenging assays together with the evaluation of the iron-reducing power. From this analysis, the ethanolic extract of red hibiscus (HV4) showed higher DPPH radical scavenging, 84.09±0.84%, and higher ferric reducing power, 52.69±1.93%. The neuroprotective activity was evaluated *in vitro* using the Ellman assay of acetylcholinesterase (AChE) inhibition and *in vivo* using neuro-vulnerable *D. melanogaster*, by negative geotaxis protocol evaluation. The results of neuroprotective activity demonstrated that the ethanolic extract of red hibiscus of

commercial origin (HVC2), had a greater ability to inhibit AChE, 96.56± 2.34%, as well as a tendency at improving neuroprotective potential *in vivo*. The red hibiscus aqueous extract showed a higher anti-glycation effect, achieving 55% of inhibition in 24 h. When it comes to the treatment with gamma radiation, sterilization was achieved at 5 kGy for HS, 10 kGy for HRS2, and 30 kGy. A dose of 30 kGy reduced TFC on HRS1 and HRS2, but antioxidant and anti-AChE properties were not affected in the same direction. IRP or DPPH increased for HRS1 (5 kGy) and HRS2 (10 kGy). γ-irradiation reduced by 30% HRS1 DPPH activity. HRS1 samples (30 kGy) showed 76% improvement in AChE inhibition. Overall, doses of 10 kGy to 30 kGy allowed high microbial loads decontamination but 30 kGy had effects on TFC. γ-radiation is an effective strategy for the microbial control of hibiscus while maintaining its beneficial properties. These results cover several biotechnological traits of hibiscus flowers with potential application in the food industry that has not been well-explored in the scientific literature. Moreover, these results open up the perspective on the use of different varieties of hibiscus, in addition to the commonly consumed red variety, as significant sources of phenolic and flavonoids, which exert antioxidant and neuroprotective roles as evidenced both by *in vivo* and *in vitro* experiments.

Key-words: Hibiscus, phenolics, gamma-radiation, antioxidant, neuroprotection, nutraceutical

1 INTRODUCTION

Teas are the most consumed beverage next to water. Although tea preparation has long been associated with traditional cultures, tea consumption is becoming more popular, especially driven by the rise in consumers' awareness of the health benefits of these plant-based beverages (THE TEA ASSOCIATION OF THE USA). In response to market demand, the food industry is urging the development of new products with functional and health claims. In this way, hibiscus, a tropical shrub from which flowers are picked for homemade tea preparation, has gained popularity and is widely explored by the food industry for natural pigment extraction (SHRUTHI; RAMACHANDRA, 2019), juices, and candy confection (DE MOURA et al., 2019). Hibiscus is a well-known source of polyphenolics with antioxidant, anti-hyperlipidemic (LONG et al., 2021), and neuroprotective compounds (ARSLAN et al., 2021), and even predicted anti-SARS-CoV-2 activity (SHAWKY; A. NADA; S. IBRAHIM, 2020). Such health benefits have increased the demand for new hibiscus-based products such as encapsulated supplements and floral powders in the American, Asian, and European markets (SILVA et al., 2016), which is projected to grow 7% by 2027 (THE EUROPEAN MARKET POTENTIAL FOR HIBISCUS | CBI, [s.d.]). Comprehensive reviews have been published on the phytochemical composition of the floral parts of the hibiscus (DA-COSTA-ROCHA et al., 2014; RIAZ; CHOPRA, 2018). Up to 70% of the crude aqueous floral extracts of hibiscus are anthocyanins, especially cyanidin-3-sambubioside, delphinidin-3-o-sambubioside, and its glycosidic derivatives, organic acids, protocatechuic acid, and eugenol, which are major sources of antioxidant capacity (WU et al., 2017)

A current discussion in the therapeutic field is the nutritional approach to antioxidant therapies (TELEANU et al., 2019). Neurodegenerative pathologies,

for instance, hold an oxidative stress component, which has gained much attention in the last decades because of the greater incidence of dementia in aging populations (SINGH et al., 2019; WU et al., 2017). Alzheimer's Disease (AD) is the main cause of dementia in elderlies (TAKIZAWA et al., 2015), and massive mitochondrial leakage decisively contributes to neurodegeneration progression (RONOWSKA et al., 2018). Meanwhile, the neuronal production of the acetylcholine intermediary, Acetyl-CoA, is lowered, contributing to gradual cognitive impairment (STANCIU et al., 2020). Some plant natural products contribute to reducing acetylcholine turnover by inhibiting acetylcholinesterase activity in the neuronal cleft, which is currently the main strategy in AD treatment nowadays (SARKAR et al., 2021).

In the manufacture of teas, leaves, flowers, and/or stems from herbal plants are hand or machine collected; however, no deep washing or cleaning occurs to preserve product integrity, color, flavor, and aroma (LANGFORD, 2021). Different origins may account for the microbial content in this class of products, including phylloplane flora, human handling, and storage conditions (MISHRA; GAUTAM; SHARMA, 2006). Gamma irradiation treatment is used by the food industry to increase the shelf life while maintaining the nutritional value of foods (VAN DOREN et al., 2013). Gamma irradiation stands out in substitution for conventional thermal methods, which promote up to 30% loss of water-soluble and thermally unstable molecules, such as phenolic compounds (VOLF et al., 2014). Artificial (80 °C) or sun-drying processes reduce water and, consequently, microbial content. However, the occurrence of spore-forming microorganisms may remain, including pathogenic bacteria or fungal species (MISHRA; GAUTAM; SHARMA, 2006). Gamma irradiation is considered a safe technology

and has been successfully used in the microbial control of dried products such as spices and teas. The United States Food and Drug Administration (FDA) limits the use up to a dose of 30 kGy (FDA 2021). However, the extension of the effects of ionizing radiation over hibiscus bioactivity is not yet known. The resulting free radicals, for instance, promote the degradation or oxidation of polyphenols with antioxidant effects (FANARO et al., 2014). In this context, the goal of the present study was to identify the optimal gamma radiation dose for microbial control in hibiscus samples as well as to evaluate the effects of the antioxidant, polyphenolic content, and antiacetylcholinesterase effects of irradiated *Hibiscus sp.* floral extracts.

2 GOALS

2.1. General goal

This work section aimed to investigate the antioxidant, neuroprotective, anti-glycoxidation, neuroprotective, and antiacetylcholinesterase effects of hibiscus floral extracts (*Hibiscus* sp.) to elucidate the effects of gamma radiation treatment on hibiscus extracts bioactivity.

2.2. Specific goals

2.2.1. Characterize different hibiscus floral samples with different floral colors, in terms of yields among aqueous and ethanolic extracts as well as compare the metabolic profiles by the use of high-performance liquid chromatography (HPLC);

2.2.2. Quantify and compare the total phenolic content (TPC) and the total flavonoid content (TFC) in the different extracts;

2.2.3. Measure the antioxidant capacity of floral extracts by different protocols including DPPH and ABTS radical scavenging methodologies, and ferric ions reducing power;

2.2.4. Evaluate the anti-glycoxidation effect by fluorimetric methods;

2.2.5. To evaluate the neuroprotective effects from the comparison of the acetylcholinesterase inhibitory capacity (*in vitro*), investigate the *in vivo* protective effects on neuro-compromised flies (*Drosophila melanogaster*).

2.2.6. Evaluate the effects of gamma radiation over the antioxidant, polyphenolic content, and antiacetylcholinesterase effects of irradiated *Hibiscus* sp. floral extracts.

3 MATERIAL AND METHODS

3.1. Biological material and extraction

Commercial hibiscus (HCV) was bought from Comércio de Ervas SP LTDA (Lot:0011, Expiration date: 02/01/2023). Samples HVC, HV, HR, HB, HA, and HL were identified as *Hibiscus rosa-sinensis* in comparison to the deposited vouchers references - BHC21598, BHC2279, BHC2374, BHC2737, BHC1101 - in the Herbário da UFMG (BHCB). After collection, the petals were separated from the pistils and dried in an oven (Fanem) at 45 °C for 24 hours. After drying, petals were ground in a blender for 1 minute and the resulting powder was stored in a desiccator. Between 5 and 50 grams, depending on availability, of each sample were used for extraction (1:1 p/v of solvent) following two methodologies: (1) aqueous extraction, 90°C for 15 minutes under agitation; (2) ethanolic extraction for 24 hours at room temperature under stirring. The resulting aqueous extracts were immediately frozen and lyophilized for 36 hours. The ethanolic

fractions were roto-evaporated (Buchi) and the remaining extracts were recovered in distilled water for lyophilization (Liotop). After lyophilization, samples were placed in a desiccator and applied to the experiments described below. The list of analyzed samples is described in table 1.

Table 1. List of samples used for the study of the antioxidant, and neuroprotective assays, and the estimation of TPC and TFC.

Sample ID	Origin	Prodominant color	Photo
HVC	Comércio de Ervas SP LTDA Lote: 0011. Valid: 02/11/2023	Red	
HV	Belo Horizonte/MG	Red	
HR	Belo Horizonte/MG	Pink	
HB	Diamantina/MG	White	
HA	Belo Horizonte/MG	Yellow	
HL	Belo Horizonte/MG	Orange	

3.2. Characterization of extracts by high-performance liquid chromatography (HPLC)

HPLC UV/VIS analyses were carried out according to the methodology presented by BORRÁS-LINARES et al. (2014) with modifications presented below. The analyzes took place in a reverse-phase C18 column (250 mm x 4.6

mm x 5 µm) (Kromasil), 20 µL of extract were injected, with a continuous mobile phase flow of 0.5 mL/min consisting of ultrapure water (Phase A) and HPLC grade acetonitrile/ultrapure water 9:1 (v/v) acidified with 1% formic acid (Phase B). The detection of phenolic compounds was carried out in the ranges of 280-370 nm. The lyophilized extracts and standards (caffeic acid, chlorogenic acid, quercetin, gallic acid, and rutin) were solubilized in analytical grade acetonitrile and ultrapure water (1:1) at a concentration of 5 mg/mL and filtered (45 nm). Analyzes were proceeded in high-performance chromatography (Shimadzu Prominence LC-20AT), coupled to a UV-Visible detector (SPD-20A) and an autosampler (SIL-20A). Elution took place according to the following separation scheme:

Table 2. Description of the gradient used for HPLC separation.

Time (min)	Acetonitrile 9:1 (%)	Water (%)
0	5	95
7	10	90
9	17	83
15	20	80
25	25	75
35	40	60
37,5	40	60

3.3. Determination of the content of total phenolics (TPC)

TFC was determined by the Folin-Ciocauteau spectrophotometric method described by ROP et al. (2012) and adapted to 96-well microplates. Extracts were solubilized in distilled water at a concentration of 1 mg/mL. A calibration curve was prepared with gallic acid, used as a standard, at concentrations from 100 to 12.5 µg/mL. Distilled water was used as a negative control. To each well, 35 µL

of standard or extract was mixed with 170 µL of Folin-Ciocauteau solution (Dynamica). The mixture was homogenized on a plate shaker for 3 min at room temperature and, after shaking, 20 µL of a saturated sodium carbonate solution was added. Plates were incubated at 25°C for 20 min. These experiments were carried out in triplicate and the absorbance reading was performed at 760 nm (Thermo Scientific Multiskan GO). Results were expressed in milligrams of gallic acid per 100g of the dry mass of petals (mg AG/g).

3.4. Determination of the content of total flavonoids (TFC)

TFC was measured using the method described by (PARK et al., 2008) adapted to a 96-well microplate. Extracts were solubilized in distilled water at a concentration of 1 mg/mL. A calibration curve was prepared with quercetin at concentrations ranging from 12.5 µg/mL to 100 µg/mL. Distilled water was as used as a negative control. The essay consisted of mixing 50 µL of extract or standard to 6 µL of sodium nitrite solution (5% w/v) and shaking for 5 minutes on a microplate shaker. Afterward, 6 µL of aluminum chloride solution (10% w/v) was added to the mixture. Plates remained at room temperature for 5 min. Then, 40 µL of NaOH solution (1 mol/L) was added. Plates remained at room temperature for 10 min. Distilled water (100 µL) was added and homogenized. Experiments were carried out in triplicate and the absorbance reading was performed at 695 nm (Thermo Scientific Multiskan GO). Results were expressed in milligrams of quercetin per 100g of dry petal mass (mg AG/g).

3.5. Antioxidant effects

3.5.1. ABTS radical scavenging

ABTS (2,2-azinobis-[3-ethyl-benzothiazolin6-sulfonic acid) radical scavenging was analyzed as described by (JOHNSTONE et al., 2006) and adapted to 96-well microplates. The ABTS stock was prepared by mixing 100 mL of 7 mmol/L ABTS solution with 12.25 mL of 20 mmolL potassium persulfate solution. This mixture was incubated for 16 h in the dark, before preparing the working solution which contained 1 mL of stock solution, and the absorbance was adjusted with ethanol (approximately 30 mL) to 0.9 at 730 nm. Both extracts and ascorbic acid (positive control) were solubilized in water to obtain a final concentration of 1 mg/mL. The same procedures were performed using distilled water as a negative control. In these experiments, 40 µL of extract or standard were distributed per well and mixed with 180 µL of working solution, this reaction was incubated at room temperature for 2 min. Absorbances were read at 730 nm. Experiments were performed in triplicate and the results with their respective standard deviations were represented in terms of inhibition of ABTS formation calculated according to the following mathematical formula, where Ab is the absorbance of the negative control and Aa is the absorbance of the extract.

$$Inibição\% = \frac{Ab - Aa}{Ab} x100$$

Equation 1: Calculation of inhibition of free radical formation.

3.5.2. DPPH radical scavenging

DPPH radical scavenging (2,2-diphenyl-1-picrylhydrazyl) was evaluated as previously described by (BRAND-WILLIAMS; CUVELIER; BERSET, 1995) and adapted to a 96-well microplate. In these experiments, both extracts and

ascorbic acid (positive control) were solubilized to obtain a final concentration of 1 mg/mL. The same procedures were performed using distilled water as a negative control. In each well, 25 µL of extract or controls and 175 µL of DPPH solution (0.1 mmol/L) were distributed. This mixture was homogenized and incubated for 30 min in the dark. After this period, the absorbance readings were taken at 490 nm. Experiments were performed in triplicate and the results are represented in terms of inhibition of DPPH formation calculated according to equation 1.

3.5.3. Iron reducing power

The evaluation of the iron(III) ion reduction capacity was estimated using the methodology described by (CHANG et al., 2011) adapted for 96-well microplates. Both the extracts and the ascorbic acid standard were diluted at a concentration of 1 mg/mL. The same procedures were performed using distilled water as a negative control. In each well, 50 µL of extract or standard were added to 125 µL of pH 6.6 phosphate buffer (0.2 mol/L) and 125 µL of potassium ferricyanide solution (1% w/v) and incubated at 50°C for 20 min. After this period, 125 µL of trichloroacetic acid solution (10% w/v) was added and homogenized and the reaction remained at rest for 10 min. A 125 µL aliquot of the resulting material was added to 125 µL of distilled water and 25 µL of ferric chloride solution (0.1% w/v). After homogenization, the corresponding absorbances were read at 700 nm. Experiments were performed in triplicate and the results with their respective standard deviations were represented in terms of inhibition of iron(III) ion formation calculated according to equation 1.

3.5.4. Anti-glycation assay

Anti-glycation tests were performed in France in the U1167 laboratories. Samples of red (Lot: 506382482, Val.: 28/02/2023) and white (Lot: 506382342, Val.: 28/09/2022) were identified by the supplier (Racines Bio) as *H. sabdariffa* (France). Extract preparation was performed similarly to what was described in item 3.1.1. Samples and reagents were diluted in phosphate buffer (pH 7.4, 100 mM). In a black 96-well plate, 50 µL of BSA (10 mg/mL) were added to 50 µL of methylglyoxal (14 mM) and 20 µL sample or controls. Phosphate buffer was used as a negative control while aminoguanidine was used as a standard glycation inhibitor. Samples and positive control concentrations ranged from 62.5 µg/mL to 1000 µg/mL. After 24 h and 48 h of incubation at 37 °C, fluorescence was measured on an Omega BMG Labtech on emission and excitation wavelengths of 355 nm and 440 nm, respectively.

3.6. Evaluation of neuroprotective effects

3.6.1. Acetylcholinesterase inhibition kinetics assay

Extracts were analyzed for their inhibitory activity on the enzyme acetylcholinesterase in 96-well microplates based on spectrophotometric methods (RHEE et al., 2001). The commercial inhibitor eserine was used as a positive control (5 mg/mL) and in the absence of an inhibitor or extract, as a negative control. Tests were performed in triplicate. To each well, 25 µL of acetylcholine iodide was dispensed together with 125 µl 5,5'-dithiobis (2-nitrobenzoic acid/DTNB); 50 µL of tris-HCl buffer pH 8 0.1% (w/v) bovine serum albumin and 25 µL of lyophilized extract (5 mg/mL) solubilized in distilled water. Plate readings were performed at 405 nm 10 times over 9 minutes. Soon after

the first reading, 25 µL of the enzyme acetylcholinesterase (0.222 U/mL) was added and 10 readings were immediately performed in 9 min at 405 nm. The interference of the spontaneous hydrolysis of the substrate was corrected by subtracting the mean absorbance measured before the addition of the enzyme. The percentage of enzyme inhibition, in terms of absorbance, was calculated (% inhibition = [(Control-Extract) x 100]/Control) for each reading performed in the periods described to monitor the temporal action of the analyzed extracts on enzyme activity.

3.6.2. Evaluation of neuroprotective activity in *drosophila melanogaster*

The neuroprotective effect of extracts HVC1 and HVC2 was evaluated *in vivo* as the methodology described by (JIMÉNEZ et al., 2017) using rotenone neuro-compromised fruit flies (*Drosophila melanogaster*) as a model. The following processes are outlined in Figure 1. The fly diet contained bananas, agar, yeast, nystatin, propionic acid, and distilled water. The procedures adopted followed the following protocol: (1) Culture of flies: Males and females of *D. melanogaster* were kept at room temperature in 50 mL glass flasks containing 30 grams of the food. After 7 days, these flies were removed, leaving only larvae and pupae that were used, for 7 days, to select age-matched males for the experiments. (2) The tested extracts, at a concentration of 5 mg/mL, were applied to the surface of the diet, and 15 male flies were distributed per flask according to the following experimental groups: Group 1: Negative control group, containing three flasks with 15 males and pure diet administration; Group 2: Positive control group: containing three flasks with 15 males each and the administration of diet containing rotenone; Group 3: Experimental group, containing three flasks with

15 males each and administration of diet containing extract. These tubes were kept for 7 days and, after this period, flies from group 2 were transferred to flasks containing diet and extract, and flies from group 3 to flasks containing diet and rotenone. The evaluation of the neuroprotective activity of the extracts was given by comparing the behavioral response of the flies of the 3 groups to the negative geotaxis test, which consisted of submitting the tubes to five successive punches. At each repetition, 10 sequential photos were taken to quantify the number of flies capable of reaching half of the tube. Test response was statistically evaluated based on the average number of flies reaching the tube seen in the sixth photo and reported about the percentage of flies per tube that reached the upper limit of the flasks.

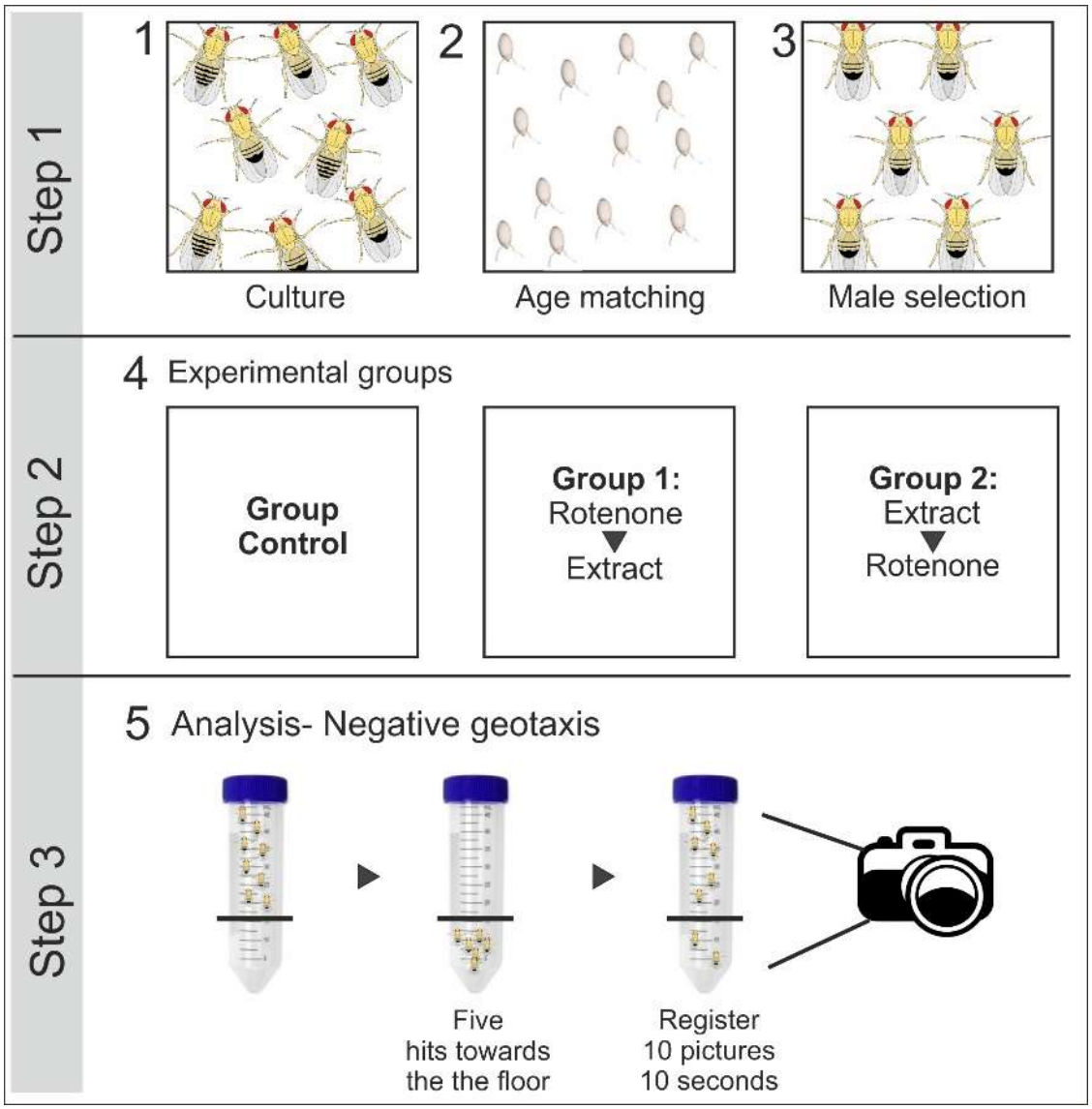

Figure 1. Neuroprotective assessment in *D. melanogaster*. Step 1: Crops, age matching, and male selection for analysis. Step 2: Distribution of males among the experimental groups. Step 3: Assessment of negative geotaxis effects. The tubes were subjected to 5 punches toward the ground and after that, the number of flies that pass the defined limit is recorded utilizing photographs.

3.7. Gamma radiation treatment

Gamma irradiation was carried in a Panoramic Multipurpose Radiator Category II MDS Nordion IR-214/GB-127 (Canada) equipped with Cobalt-60 source part of the Laboratório de Irradiação Gama (LIG), Centro de Desenvolvimento da Tecnologia Nuclear (CDTN, MG, Brazil). Powdered (1 g)

dried flowers were packed in triplicates and submitted to 5 kGy, 10 kGy, and 30 kGy.

3.7.1. Microbiological analysis

Microbial loads were estimated by direct bacterial and fungal colony-forming counts in Petri dishes. Briefly, samples submitted to gamma radiation (1 g) were resuspended in 50 mL (50x dilution) sterile distilled water under aseptic conditions. For each sample, 1 mL of this suspension was plated in Petri dishes containing Plate Counting Agar (PCA, Himedia Labs), for bacterial colony-forming units (CFU) estimation, and Potato Dextrose Agar (PDB, Himedia Labs), for fungi (filamentous and yeast fungi) CFU counts. Plates were incubated at 37 °C and 28 °C for bacteria and fungi growth, respectively, for 48 h. CFU was assessed by direct counting of visible colonies in the respective culture media plated in triplicates.

3.8. Statistical analysis

Statistical analysis of variance, one-way ANOVA, and multiple comparison tests (Tukey), as well as Pearson's correlation analysis, were performed in a MiniTab 18 and GraphPad Prism 9, considering $\alpha \leq 0.05$ at a 95% confidence interval, except when indicated.

4 RESULTS AND DISCUSSION

4.1. Content of total phenolics and flavonoids

Samples shown in Table 1 were subjected to extractions with two polar solvents, water, and ethanol. The extraction efficiency of the main phenolic compounds present in hibiscus flowers was previously demonstrated by TSAI et

al. (2002) and (SINDI; MARSHALL; MORGAN, 2014). Furthermore, the use of these two methods approached common forms of extraction practiced both in the food industry and in households, in the production of teas, dyes, or alcoholic beverages such as hibiscus-based beers and wines (DA-COSTA-ROCHA et al., 2014). The identifications of the resulting extracts, as well as the extraction yield percentages and the total phenolic and flavonoid contents of each sample, are shown in table 3. The absence of ethanolic extracts for HB and HL samples is due to the limitation in the sample amount.

The total phenolic content was determined in relation to the calibration curve of the gallic acid analytical standard (R2=0.9997, y=0.0052x+0.0486). The aqueous extract derived from pink hibiscus (HR5) had the highest total phenolic content, 31.28±1.24 mg EAG/100 g, followed by the aqueous extract of commercial red flowers (HVC1), which presented 29.16±1, 54 mg EAG/100 g. Values presented by HR5 and HVC1 are statistically ($p<0.05$) higher than their respective ethanolic extracts (same floral origin), HR6 and HVC2, suggesting the greater efficiency of phenolic extraction by using water in relation to the use of ethanol as a solvent. This fact was corroborated by the higher phenolic content in the aqueous extract HA8 in relation to the corresponding ethanol extract (HA9). The extracts HV3, HB7, HA9, and HL10, orange hibiscus, showed lower phenolic contents, below 12 mg EAG/100 g. The following decreasing order of total phenolic content can be assumed: HR5>HVC1>HA8>HR6>HVC2>HV4>HB7>HV3>HA9>HL10.

Table 3. Relationship between extracts obtained and their corresponding hibiscus petal samples.

Floral sample	Extract	Solvent	Yield (%)	TPC (mg GAE/100 g)	TFC (mg QE/100 g)
HVC	HVC1	Water	21.87%	29.16±1.54^{a}	156.62±3.23^{d}
	HVC2	Ethanol	17.37%	18.10±1.29^{c}	138.30±7.67^{de}
HV	HV3	Water	7.69%	6.53±0.17^{fg}	95.73±10.83^{fg}
	HV4	Ethanol	15.13%	16.20±0.58^{d}	273.97±11.49^{bc}
HR	HR5	Water	15.72%	31.28±1.24^{a}	469.20±3.24^{a}
	HR6	Ethanol	8.01%	19.80±0.53^{c}	260.42±7.21^{c}
HB	HB7	Water	19.55%	12.39±0.35^{e}	298.35±7.02^{b}
HA	HA8	Ethanol	20.49%	22.61±0.45^{b}	108.14±9.53^{ef}
	HA9	Water	5.17%	7.90±0.43^{f}	77.19±5.86^{gh}
HL	HL10	Ethanol	8.56%	4.69±0.22^{g}	66.53±11.55^{h}

Units: Extraction yields (% mass of extract/mass of dried flowers in grams); phenolic content (mg GAE/100 g dry mass of flowers) and total flavonoids (mg QE/100 g dry mass of flowers). The significance of the differences in contents between the samples was verified by Tukey's test (α = 0.05). Different letters indicate statistically significant differences between the means in the same column. HVC: commercial red hibiscus; HV: red hibiscus; HR: pink hibiscus; HB: white hibiscus; HA: yellow hibiscus; HL: orange hibiscus.

As for the determination of total flavonoids, a quercetin calibration curve (R2=0.9942, y=0.0006x+0.0492) was used to calculate the flavonoid content in equivalence. The HR5 extract had the highest TFC, 469.20±3.24 mg QE/100 g. As for the total phenolic content, the aqueous extracts showed a higher content of flavonoids such as HVC1 and HA8, in relation to the ethanolic extracts of the same floral origin. Both HA9 and HL10 extracts showed the lowest levels of flavonoids. The following decreasing order of total flavonoid content can be assumed: HR5>HB7>HR6>HVC1>HVC2>HA8>HV3>HA9>HL10.

Given the proportionality of the levels presented by the HR5, HA9, and HL10 extracts, it was observed a positive correlation between the TPC and TFC levels (r = 0.604) among samples (Figure 2).

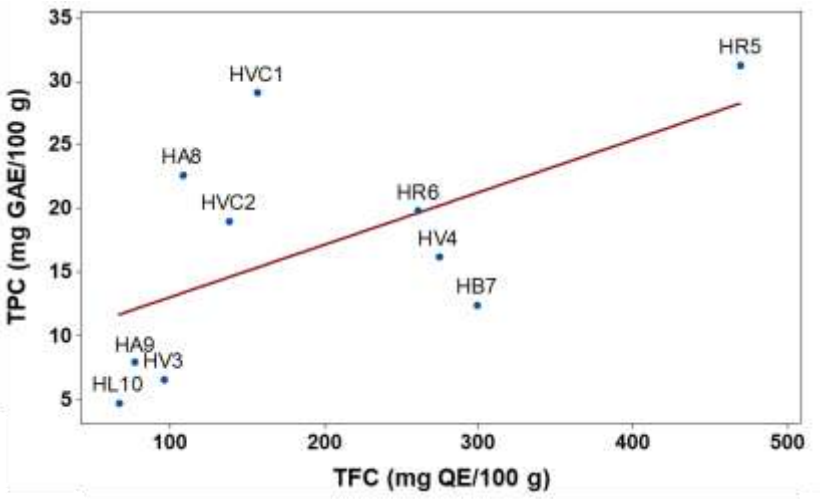

Figure 2. Pearson correlation (r=0.604) was established between total phenolic and total flavonoid contents considering α = 0.05, p=0.002. HVC1: aqueous commercial red hibiscus; HVC2: ethanolic commercial red hibiscus; HV3: aqueous red hibiscus; HV4: ethanolic red hibiscus; HR5: aqueous pink hibiscus; HR6: ethanolic pink hibiscus; HB7: aqueous white hibiscus; HA8: aqueous yellow hibiscus; HA9: ethanolic yellow hibiscus; HL10: aqueous orange hibiscus.

The levels of phenolics and total flavonoids described for hibiscus in the scientific literature are quite discrepant. Several factors may be related to this fact, such as different cultivation conditions, geographic location, plant genotype, and analysis methodologies (DA-COSTA-ROCHA et al., 2014). The quantification of total phenolics in petals of *H. sabdariffa* of Taiwanese origin, for example, showed 23 mg EAG/100 g dry mass (TSAI et al., 2002), a value quite similar to that presented in table 3. Another study, which took into account 24 Mexican varieties of hibiscus, reported great variation in the TPC, between 30±6 and 100±4 mg EAG/100 g, and flavonoids, between 429±5 and 2260 ± 70 mg EAG/100 g (BORRÁS-LINARES et al., 2015). This fact is directly related to the content of anthocyanins that give color to these flowers, suggesting that the agreement in the low levels of phenolics and flavonoids in varieties such as white hibiscus is related to the absence of pigmentation (HB7 extract).

4.2. Antioxidant activity

The antioxidant potential of hibiscus extracts was evaluated according to three complementary methods that allowed a more accurate measurement of antioxidant activity. The ABTS and DPPH radical scavenging spectrophotometric tests are based on the ability of these organic radicals to be reduced by a candidate antioxidant compound through the transfer of electrons or hydrogen atoms; another applied method, called ferric reducing power (ferricyanide protocol), is based on the reduction of inorganic ions, Fe(III) to Fe(II), from electron transfer (PISOSCHI; NEGULESCU, 2011).

The results of the antioxidant activity assays of the aqueous and ethanolic extracts evaluated by the three methods considered above are shown in Figure 3. The radical scavenging capacities (RS) ABTS (Figure 3a) and DPPH (Figure 3b) exceeded 66% and 55 %, respectively. The antioxidant capacity of the analyzed extracts was very similar for both methods, with a variation between extracts of ±5.48, for capturing ABTS radicals, and ±9.15, for DPPH radicals.

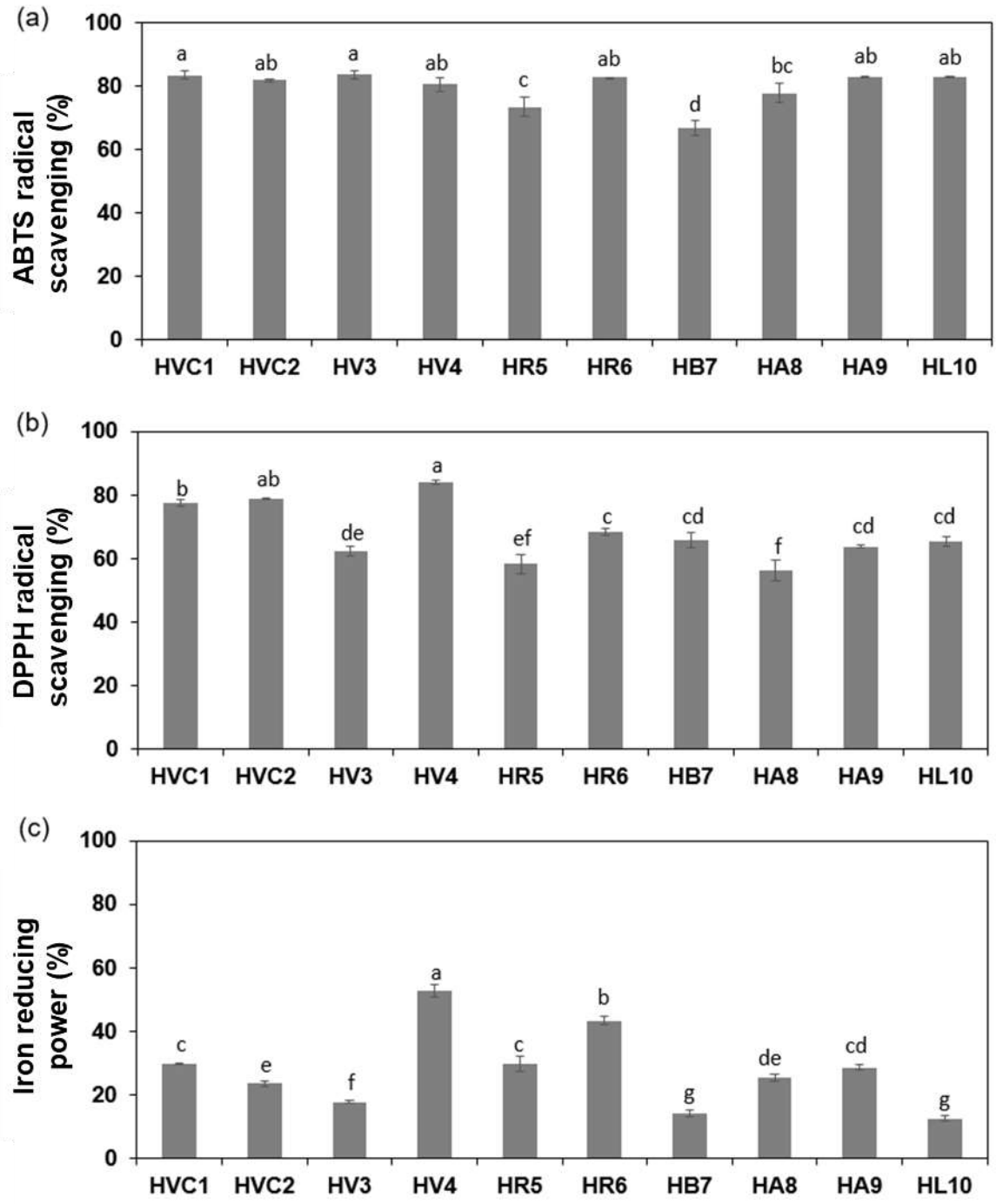

Figure 3. Antioxidant capacity from the scavenging of radicals (SR) (a) ABTS and (b) DPPH and from the capacity of (c) reduction of ferric ions. The bars indicate the mean percentages followed by their standard deviations. The significance of differences in antioxidant activity between extracts was verified by Tukey's test (α = 0.05). Different letters indicate statistically significant differences in the same experimental assay (ABTS, DPPH, or ferric reducer). Identification of extracts: HVC1: aqueous commercial red hibiscus; HVC2: ethanolic

commercial red hibiscus; HV3: aqueous red hibiscus; HV4: ethanolic red hibiscus; HR5: aqueous pink hibiscus; HR6: ethanolic pink hibiscus; HB7: aqueous white hibiscus; HA8: aqueous yellow hibiscus; HA9: ethanolic yellow hibiscus; HL10: aqueous orange hibiscus.

For both experiments, there are significant differences between the means of antioxidant activities of ethanol extracts in relation to aqueous extracts. For RS ABTS, the mean of ethanolic extracts, 81.93±1.33%, was higher than the mean of aqueous extracts, 77.91±6.60% ($p=0.048$), the same occurred for SR DPPH, 73, 85±8.42% for ethanolic extracts, and 64.35±7.27% ($p=0.003$) for aqueous extracts.

Individually, regarding the experiments with ABTS radicals, the aqueous extract HV3 showed the highest antioxidant activity, reaching 83.46±1.43% of inhibition of radical formation. Regarding the capture of DPPH radicals, the HV4 ethanolic extract showed the highest antioxidant activity with 84.09±0.84% inhibition. These results demonstrate the significant antioxidant power of red hibiscus extracts (HV) through the scavenging of organic radicals. There is no statistically significant correlation ($r=0.310$, $p=0.384$) between these two experiments (ABTS and DPPH), despite sharing the same antioxidant action strategies.

As for the reduction capacity of Fe(III) ions (Figure 3c), a greater variation was observed between extracts (±12.51). In agreement with the previously described antioxidant experiments, the Fe(III) ion reduction potential was higher, on average, among ethanolic extracts (37.14±12.13%) in relation to aqueous extracts (21.72±7 .37%) ($p<0.05$). In these experiments, the ethanolic extract HV4 showed the highest antioxidant activity 52.69±1.93%. While the HB7 and

HL10 extracts showed lower Fe(III) reduction activities, 14.19±1.00%, and 12.68±0.73%, respectively.

Considering the previously presented results (phenolic and total flavonoid contents), there is a positive and significant correlation ($p<0.05$) between the Fe(III) ion reduction capacity and the phenolic ($r=0.387$) and total flavonoid contents ($r=0.441$), suggesting that the ferric reducing capacity is directly proportional to the content of phenolic compounds in the analyzed extracts.

The results described for red hibiscus extracts are in agreement with studies on this floral variety that demonstrated that antioxidant activity exceeds 80% inhibition in relation to the formation of DPPH (AWE et al., 2013). Another study demonstrated that the aqueous extracts of *H. sabdariffa* (red) achieved up to 70% ferric-reducing power (TSAI et al., 2002).

4.3. Anti-glycation effects

The anti-glycation effect was evaluated *in vitro* from fluorescent measures on 355 nm excitation and 440 emission wavelengths. Samples were incubated in a BSA-methylglyoxal system. Measures were taken after 24 h and 48 h of incubation at 37°C. The red variety had a higher anti-glycation effect (57.24 ±1.32 %) when compared to the white one (37.39 ±3.81 %) in concentrations equivalent to 1 mg/mL after 24 h (Figure 4). Such a difference was statistically significant ($p<0.0001$). Results showed that the red and white hibiscus extracts performed a better anti-glycation effect within 24 h compared to 48 h of incubation. Comparatively, a 50-60% reduction in the anti-glycation effect was observed after 48 h for both tested samples. The such effect must be associated with mechanisms such as the degradation of potential anti-glycation compounds,

and/or the high reactivity of methylglyoxal towards the formation of AGEs ((DE OLIVEIRA et al., 2020)).

Advanced glycation End-products are a group of heterogeneous compounds derived from the non-enzymatic modification of amino acids, especially lysine, and arginine. Several AGEs are auto-fluorescent (*e.g.* argypirimidine, pentosidine, HA-triosidine) and have been proposed as biomarkers of skin aging and diabetes progression, for instance (OLIVEIRA et al., 2013). The *in vitro* screening of natural compounds takes advantage of this feature to evaluate the anti-glycation potential by simple fluorometric methods (VILLA et al., 2017). Methylglyoxal is a highly reactive dicarbonyl derived from the autoxidation of glucose and implicated in the progression of diabetes and obesity, for instance. Argypirimidine is one example of fluorescent AGE resulting from methylglyoxal glycation (TWARDA-CLAPA et al., 2022).

Glycation has been previously presented as an oxidative process. Some phenolic compounds are well-known antioxidant compounds and the mitigation of glycation has been described for some molecules as rutin and curcumin (AL-MUSAYEIB et al., 2011). Unfortunately, the study of hibiscus as an anti-glycation agent is poorly explored in the literature. In a similar approach, (SANTHOSH; VEERESHAM; RAMA RAO, 2017) reported that 100 µg/mL of the crude extract of hibiscus inhibited 68% of inhibition of a BSA-glucose system. Compared to the most consumed tea plant in the world, *Camelia sinensis*, on a BSA-methylglyoxal system, black tea extract reached 55% of inhibition at 200 µg/mL (ABEYSEKERA et al., 2016). *In vivo*, the administration of hibiscus extract to diabetic mice was demonstrated to inhibit AGE formation compared to animals under no hibiscus consumption (PENG et al., 2011). The authors suggested that the control of

oxidative stress related to AGE formation and the higher levels of glucose was key to the positive role of the hibiscus on these pathways.

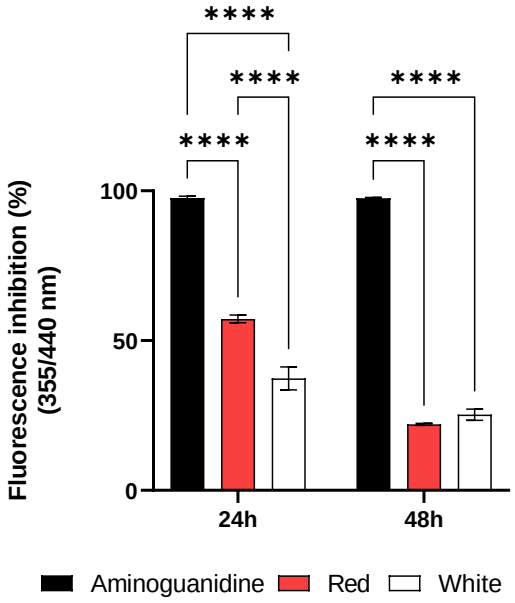

Figure 4. Fluorescence inhibition percentages of red and white hibiscus aqueous extracts (1 mg/mL) after 24 h and 48 h.

4.4. Chromatographic profile characterization

The ten extracts described in table 3 were analyzed by HPLC to characterize their metabolic profiles. For comparison purposes, a pool of phenolic compounds, composed of gallic acid (Peak 1), chlorogenic acid (Peak 2), caffeic acid (Peak 3), quercetin, and rutin (not detected) was used. These substances are described in the literature as part of the composition of hibiscus flowers (PENG et al., 2011). The resulting chromatograms are shown in Figure 5.

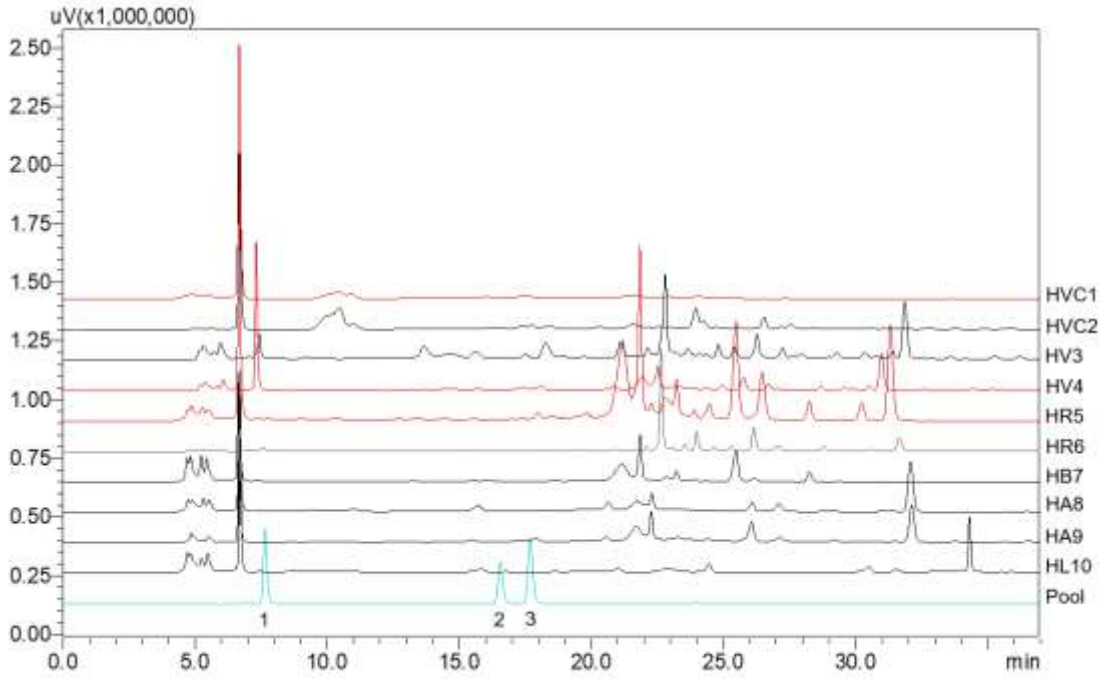

Figure 5. Chromatograms obtained from 10 hibiscus extracts of different colors were analyzed at 280 nm. Peak 1: Gallic acid; Peak 2: chlorogenic acid; Peak 3: caffeic acid. Identification of extracts: HVC1: aqueous commercial red hibiscus; HVC2: ethanolic commercial red hibiscus; HV3: aqueous red hibiscus; HV4: ethanolic red hibiscus; HR5: aqueous pink hibiscus; HR6: ethanolic pink hibiscus; HB7: aqueous white hibiscus; HA8: aqueous yellow hibiscus; HA9: ethanolic yellow hibiscus; HL10: aqueous orange hibiscus.

In general, in the 280 nm detection range, no great metabolic diversity was observed for the evaluated extracts, nor were notable differences between the chromatographic profiles of aqueous and ethanolic extracts of the same floral origin. An exception occurred with the HR5 extract, which presented the profile with the highest metabolic diversity, with 11 main peaks, corroborating the relationship between the number of detected peaks and the highest levels of total phenolics and flavonoids, as discussed in item 5.1.

Observing the chromatograms, the region between 4 and 7 min presented peaks common to all extracts, suggesting a metabolic fingerprint inherent to extracts originating from hibiscus flowers. The identification of similar profiles

between extracts is reported in other studies that evaluate the effect of the collection site on the phytochemical composition of *H. sabdariffa* flowers (BORRÁS-LINARES et al., 2015) and *H. mutabilis* L. leaves (LIANG; MA; SU, 2013). These works pointed out metabolic similarities between individuals from different regions in Mexico and China, respectively.

Comparing these chromatograms to the profile of analytical standards evaluated, it was observed that only the extracts of HV3 and HV4 had peaks in common with the standard of gallic acid with an approximate retention time of 8 min. This suggests that, for the other extracts, these components are absent or in undetectable amounts under the conditions considered for analysis. Through CLAE-DAD-ESI-TOF, BORRÁS-LINARES et al. (2015) identified 37 characteristic peaks in hibiscus extracts, corresponding mainly to hibiscus acid and its derivatives. In agreement with PENG et al. (2011) who, using HPLC/UV-Vis, detected 16 peaks in the chromatogram of the aqueous extract of hibiscus, identifying the main derivatives of hibiscus acid and, to a lesser extent, the occurrence of phenolic compounds such as chlorogenic acid, quercetin, and caffeic acid, justifying the absence of these metabolites in the analyzes presented in this work.

Another important feature that emerges from the chromatographic profiles above is the greater diversity of metabolites with retention times greater than 20 min. Considering that these analyzes were performed in a C18 column (nonpolar stationary phase), less polar molecules interact with greater affinity with the column and have longer retention times, suggesting that extracts such as HR5 have fewer polar compounds.

4.5. Acetylcholinesterase inhibition effect

Aqueous and ethanolic extracts of hibiscus were tested *in vitro* for their ability to inhibit AChE. The inhibitory activities were compared to eserine, a positive control, a reversible AChE inhibitor applied in the treatment of Alzheimer's disease which is commercially available. The average percentages of AChE inhibition, obtained at the sixth minute of the reaction, are shown in figure 6a.

The inhibitory activities of ethanol extracts of red hibiscus (HVC2), 96.56±2.34%, and yellow (HA9), 87.93±2.40%, above 85% inhibition, are statistically equivalent ($p< 0.05$) to the inhibition promoted by eserine (98.45±1.41%). Furthermore, the inhibition potential of the HVC2 ethanol extract is 1.7 times more potent than the aqueous extract (HVC1) of the same floral origin, while the inhibition potential of the ethanolic extract (HA9) of yellow hibiscus is 4.5 times higher. to the aqueous extract (HA8). The only aqueous extract that overcame 50% inhibition is derived from commercial red hibiscus (HCV1), reaching 56.64±8.84%. For the other extracts, the inhibition values varied between 12.58±3.60% (HB7) and 39.48±16.32% (HV3) with no significant difference ($p<0.05$) between the different extracts. These results suggest that both red hibiscus (commercial origin) and yellow hibiscus have potential AChE inhibitor molecules and that the ethanol extraction process is more efficient in the recovery of these molecules.

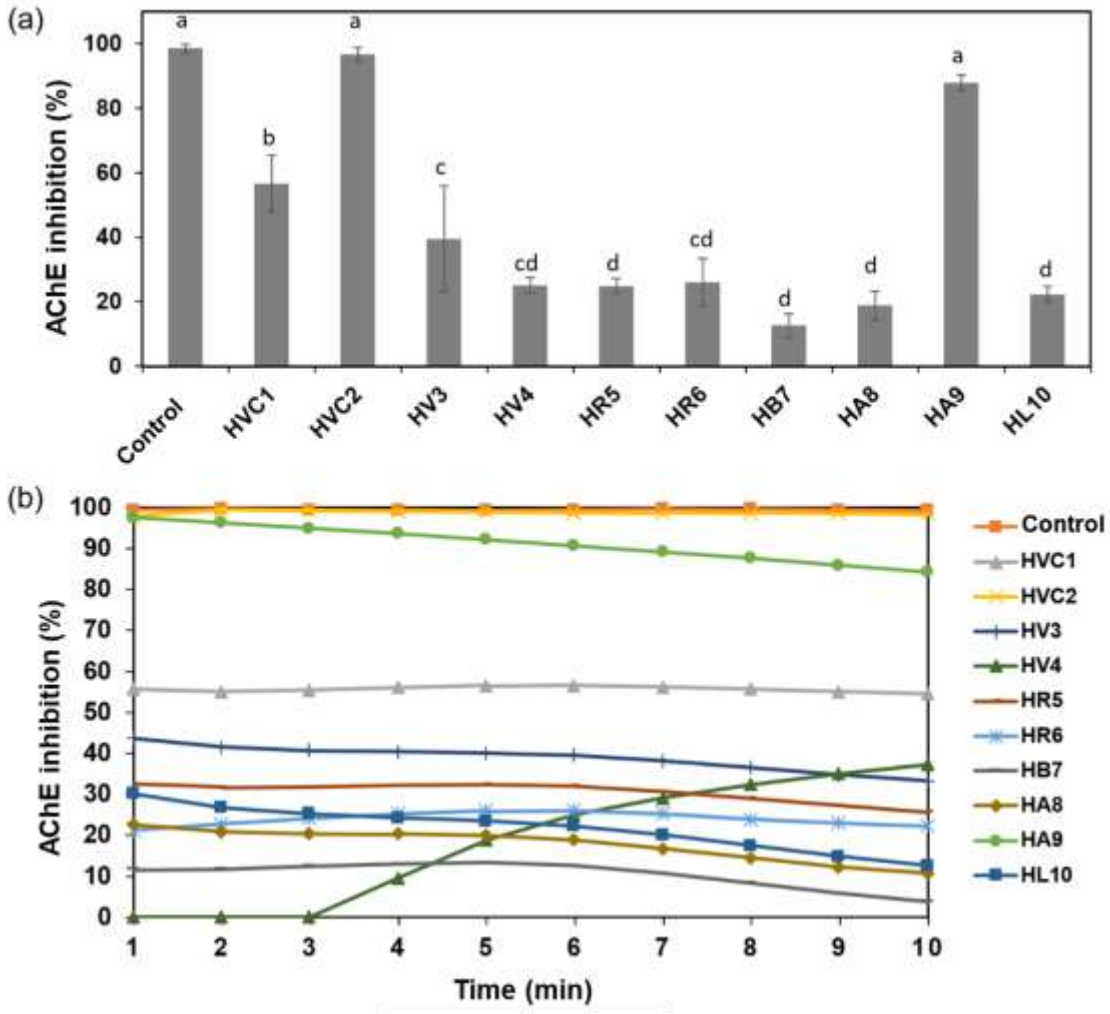

Figure 6. Assessment of acetylcholinesterase (AChE) inhibition potential. (a) Mean AChE inhibition values were determined at the sixth minute of the reaction. The bars indicate the mean percentages of inhibition followed by their standard deviations. The significance of differences in inhibitory activity between extracts was verified by Tukey's test ($\alpha = 0.05$). Distinct letters indicate significant statistical differences; (b) Kinetics of the inhibition reaction over 10 minutes. Control: Eserine. Identification of extracts: HVC1: aqueous commercial red hibiscus; HVC2: ethanolic commercial red hibiscus; HV3: aqueous red hibiscus; HV4: ethanolic red hibiscus; HR5: aqueous pink hibiscus; HR6: ethanolic pink hibiscus; HB7: aqueous white hibiscus; HA8: aqueous yellow hibiscus; HA9: ethanolic yellow hibiscus; HL10: aqueous orange hibiscus.

Figure 6b presents a graph showing the progression of inhibition reactions over 10 minutes. The extract with the highest percentage of inhibition, HVC2, has

a temporal enzyme inhibition profile similar to that of eserine, with consistent inhibition over time (±0.45 variation between the indicated points over the 10 min). The same temporal profile is observed for its aqueous counterpart, HVC1 extract, despite the lower inhibitory activity. For HA9, there is a decrease in inhibitory activity over time. For the other extracts, the temporal profile of inhibition is similar to decay, although small, from the fifth minute of the reaction. Except for the HV4 extract which shows significant growth over time.

Little is explored in the scientific literature on the inhibition capacity of varieties of hibiscus extracts on AChE. Recently, the inhibition potential of *H. sabdariffa* methanolic extract was demonstrated as a function of concentration, presenting EC50 equivalent to 46.96 µg/mL (OBOH; ROCHA, 2008). A similar approach was produced for the species *Hibiscus rosa-sinensis*, which showed inhibition of 62.02±0.03% of AChE (NAZOOL; KUMAR, 2015). Another study that evaluated the *in vivo* inhibition potential was evaluated in mice in which aqueous extracts of hibiscus were administered at concentrations of 100 and 200 mg per kilogram of body mass. Both concentrations showed similar effects and significantly decreased AChE activity in model brains (JOSHI; PARLE, 2006).

4.6. Neuroprotective effect on *D. melanogaster*

The aqueous (HVC1) and ethanolic (HVC2) extracts derived from red hibiscus were evaluated for their *in vivo* neuroprotective potential in *D. melanogaster*. This test evaluates the effect of plant extracts on neuro-compromised flies by the use of rotenone, an insecticide. The dopaminergic neuronal impairment caused by rotenone (JIMÉNEZ et al., 2017) promotes a reduction in the motor capacity of flies, which can be assessed by negative

geotaxis, as described in item 4.3.2. The results are represented in the number of flies in figure 7.

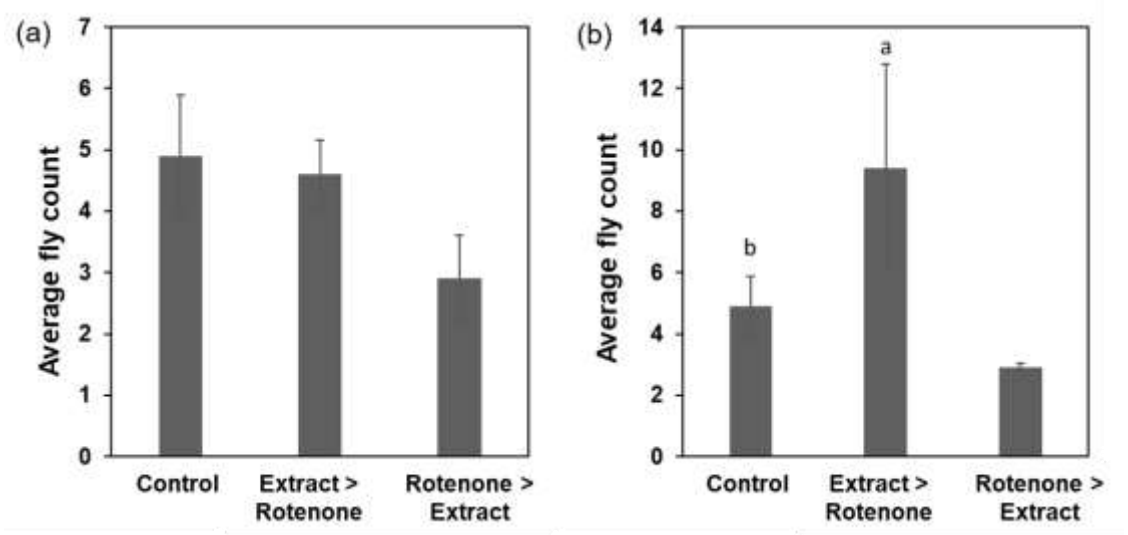

Figure 7. Negative geotaxis evaluation of *D. melanogaster* under the effect of extracts (a) HVC1 and (b) HVC2. The bars indicate the mean number of flies in each experimental group accompanied by their standard deviations. The significance of the differences in the number of flies between the extracts was verified by Tukey's test (α = 0.15).

With the establishment of experimental groups, two effects of extracts on flies can be estimated: the neuroprotective effect, evaluated within the group initially exposed to the extract and later exposed to rotenone; and the therapeutic effect, related to the ability to reduce the neurodegenerative effect of the insecticide within the group initially exposed to rotenone.

Under the experimental conditions, there was great variability within the experimental groups. For the HVC1 extract (Figure 7a) there was no statistically significant difference even within an error statistic with α = 0.15. However, there was a tendency to reduce the number of flies in the group that was initially submitted to rotenone and later to the extract, but there is no therapeutic effect inherent to this extract. On the other hand, the group initially submitted to the

extract showed similar crude values to the control, suggesting a protective effect against the damage caused by rotenone.

For the analysis of HVC2 (Figure 7b), the number of flies was significantly higher ($p<0.05$, $\alpha = 0.15$) within the Extract-Rotenone group (counting more than 2 times the number identified in the other groups) compared to the control and, in particular, to the neuro-compromised group (Rotenone-Extract), suggesting a significant neuroprotective effect, but no therapeutic effect. The relationship between the number of flies in the Extract-Rotenone group and the control group was not clear and, due to the large deviations, needs to be better defined.

In comparison to the inhibition effect of AChE, the results presented for both experiments in relation to the ethanolic extract HVC2 suggest a potential neuroprotective effect demonstrated *in vitro* through antiacetylcholinesterase activity and, *in vivo*, through the promotion of neuroprotection in *D. melanogaster*.

4.7. Effects of gamma radiation over hibiscus microbial load and extract bioactivity

Characterizing the effects of gamma radiation on the postharvest control of potential pathogens in tea-producing plants is of great interest as this technology has been extensively used in the agri-food field (LIMA et al., 2018). In addition to being an effective and secure technology, ionizing radiation can promote bioactivity loss (KUMARI et al., 2009). The bioactive component content is an important aspect that influences hibiscus quality and market value. Therefore, the optimization of their content during production and processing is key to obtaining a product with better functional properties. In this study, a combination of microbiological analysis of hibiscus samples, irradiated with

different doses, and bioassays (antioxidant and antiacetylcholinesterase activities, TPC, and TFC) were used to investigate the effectiveness of gamma radiation in guaranteeing the microbiological quality and bioactivity of this food matrix. It is worth noting that sample differences are likely related to manufacturing (*e.g.,* management, production, and postharvest handling systems employed), environment (*e.g.,* soil, drought), and genetic traits (JULIANI et al., 2009).

4.7.1. Microbial decontamination

Microbial contamination was investigated using colony-forming unit (CFU) counts from direct counting agar plates of non-irradiated and irradiated samples (HS, HRS1, and HRS2) (Figure 8b). Specific culture media were used for bacterial and fungal CFU counts *per* gram of tea. This experiment measured the survival of the major microorganisms colonizing hibiscus flowers according to increasing irradiation doses.

As expected, under no irradiation treatment, the lowest microbial load was found in the commercial sample (10^2 CFU/g) after flowers were subjected to reduced microbial growth after drying (*e.g.* reduced water activity, stocking conditions). It is important to highlight that no irradiation treatment or specific microbiological control methods were indicated on the original packaging label. Comparing bacterial and fungal counts, bacterial CFUs were more abundant in HS samples compared to the number of fungal colonies. However, both groups of microorganisms were found on HRS1 and HRS2 (10^4 CFU/g).

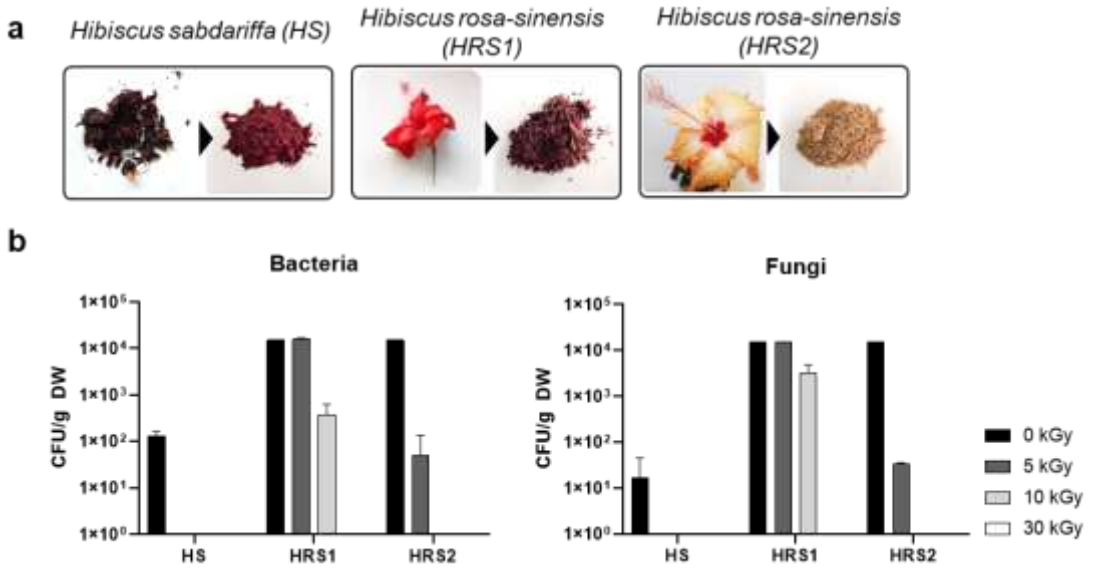

Figure 8. (a) Aspect of fresh and dry powdered hibiscus samples, HS (*Hibiscus sabdariffa*), HRS1, and HRS2 (*Hibiscus rosa-sinensis*); (b) Bacterial and fungal loads on hibiscus samples before and after-gamma radiation treatment. (i) HS, (ii) HRS1, and (iii) HRS2 counts.

We then subjected the packed powdered hibiscus samples to three different gamma radiation doses (5 kGy, 10 kGy, and 30 kGy). The commercial sample was the only one to have complete eradication of microorganisms under a 5 kGy dose, due to the lower initial microbial counts. Hand-picked samples required higher radiation doses. Microbial decontamination was achieved at a dose of 10 kGy for HRS2, but a dose of 30 kGy was needed to sterilize HRS1, possibly due to the occurrence of a higher microbial load and/or the presence of more resistant microorganisms. These results corroborate that the initial microbial load in dry hibiscus is an important factor in the efficiency of microorganism elimination by gamma radiation.

The presence of microorganisms in teas may have different origins including phylloplane flora (associated with the cultivation environment), human manipulation, or microbial growth during storage (MISHRA; GAUTAM; SHARMA, 2006). In hibiscus, the occurrence of bacteria of mammal origin and aflatoxins-

synthesizing fungi species have been reported, emphasizing the need for microbial quality control. The irradiation effectiveness on microbial decontamination depends on the substrate, dose, and microbial load (KUMARI et al., 2009). From our results, we observed that a higher initial microbial load requires higher irradiation doses for hibiscus (powder) decontamination. Irradiation treatments of 10 or 30 kGy were found to be effective for sterilization even at higher microbial counts, ensuring hibiscus safety, while respecting the established international recommendations on the limit of gamma radiation used in food products (30 kGy limits).

To our knowledge, this study is the first to investigate the effect of gamma irradiation on hibiscus samples. In comparison, microbial contamination levels were similar in commercial green tea samples where the microbial load ranged from 10^1 to 10^2 CFU/g, and a dose of 5 kGy was reported to be optimal for complete sterilization (MISHRA; GAUTAM; SHARMA, 2006) as found in our results concerning the commercial sample, HS. Relative to other dry products, complete elimination of microorganisms from dry food matrices such as rosemary, and medicinal herbs, with a gamma radiation dose of up to 10 kGy (PÉREZ; CALDERÓN; CROCI, 2007).

4.7.2. TPC and TFC

The effects of gamma radiation on TPC and TFC parameters are shown in Figure 9. In control conditions (0 kGy), TPC in the commercial sample (HS), 0.40±0.02 mg GAE/g DW, was significantly lower ($p<0.05$) relative to 1.95±0.20 mg GAE/g DW and 2.09±0.20 mg GAE/g DW in the non-commercial samples, HRS1 and HRS2, respectively. Screening for TFC showed similar results with

statistically significant ($p<0.05$) lower contents in HS (7.92±1.14 mg QE/g DW), compared to HRS1 (31.95±1.53 mg QE/g DW) and HRS2 (36.86±4.52 mg QE/g DW). Overall, the TPC and TFC values determined under control conditions (0kGy) fit well the range of polyphenol levels described in the literature for some hibiscus varieties from different countries (BORRÁS-LINARES et al., 2015; GAMAGE et al., 2021). Differences between the phenolic contents *H. sabdariffa* and *H. rosa-sinensis* have already been reported, suggesting that the lower TPC and TFC in HS are species-specific (PULGARÍN; BERMEJO; CARRASQUERO, 2016).

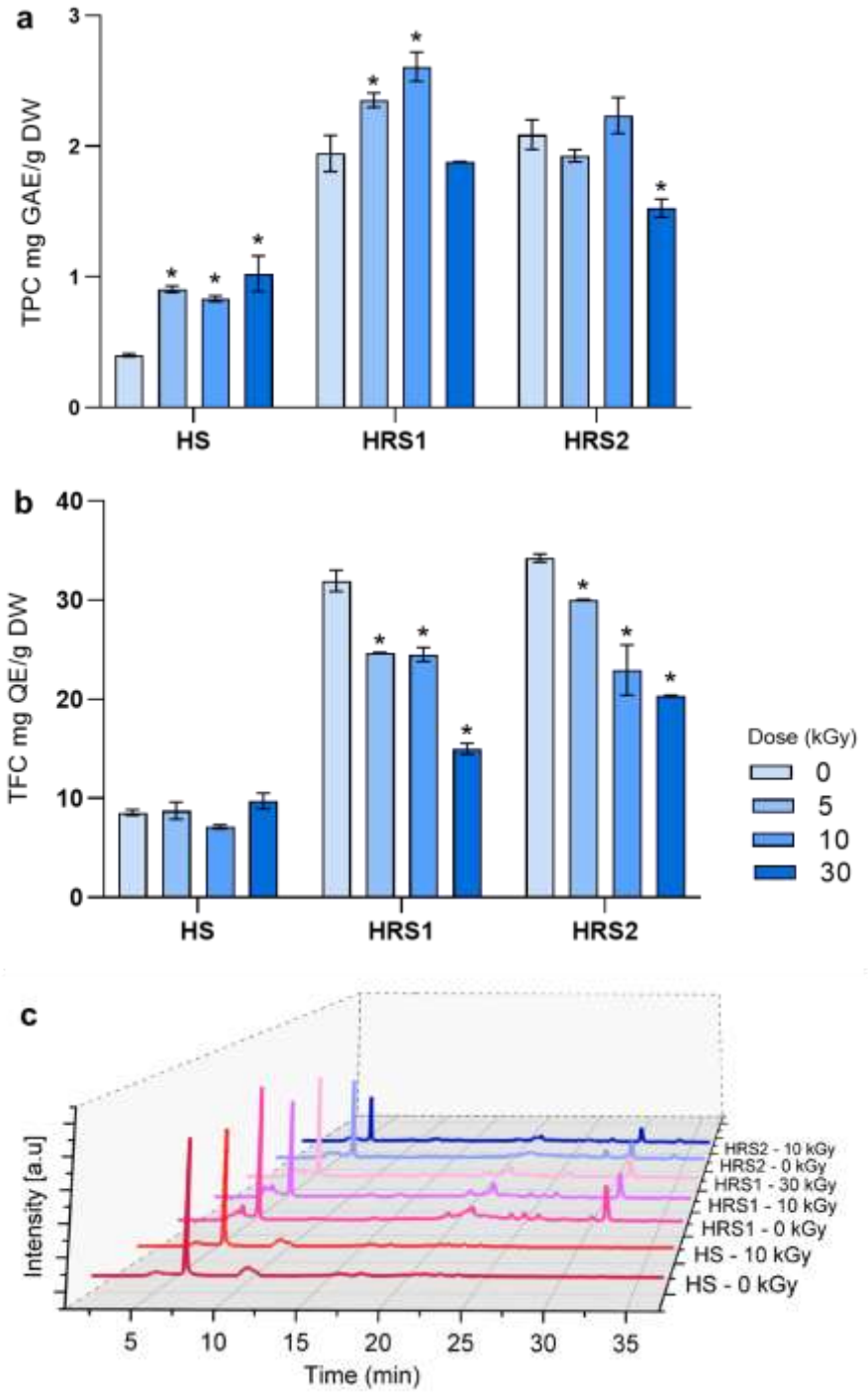

Figure 9. (a) TPC and (b) TFC of irradiated (5-30 kGy) and non-irradiated (control) hibiscus samples. The bars indicate the average percentages accompanied by their standard error of the mean (SEM). The significance of differences -indicated by asterisks - in TPC or TFC within the same sample was verified by Dunnett's test ($\alpha = 0.05$) having the control condition (0 kGy) within each sample as reference. (c) Metabolic profiles of HS, HRS1, and HRS2 from

HLPC UV-Vis analysis (280 nm). Controls (0 kGy) and irradiated samples are indicated.

The total phenolic and flavonoid contents of the HS were less likely to be negatively affected when exposed to radiation. An increase ($p<0.05$) in TPC was observed from the minimal radiation dose ahead for HS extracts, but no change was observed for TFC (Figure 9a). HRS1 showed a similar increase at 5 kGy and 10 kGy, but the phenolic content at 30 kGy remained the same as that in the control condition (Dunnett's test, $\alpha=0.05$). Expressive losses occurred in HRS1 and HRS2 TFCs when subjected to 30 kGy, accounting for almost 50% of flavonoid content reduction. Variations in phenolic levels could be explained by opposing effects associated with ionizing radiation which can interact with TPC and TFC. First, the disruption of glycosidic bonds can make phenolic compounds more reactive than the glycoside forms themselves. However, gamma radiation creates a source of oxidative stress that may contribute to the oxidation of several bioactive compounds in hibiscus.

Hibiscus metabolic profile was assessed by chromatographic analysis using HPLC UV/VIS (280 nm) based on the methodology for polyphenol detection proposed by Borrás-Linares et al. (2014). Comparative analysis of the resulting chromatograms did not reveal consistent changes in the metabolome in any experimental condition, even at higher doses (Figure 9c). Thus, samples kept metabolic signature despite radiation dose and, although the irradiation promoted some effects in phenolic and flavonoid contents, these alterations did not result in qualitative variation in the respective metabolic profiles. This result contributes to the understanding of the effects of gamma irradiation on hibiscus samples. Preservation of the phenolic compounds after irradiation was also supported by the literature (PEREIRA et al., 2017).

4.8. Antioxidant activity

Hibiscus has received considerable attention because of its well-known antioxidant potential. Therefore, the effect of irradiation was further tested for antioxidant activity. The results of the ABTS and DPPH radical scavenging activities as well as the Iron Reducing Power (IRP) of irradiated and non-irradiated extracts of hibiscus are shown in Figure 10. Overall, non-irradiated samples showed higher ABTS and DPPH radical activities than IRP. All samples had similar ABTS scavenging (around 80%) across all treatments. For DPPH, HRS1 samples showed the greatest ($p<0.05$) initial (0 kGy) scavenging potential, 92.80±0.23%. While HRS2 had a higher ($p<0.05$) IRP effect, 40.03±2.07%.

When subjected to radiation, greater variations were observed for the DPPH and IRP assays. The results indicated a higher HS stability across the radiation doses applied (similar to that described for TPC and TFC). Among the different methods, the antioxidant activity of HS was only affected by the IRP effect, which increased at 10 kGy. An increase in IRP or DPPH effects was observed when subjected to 5 kGy or 10 kGy for HRS1 and HRS2, respectively. Initial antioxidant levels were maintained at 30 kGy. An exception was made for HRS1, in which gamma irradiation processing reduced by 30% ($p<0.05$) DPPH radical scavenging compared to the non-irradiated control. The different antioxidant responses within each sample across different antioxidant protocols highlight the importance of multiple measures of this trait.

It is important to highlight that the mechanism of glycosidic bond breakdown (previously discussed) could account for the increased antioxidant activity (also to the antiacetylcholinesterase effects described ahead). In soybeans seeds, for instance, 5 kGy radiation dose led to aglycone release,

which consequently enhanced the antioxidant capacity. On the other hand, ionizing energy can interact with food constituents (*e.g.* water, lipids) creating an oxidant environment. Free radicals resulting from water molecules under irradiation conditions could promote the degradation or oxidation of polyphenols with antioxidant effects, reducing the effectiveness of antioxidant effects (KUMARI et al., 2009).

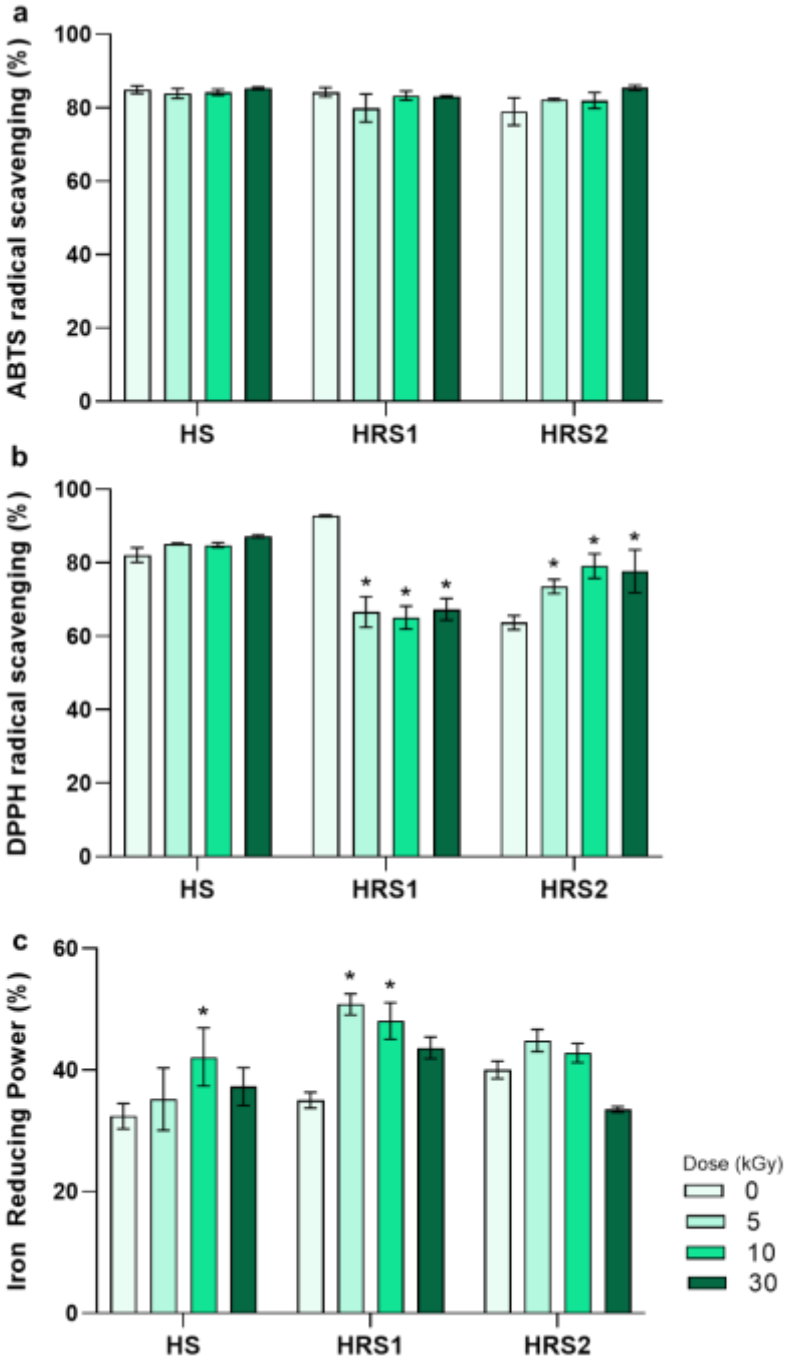

Figure 10. Antioxidant analysis of irradiated (5-30 kGy) and non-irradiated (control) hibiscus samples. (a) ABTS radical scavenging; (b) DPPH radical scavenging; (c) Iron Reducing power analysis. Bars indicate the average percentages accompanied by their standard error of the mean (SEM). The significance of differences -indicated by asterisks - in TPC or TFC within the same sample was verified by Dunnett's test ($\alpha = 0.05$) having the control condition (0 kGy) within each sample as reference.

4.9. Antiacetylcholinesterase activity

The neuroprotective effect of hibiscus extracts was assessed using an *in vitro* colorimetric antiacetylcholinesterase kinetic assay. The results are summarized in Table 4. Without radiation treatment, the highest antiacetylcholinesterase potential was observed for HS and HRS2, 29.05±1.54 % and 24.42±0.77 %, respectively ($p<0.05$) compared to HRS1, 3.98±0.55 %. Gamma irradiation had no significant effect on HRS2. On the other hand, a 30% decrease in acetylcholinesterase inhibition was observed for HS when subjected to 10 kGy at both start and endpoints. However, the enzyme inhibitory effect was significantly improved in HRS1 samples irradiated with 30 kGy compared to those irradiated with 0 and 10 kGy. This improvement was 76% when comparing reaction starting points, and 40% when comparing ending points. The enzyme kinetics presented in Figure 11 for the first 10 min of reaction suggests a tendency for temporal reduction of the inhibitory effect once there is a significant difference ($p<0.05$, underlined numbers) between the starting and endpoints within the same irradiation dose. Only HRS1 showed temporal stability under irradiation treatments.

Table 4. Initial (0 min) and final (10 min) antiacetylcholinesterase inhibition effect of hibiscus (HS, HRS1, HRS2) aqueous extracts.

Sample	Reaction time (min)	Dose (kGy)		
		0	10	30
HS	0	29.05±1.54%	20.95±2.73*%	-
	10	17.05±1.05%	12.29±1.36*%	-
HRS1	0	3.98±0.55%	5.15±1.09%	17.10±2.73*%
	10	7.09±0.21%	4.71±1.47%	11.69±0.84*%
HRS2	0	24.42±0.77%	24.68±2.36%	-
	10	13.48±2.43%	13.28±1.89%	-

The significance of differences within the same row is indicated by asterisks ($p<0.05$). The significance of differences within the column and sample is indicated by underlined numbers ($p<0.05$). –, not analyzed.

Little is known about the hibiscus antiacetylcholinesterase effect. Recently, the methanolic extract of *H. sabdariffa* was reported to have an EC_{50} equivalent to 46.96 µg/mL (OBOH et al., 2018). A similar approach was demonstrated for *Hibiscus rosa-sinensis* species, which showed inhibition of 62.02±0.03% of AChE on aqueous extracts (NAZOOL; KUMAR, 2015). Another study assessed the potential *in vivo* inhibition was evaluated in mice to which extracts were administered hibiscus solutions at concentrations of 100 and 200 mg/kg of body weight. Both concentrations had similar effects at significantly decreasing AChE activity in model mice (JOSHI; PARLE, 2006). Here, we observed a beneficial effect while increasing by almost 76 % the inhibitory effects which require an investigation of the mechanism and the compounds contributing to the neuroprotective effect that could help mainly the formulation of more accessible supplements based on hibiscus.

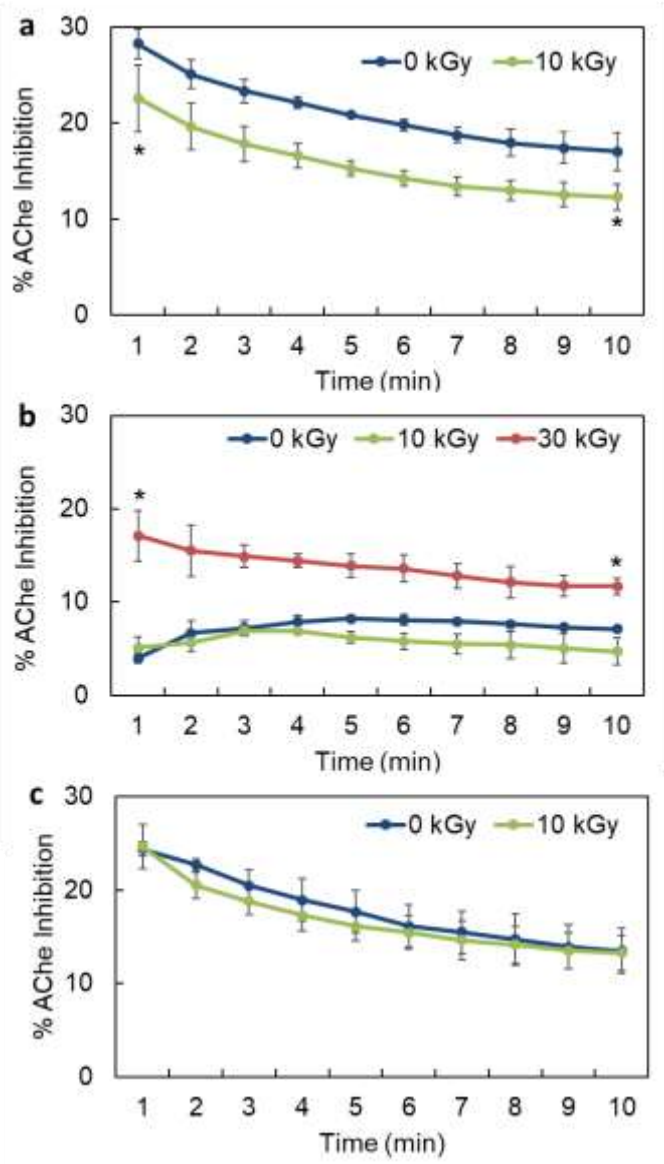

Figure 11. Kinetics of the antiacetylcholinesterase inhibition reaction of hibiscus samples (a) HS; (b) HRS1; (c) HRS2 over 10 minutes reaction. Control: Eserine -The reference value was set to 100 % of inhibition – value not shown. The significance of differences -indicated by asterisks - within the same reaction time ($p<0.05$).

5 FINAL CONSIDERATIONS AND PERSPECTIVES

The results obtained so far regarding the hibiscus varieties analyzed in this study have shown that there are significant differences between samples in terms

of total phenolic and flavonoid content, antioxidant capacity, and neuroprotective potential *in vitro* and *in vivo* evaluations.

The ethanolic extracts showed higher antioxidant activity, while the ethanolic extracts showed higher AChE inhibition and neuroprotective activity, indicating a difference in the solubility of bioactive molecules between the solvents used. The aqueous extract of pink hibiscus, HR5, stands out for its higher levels of phenolics and total flavonoids and greater metabolic diversity presented by the chromatographic profile. On the other hand, the extracts of hibiscus red HVC2 and yellow HA9 stood out for their ability to inhibit AChE similar to the positive control, eserine, as well as the neuroprotective capacity, demonstrated for HVC2 *in vivo*.

Regarding the processing of hibiscus samples using gamma radiation, the results indicated that there was no significant change in the flavonoid and phenolic contents, antioxidant, and antiacetylcholinesterase capacity at the doses used for sterilization of the material. Therefore, the use of this technique can be an effective way of conserving this floral material, through the elimination of microorganisms, without changing the content of metabolites with bioactive capacity.

Regarding the primary aim of irradiation use, 10 kGy and 30 kGy were demonstrated to be optimal for microbial eradication even at higher microbial counts, respecting the standard international recommendations for gamma irradiation in food for human consumption. Overall, irradiation had a specific effect on hibiscus phenolic contents or bioactivity. Pushing doses up to 30kGy demonstrated to have adverse effects on TFC, but no direct relationship was observed with other parameters described in this paper. Some specific

treatments resulted in a significant reduction of TPC, for instance, but this effect did not result in a direct reduction of bioactivity for all samples. However, decreasing tendencies deserve attention, especially regarding the investigation of correlated features such as sample matrix composition, or even the association of water content which could corroborate with the degradation of important functional target molecules. This leads to the conclusion that irradiation is an effective way of guaranteeing hibiscus microbial quality but reaching 30 kGy results in observable negative effects. From a perspective, antioxidant effect maintenance and the increase in almost 80% of the antiacetylcholinesterase effect highlights gamma irradiation as an important tool to improve hibiscus bioactivity and opens possibilities to investigating it as a source of neuroprotectants, which could lead to interesting therapeutic and market valuable uses.

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CHAPTER 3

Evaluation of HUVECs (Human Umbilical Vein Endothelial Cells) as a model for the study of the cellular inflammation mediated by the Receptor of Advanced Glycation End-Products (RAGE)

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ABSTRACT

RAGE, the Receptor for Advanced Glycation End-Products, has been associated with human aging and the development of several age-related pathologies owing to its pro-oxidative and pro-inflammatory signaling. RAGE is emerging as a much more promiscuous receptor for which several molecules have been identified as ligands, TNFα (Tumor Necrosis Factor Alpha), HMGB1 (High-Mobility Group Box1) proteins, and dietary AGEs (largely generated in thermally processed foods). Endothelial cells function as the first barrier lining blood vessels and regulating the exchanges between the bloodstream and tissues. Endothelial cells express RAGE and have been used as a model in inflammation studies, vascular injury, diabetes, and angiogenesis. The present work section aimed to characterize Human Umbilical Vein Endothelial Cells (HUVECs) as a model for inflammation. *TNFα*, *VCAM1*, *IL6*, and *RAGE* relative expression (rt-PCR) were evaluated under TNFα (10 ng/mL) or HMGB1 stimulation for different periods. RAGE and NF-κB protein expressions were evaluated through immunofluorescence staining. TNFα elicited *TNFα*, *VCAM1*, and *IL6* relative expression increased in a time-dependent manner up to 9 h of stimulation. Only *IL6* expression increased up to 24 h of stimulation, while e *TNFα* and *VCAM1* were significantly reduced between 9 h and 24 h. *RAGE* expression increased over time, but no response to TNFα or HMGB1 was observed. Similarly, RAGE protein expression was not noticeable from the immunostaining assay, but NF-κB nuclear translocation occurred within 3 h of exposition to TNFa. Results suggest that inflammation activation is independent of variations of *RAGE* expression both at the transcriptional and translational levels. Further investigation is required to delineate the precise participation of RAGE in inflammation activation on HUVEC cultures and to better define the usefulness

of this model for such a purpose. Such definition can corroborate with the elaboration of a cell culture model to screen important dietary neo-formed contaminants which interact with RAGE.

Keywords: HUVECs; inflammaging; cell model; TNFα; HMGB1; RAGE

1 INTRODUCTION

1.1 Endothelial cells as a model to characterize dietary pro-inflammatory compounds

Endothelial cells are the first protective layer lining blood vessels. Such a position is key in regulating exchanges between the bloodstream and tissues (POBER; SESSA, 2007). As a barrier, the vascular endothelium takes part in the regulation of inflammatory responses mediated by different cell receptor families as TNFRSF, Toll-like receptors (TLR) cell membrane receptors, and RAGE (BOULANGER et al., 2002; JEUCKEN et al., 2019; SALVADOR et al., 2016; SCHMIDT et al., 1992). For this reason, endothelial cells have been used as models for the investigation of inflammatory implications in angiogenesis, diabetes, and the inflammatory component linked to aging “inflammaging” (FRANCESCHI et al., 2018; SULTAN et al., 2015). HUVECs (Human Umbilical Vein Endothelial Cells) is a model for the functional study of inflammatory diseases and microvascular injury (MAY et al., 2018, p. 1). First isolated during 1970, HUVECs became popular due to the relative easiness of extraction and culture in laboratory conditions compared to other vessel cell types. Moreover, HUVECs were discovered to be responsive to stress stimuli such as LPS or glucose, which modulate the expression of important endothelial biomarkers (*e.g.* VCAM1 and selectins) (CAO et al., 2017).

RAGE is expressed in endothelial cells and is believed to control endothelial permeability, which is crucial to inflammation susceptibility (JEONG et al., 2019), and vessel integrity (GROSSIN et al., 2015). More recently, RAGE turned to be treated as a sensor able to respond to cytokines, pathogen particles, and even to AGES of alimentary origin (HUDSON; LIPPMAN, 2018). AGEs are both formed at physiological conditions (37 °C) and during the thermal processing

of foods (SHARMA et al., 2015). Considering the frequent intake of dietary AGES (dAGES) from westernized ultra-processed foods and the potential association of these neo-formed compounds to inflammation initiation, the constant consumption of dAGES would expose the immune system to chronic inflammation and oxidative stress (FOUGÈRE et al., 2017; LÓPEZ-OTÍN et al., 2013).

Considering the health implications of the RAGE-dAGE axis, it is quite important for the food industry to understand the extension of the impacts these neo-formed compounds could have on human health. Carboxymethyl-lysine (CML), for instance, is one of the main studied AGEs due to its abundance in food matrices, and stability as a biomarker (SCHEIJEN et al., 2016). CML was demonstrated to affect calcium homeostasis on HUVECs by the activation of endoplasmic reticulum stress, the activation of apoptosis, and cell injury (LEE et al., 2014). More recent discoveries have shown that a 24 h exposure of HUVEC to CML-albumin was able to reduce the expression of paraoxonase (PON2) enzyme, incrementing the oxidative stress, endoplasmic reticulum stress, and inflammation activation (RAVI; RAGAVACHETTY NAGARAJ; SUBRAMANIAM RAJESH, 2020).

That being said, composing a functional experimental model for the screening of dietary compounds is of great industrial/therapeutic value. However, as a model, several factors may modulate the effects attributed to RAGE *in vitro*, such as medium composition, culture period, or even the occurrence of the cell membrane as heparin or heparan sulfate (XU; ESKO, 2014). Following, a brief review is done of RAGE signaling modulation and the interactions with glycosaminoglycans.

2 UNDERSTANDING RAGE CELL SIGNALING AND FACTORS IMPLICATED IN ITS MODULATION

2.1. RAGE cell signaling

RAGE cell signaling cascade is an intricate network. RAGE-dependent mechanisms are associated with NF-κB which modulates gene transcription under inflammatory, oxidative stress, high-glucose, or AGEs (Figure 1) (TÓBON-VELASCO; CUEVAS; TORRES-RAMOS, 2014). Increases in the expression of intracellular oxidative, pro-inflammatory, and pro-angiogenic signals as NADPH oxidase, and cytokines as interleukin 6, NF-κB, TNFα, and TNFβ, have been linked to RAGE activation (SERBAN et al., 2015). The effects of AGEs for instance have been demonstrated over VCAM1 transcription. The expression of VCAM1 was shown to be disrupted within the administration of glycated albumin in endothelial cells. The role of RAGE in this interaction was evidenced by mAb RAGE blocking, which prevented the increase of VCAM1 expression in the presence of the glycated proteins (BOULANGER et al., 2002). Later, the same author demonstrated that glycated albumin induced vascular endothelial growth factor (VEGF) expression in mesothelial cells and inhibit capillary tube formation on HUVECs. Similarly, the use of RAGE antibody blockage was demonstrated to inhibit such effects (BOULANGER et al., 2007).

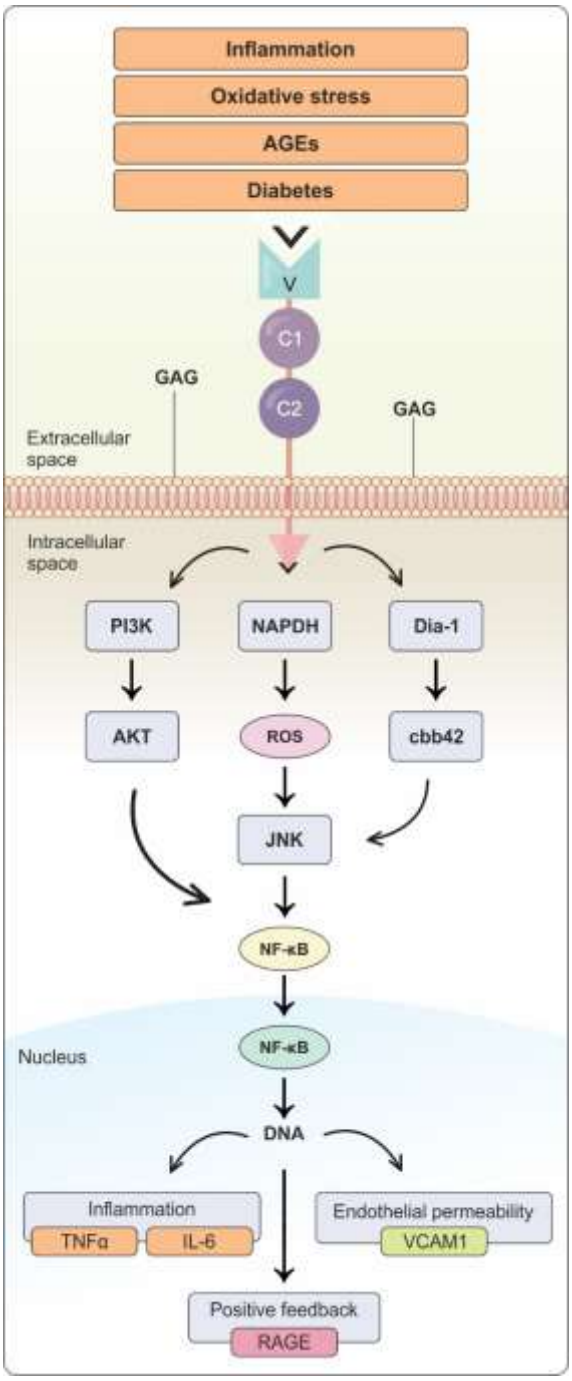

Figure 1. RAGE is known as a promiscuous receptor that interacts with different ligands and responds to different pathological conditions such as inflammatory conditions, hypoxia, oxidative stress, and dietary advanced glycation end-products. RAGE signaling is linked to gene expression modulation especially mediated NF-κB. Different pathways may contribute to NF-κB translocation into the nucleus as ROS increases, Dia-1 activation, or P13k/AKT pathway trigger. In the nucleus, NF-κB is able to activate the expression of cytokines, endothelial

markers of cell membrane integrity as VCAM1, added to the positive feedback of RAGE expression.

Despite its putative implication in human health above described, RAGE-mediated signal transduction is not completely understood due to the intrinsic complexity of interactions between a cell receptor and its ligands, and the many different pathophysiological contexts in which these interactions may arise. A further complication is emerging from recent discoveries of RAGE's conformation, based on results from NMR, crystallography, and exploratory RAGE-ligand research, which have revealed significant interactions between the receptor, its ligands, and a vast family of glycosaminoglycans (GAGs) (SHUJI; JUN; KAZUYUKI, 2012). Contrasting events have been observed associating the RAGE-ligand axis with GAGs, leading to both RAGE-mediated cell activation and anti-inflammatory responses. Two main effects are observed: (1) the interception of RAGE-ligand interaction, resulting in signal blocking, and; (2) promotion of RAGE oligomerization and strong signal-transduction (Table 1).

2.2. RAGE activation is modulated by glycosaminoglycans (GAGS)

Heterogeneity is a key characteristic of the GAG family (POMIN; MULLOY, 2018). GAGs are polysaccharides composed of a basic disaccharide unit made up of an amino sugar (N-acetylgalactosamine or N-acetylglucosamine) and uronic acid (glucuronic or iduronic acid), and the family comprises several molecules such as heparin, heparan sulfate, and chondroitin sulfate (Figure 2). GAGs are expressed in different cell types and are commonly associated with proteins such as cytokines, intra- or extracellular proteins, and even membrane receptors (MIZUMOTO; SUGAHARA, 2013). Heparin and heparan sulfate is the

best-described GAGs, naturally present in cell membranes, and commonly associated with proteins.

Heparin has been used for decades as an efficient antithrombin agent: it is a highly sulfated GAG, has a high iduronic acid content, and is produced in connective tissue mastocytes. It has a lower molecular weight (12-14 kD) but greater iduronic acid and sulfation levels (>80%) compared with heparan sulfate (JIN et al., 1997; XU; ESKO, 2014). Heparan sulfate (10-70 kD) is expressed by every animal cell and is a linear, sulfated compound capable of interacting with a great number of proteins that constitute a heparan sulfate “interactome”. This group of molecules is involved in the key pathophysiological process: heparan sulfate and chondroitin sulfate, for instance, are overexpressed in Lewis Lung Carcinoma (LLC) cells and are thought to play an important role, mediated by RAGE, in the proliferation of metastatic lung cells (SHUJI; JUN; KAZUYUKI, 2012).

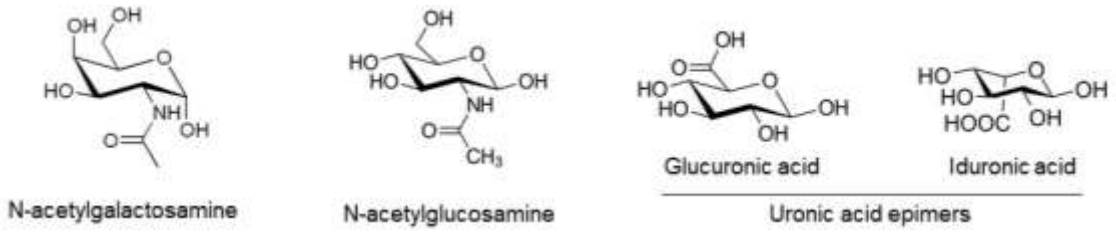

Figure 2. Chemical structures of glycosaminoglycans’ constituent polymeric units.

Some other GAGs described below are the result of biotechnological approaches to investigate the potential therapeutic potential of GAGs. The synthesis of desulphated variants of heparin (*e.g.* ODSH) or the use of bacteria

for high-volume production (*e.g.* K5 polysaccharides) are examples of these biotechnological investigations of therapeutic GAGs (LINDAHL et al., 2005).

Table 1. Glycosaminoglycans (GAGs) and their effects on the RAGE-ligand axis.

Mechanism	GAG	Effect	Reference
Blocking of RAGE-ligand interaction	Heparin	Heparin changes HMGB1 conformation reducing its affinity to RAGE	(LING et al., 2011)
		Heparin reduced HMGB1 protein and mRNA expression on Wistar Rat ischemia and reperfusion models	(LIU et al., 2009, p. 12)
	2-0, 3-0 Desulfated Heparin (ODSH)	ODSH blocks HMGB1 binding to RAGE	(RAO et al., 2010)
	K5 Polysaccharide	K5OSH molecules inhibit biotinylated HMGB1 and Aβ-peptide binding to RAGE	(HEIZMANN, 2013)
	Low Molecular Weight Heparin (LMWH)	LMWH acts by inhibiting HMGB1 mediated NF-κB activation	(TAKEUCHI et al., 2013)
		Anti-inflammatory effect through RAGE-HMGB1 blocking in placental explants reduces TNFα and IL-6 expression	(ZENERINO et al., 2017)
		LMWH reduced S100A12 RAGE binding in a dose-dependent manner (0-100 µg/mL)	(LIU et al., 2009)
Supporting RAGE oligomerization	Heparin	Heparin inhibits RAGE homophilic interaction Live-Cell Protein-Fragment Complementation Assay	(ROUHIAINEN et al., 2018)
	Heparan sulfate	Heparan sulfate interacts with RAGE amplifying HMGB1 cell activation	(XU et al., 2011)
		Heparan sulfate promotes the orientation of V-C1 hydrophobic regions required for dimer stabilization	(XU et al., 2013)

2.3. GAGS and RAGE ligand interactions

Among the known RAGE ligands, HMGB1 and S100 proteins stand out owing to their involvement in cancer development, diabetes, cardiovascular disorders, and inflammation (CHEN; XU; LIU, 2014; TRIPATHI; SHRINET; KUMAR, 2019). Both proteins are described as Damage-Associated Molecular Patterns (DAMPs) and HMGB1 (Figure 3) is a ubiquitous, DNA-binding protein involved in chromatin remodeling. In addition, HMGB1 has been identified as a heparin-binding molecule with a conserved heparin-interaction motif over the N-terminal tail (RAUVALA; ROUHIAINEN, 2007). Owing to its physiological function, HMGB1 is mainly found in the cell nucleus but may also be found in the cytoplasm or the extracellular space when released from necrotic cells or following inflammatory responses (YAMADA; MARUYAMA, 2007). In their turn, S100/calgranulin proteins are part of a calcium-binding family with several functions besides calcium homeostasis, including cell migration and inflammation signaling. The S100 proteins play a role in both the intracellular as well as extracellular compartments. Both HMGB1 and S100 play a role in immune responses and inflammation initiation, therefore, working as cytokines (ROH; SOHN, 2018; YANG et al., 2005, p. 1). HMGB1, as well as S100 proteins and other RAGE ligands, may form complexes with GAGs that engender contrasting effects as a result of their interaction with RAGE (XU et al., 2011).

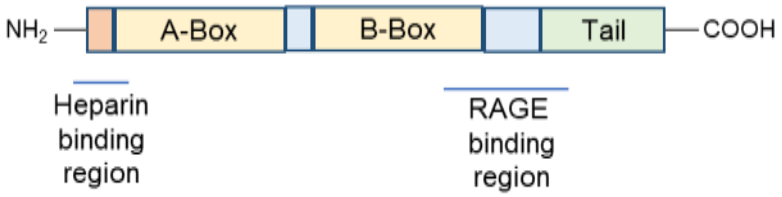

Figure 3. Simplified representation of HMGB1 structure. Emphasis is given to heparin and RAGE binding regions.

A series of studies on the effects of HMGB1's interaction with heparin have been published demonstrating that heparin is capable of modifying HMGB1's structure and inhibiting, both *in vitro* and *in vivo*, its interaction with RAGE. Some of these effects are schematically represented in Figure 4. The inhibitory mechanisms were elucidated by (LING et al., 2011). Using a circular dichroism spectroscopy assay, a conformational shift in HMGB1's structure was observed in the presence of heparin which reduced the number of β-sheets and increased the α-helix content. These conformational modifications were mediated by heparin-binding to the specific, heparin-binding motif in the N-terminal region of HMGB1, thereby reducing its affinity to the RAGE receptor by a factor of almost 50 (heparin present at 50 U/mL). A non-linear decrease in TNFα and IL-6 protein expression was observed with a maximum following the addition of 50 U/mL of heparin as a result of this reduced HMGB1-RAGE affinity (LING et al., 2011). *In vivo*, a similar effect was observed using a Wistar rat model where heparin (at a dose of 150 U/kg) reduced both HMGB1 protein synthesis and mRNA expression in the ischemic (~3-fold reduction in protein expression and a ~2-fold reduction in HMGB1 gene expression over a 12h treatment) and reperfusion groups (~4-fold reduction in protein expression and a ~2.5-fold reduction in HMGB1 gene expression over a 12h treatment) (LIU et al., 2014).

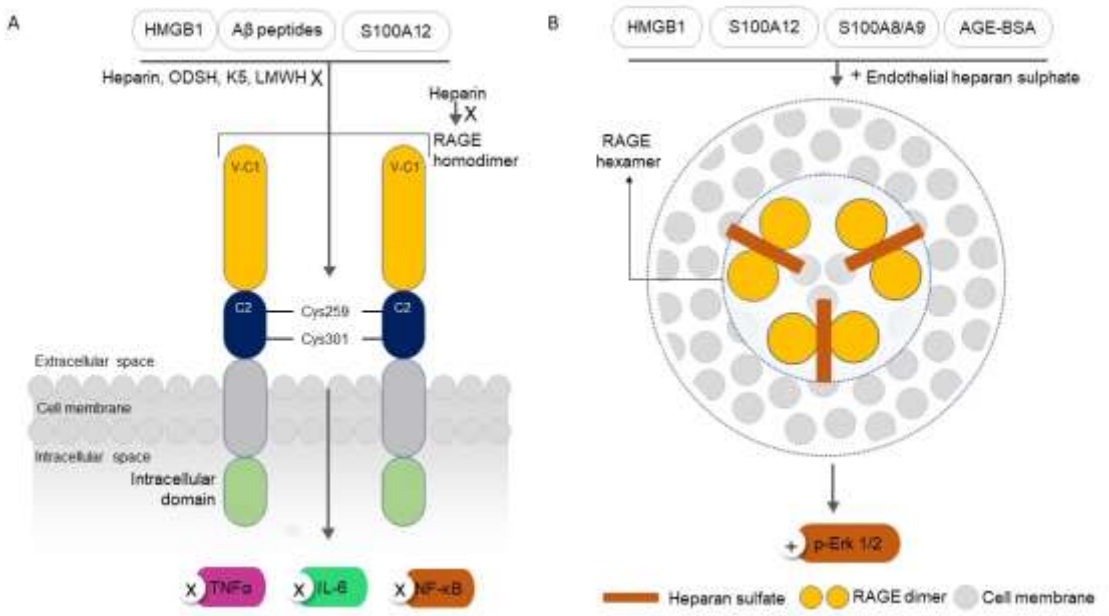

Figure 4. RAGE-ligand binding and RAGE dimerization are blocked by some GAGs (A), while heparan sulfate stabilizes the formation of RAGE hexamers (B). (A) RAGE-ligand interaction and homodimerization blockage by heparin, ODSH, K5 polysaccharides, and LMWH. A cross (x) highlights the inhibitory effects of these GAGs, which act by reducing ligands' affinity for RAGE. Heparin modifies the tertiary protein conformation of HMGB1 and also reduces RAGE's self-recognition that contributes to dimerization (RAGE dimerization is considered to be required for signal transduction). The effect is the suppression of inflammatory responses as indicated by a reduction in some inflammation biomarkers such as TNFα, IL-6, and NF-κB. (B) The composition of a robust RAGE structure composed of dimers in a hexameric confirmation is related to the signal cascade trigger. RAGE hexameric stabilization by endothelial heparan sulfate is essential for strong phosphorylation of Erk1/2. The plus sign (+) indicates an effect on the activation of RAGE-mediated pathways. Heparan sulfate stabilizes the hydrophobic interface established between the positively charged V-C1 domains, allowing oligomerization and better signal amplification.

TNFα, IL-6, and NF-κB are part of the inflammatory response modulated by the RAGE-ligand axis. Cytokine downregulation has been experimentally demonstrated as a result of GAGs' interaction with RAGE-ligands. In the case of heparin, Low Molecular Weight Heparin (LMWH) – a fractioned version of heparin

– has been shown to reduce TNFα and IL-6 production: using placental explants, in both physiological and pre-eclamptic conditions, the administration of LMWH (0.5 U/mL) reduced IL-6 and TNFα expression over 48 h, mediating an anti-inflammatory effect by blocking RAGE-HMGB1 binding (ZENERINO et al., 2017). Similarly, in fibrosarcoma cells (HT1080), LMWH acted by inhibiting the HMGB1-mediated NF-κB activation pathway, reducing inflammation and tumor progression (TAKEUCHI et al., 2013). Besides the fact that HMGB1 has been used as a prototype ligand model, (LIU et al., 2009) also found that LMHW reduced the affinity of RAGE for S100A12 in a dose-dependent fashion (0-100 µg/mL), however, this effect was of lower intensity compared with its impact on the binding of HMGB1 to RAGE.

Taken together, these results demonstrate an important anti-inflammatory effect of heparin, in addition to its established use for nearly a century as a long-lasting anti-coagulant. Such an effect is of potentially great importance in a therapeutic context, and other GAGs also exhibit this anti-inflammatory effect (see Table 1). ODSH, with less anticoagulant activity than heparin, was shown to block HMGB1 binding to RAGE more effectively than heparin (ODSH IC_{50}= 0.23 µg/mL; Heparin IC_{50}= 0.04 µg/mL). Also, ODSH reduced *in vivo* inflammatory progression induced with an HMGB1 injection (50 µg) into Balb/C mice's lungs (RAO et al., 2010). The administration of ODSH (100 µg) reduced not only neutrophil infiltration (HMGB1: 12.0 ± 0.8 x 10^4/mL; HMGB1+ODSG: 10.2 ± 0.6 x 10^4/mL in cell number), but also TNFα levels compared with the control group (HMGB1: 100.4 ± 50.7 pg/mL and HMGB1+ODSG : 24.1 ± 19.0 pg/mL) (Rao et al., 2010). In contrast to ODSH, K5OSH molecules (highly sulfated K5 GAG) were more efficient inhibitors of HMGB1 and Aβ-peptide binding to RAGE than K5

polysaccharide, a chemical analog of heparin (IC_{50}= 0.9 µg/mL for K5OSH, IC_{50}= 6837 µg/mL for K5). These observations have prompted the investigation of sulfated glucuronic acid in K5 GAGs as a starting point for the development of potential RAGE antagonists (ROUHIAINEN et al., 2018; XU; ESKO, 2014).

2.4. GAGS and RAGE oligomerization

Besides the influence of GAGs on RAGE-ligand interactions, some glycosaminoglycans also have an impact on the formation of RAGE oligomers. In general, protein oligomerization of soluble and cell-bound proteins is considered a *bona fide* evolutionary strategy to naturally create novel protein functions based on pre-existing building blocks (GOODSELL; OLSON, 2000). When it comes to RAGE, the downstream signaling cascade is thought to be triggered by a more robust, oligomeric structure, such as receptor dimers and/or hexamers, rather than the monomer (Figure 2a and 2b) (XU et al., 2013; ZONG et al., 2010). RAGE dimerization had been described as essential for triggering cell-signaling by proper recruitment of Protein Diaphanous Homolog 1 (DIAPH-1) (XUE et al., 2016) while blocking dimer formation with sRAGE (the soluble forms of RAGE work as antagonists and reduce its dimerization) results in no signal transduction (ZONG et al., 2010).

When in its full-length variant (flRAGE), RAGE contains an extracellular region composed of a V-type, ligand-binding domain (amino acid residues from 23 to 119) and two C-type domains (C1 and C2, residues from 120 to 325), a spanning transmembrane helix and an intracellular domain that lacks kinase activity (SPARVERO et al., 2009; XUE et al., 2016). Structural evidence, based on experiments with soluble RAGE (sRAGE), suggests that the V-C1 and C2 domains are independent structures that allow receptor homodimerization.

Intermolecular linkages established through the C2 region, for instance, occur through two cysteine residues - Cys259 and Cys301 (Wei et al., 2012) (Figure 2A). The necessity of such crosslinks for RAGE homodimerization was highlighted with the production of C2-RAGE (Cys>Ala) mutants, expressed in HEK293 cell cultures, which resulted in a reduction of C2 interaction and a consequent decrease in NF-κB pathway activation (ZONG et al., 2010). Similarly, this effect was also observed with heparin (30 µg/mL), which dramatically suppressed RAGE homophilic interaction (60%) in a Live-Cell Protein-Fragment Complementation Assay (PCA) (ROUHIAINEN et al., 2018).

The effects of heparan sulfate on the conformation of RAGE have been refined over the last decade by Xu and colleagues, their studies elucidating the role of these compounds in RAGE cell activation, in promotion of the hexamer formation, and the regulation of ligands' binding to the receptor (LIU et al., 2014). A hexameric receptor conformation, composed of three RAGE dimers and stabilized by heparan sulfate, is required for Erk1 phosphorylation as proposed by XU et al. (2013) (Figure 2B). Structural studies have indicated the presence of a hydrophobic region on the V-C1 domain, resulting from basic amino acids, thought to be responsible for interacting with endothelial heparan sulfate through electrostatic forces. Examinations of the conformation of RAGE hexamers in the presence of heparin-dodecasaccharides revealed that the electrostatic interaction between heparan sulfate and RAGE dimers permits the correct orientation of the V-C1 hydrophobic regions required for the stabilization of both dimers and hexamers (XU et al., 2013), and show that dodecasaccharide works to stabilize sRAGE dimers. Using size exclusion chromatography, sRAGE (~32

kDa) was eluted as a 200 kDa oligomer in the presence of dodecasaccharides at a ratio of 2:1 (sRAGE: dodecasaccharide) (XU et al., 2013).

The effects of changing the charge distribution of the V-C1 domain were experimentally investigated using site-directed mutagenesis over seven V-C1 peptides, substituting basic amino acids (lysines or arginines) for alanines. These mutations reduced the affinity of all tested peptides to column-bound heparan sulfate demonstrating that this region is key for RAGE-heparan sulfate binding (XU et al., 2013). Notwithstanding these observations, these same RAGE mutated fragments retained an affinity for HMGB1 and S100b indicating that the heparan and ligand-binding regions are distinct. Erk 1/2, an extracellular signal-regulated kinase, is an important intermediate in the RAGE cell-signaling pathway, directly docking at the AGE cytoplasmic tail (HEIZMANN, 2013). Endothelial heparan sulfate thus seems to be an essential conformational element for RAGE-mediated signaling to occur – indeed, reduced Erk1/2 phosphorylation was observed when heparin lyases were added over primary human endothelial cells, even under the stimulus of several well-known RAGE ligands such as HMGB1, S100B, AGE-BSA, S100A8/A9, and S100A12 (XU et al., 2013).

3. GENERAL GOAL

This work section aimed to characterize the use of HUVECs (Human Umbilical Vein Endothelial Cells) as a cell model for RAGE-mediated inflammation analysis by the assessment of *TNFα*, *IL-6*, *VCAM1*, and *RAGE* expression to evaluate the usefulness of this model as a functional platform of analysis.

3.1. Specific goals

3.1.1. Evaluate the effect of TNFα, a model cytokine, on the activation of inflammation and the expression of *TNFα*, *IL-6*, *VCAM,* and *RAGE* by rt-PCR and immunocytochemistry, respectively;

3.1.2. Investigate the effect of HMGB1, a notable RAGE ligand, on the activation of inflammation and the modulation of RAGE expression.

4. MATERIAL AND METHODS

4.1. HUVECs culture and stimulation

HUVECs were isolated from freshly obtained human umbilical cords donated by the Jeanne de Flandre Maternity (Lille, France). Extractions proceeded within 48 h after birth. Cells were cultivated at 37°C at 5% CO_2 in M199 complete medium including, penstreptomicin (1%), HEPES (15 mM), glutamax (1x), amphotericin B (500 ng/mL) - all supplied by Gibco-, and 10 % heat-inactivated fetal bovine serum (FBS) supplied by Dutscher. Extra supplementation was used with the addition of 20% of EBM2 medium (Lonza) to boost cell growth as indicated by (MAY et al., 2018). HUVECs were cultured up to the third passage (P3) until reaching confluency. Cells (200.000 cells/well) were seeded in 6 well-plates. Confluent cells were exposed to TNFα (10 ng/mL for 3 h, 6 h, 9 h, and 24 h. A similar approach was performed using HMGB1 (1000 ng/mL) as a stimulator.

4.2. Real-time PCR analysis

After stimulation, cells were washed 3 times with cold phosphate buffer. Total RNA extraction was performed with TRIzol™ Reagent (Invitrogen) protocol. Reverse transcription was performed with the High-Capacity cDNA RT Kit

(Applied Biosystems) from 1000 ng of total RNA. After cDNA synthesis, the relative expression of candidate genes was evaluated by real-time PCR using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) on QuantStudio 3 System (Applied Biosystems). Primers (Table 2) were designed according to the following parameters: PCR products with lengths between 80-20 nucleotides; primer dissociation temperature of 58-62 °C with a maximum difference of 2°C; CG content between 40 and 60% and oligonucleotide size between 18 and 23 nucleotides. For the analysis of relative expression, cDNA samples were diluted 10x for the quantification of the expression of target genes relative to the endogenous control gene, actin-β. Reactions (10 µL), conducted in two technical replicates, contained 2.5 µL of diluted cDNA, 5 µL of SYBR, 0.5 µL of primers mix (5 mM each), and 2 µL of deionized water. Relative expression was assessed using the ΔΔCt method (LIVAK; SCHMITTGEN, 2001).

Table 2. List of rt-PCR primers used on gene expression tests.

Gene	**5'-3'**	**Sequence**	**Length** (bp)
VCAM1	F	TCAGATTGGAGACTCAGTCATGT	23
	R	ACTCCTCACCTTCCCGCTC	19
RAGE	F	TGCTGATCCTCCCTGAGATAG	21
	R	CTCCTCGCCTGGTTCGAT	18
TNFa	F	TCTGGGCAGGTCTACTTTGG	20
	R	TGAGCCAGAAGAGGTTGAGG	20
IL6	F	AGACAGCCACTCACCTCTTC	20
	R	AGTGCCTCTTTGCTGCTTTC	20
Act-β	F	AAAGACCTGTACGCCAACAC	20
	R	TGATCTCCTTCTGCATCCTGTC	22

F: Forward; R: Reverse

4.3. Immunofluorescence staining assay

Cells (200.000 cells/well) were seeded over round glass layers in 6 well-plates. Confluent cells were exposed to TNFα (10 ng/mL for 3 h, 6 h, and 9 h).

Briefly, cells were fixed with 400 µL of 4 % paraformaldehyde for 10 min at room temperature and following 3 washes with PBS. Proteins were blocked by the addition of 500 µL of 2 % BSA solubilized in PBS. Blocking took place for 60 min. The blocking solution was removed and 500 µL of appropriate antibodies were added and incubated for 3 h at room temperature. Afterward, fluorescent secondary antibodies (Table 3) were incubated for 45 min in the dark at room temperature. DAPI (DAPI 1 µg/mL) was used for the nucleus counterstain. Before analysis on a confocal microscope, secondary antibody solution was washed, and slides were mounted in antifade Vectashield. Slides were analyzed by confocal microscopy (Zeiss LSM 710) in the Microscopy Center of the University of Lille (BiCel, CHU, Lille).

Table 3. Primary and secondary antibodies for immunofluorescence analysis of RAGE and NF-κB.

Protein	Primary antibody [Ratio]	Secondary antibody [Final concentration]
RAGE	Anti-RAGE ab89911 (Mouse) [1/500]	ThermoFisher Alexa Fluor® 488 A32723 (Anti-Mouse) [5 µg/mL]
NF-κB	Anti-NF-kB 4764S (Rabbit) [1/500]	ThermoFisher Alexa Fluor® 555 A21428 (Anti-Rabbit) [5 µg/mL]

5. RESULTS AND DISCUSSION

This study was dedicated to characterizing HUVECs as a model for RAGE-dependent inflammation analysis. HUVECs are explored in the literature for the functional characterization of different molecules, including RAGE ligands such as S100 A8/A9, HMGB1, and LPS (DAYANG et al., 2019; EHLERMANN et al., 2006; LIANG et al., 2015). However, from our experience, published results inferred to the RAGE expression pattern seem to be not reproducible. Properly

addressing RAGE expression in the inflammation context is needed to define the extension of the AGE/RAGE activation, for instance. Accordingly, following we express the results and literature discussion on different biomarkers, and techniques (rt-PCR, immunofluorescence). An effort had been done to explore distinct biological samples (umbilical cordon from different donors), so biological variation could be considered in our results rather than technical repetitions.

5.1. TNFα effects over HUVEC cell culture

To understand the behavior of HUVECs as an inflammation model, cellular stimulation started with the use of TNFα as a prototype inflammation inducer. Three independent assays were performed within the same day, but with different cordon donors, to better represent the biological variations associated with this analysis. Figure 5 shows the expression of the four biomarkers in response to TNFα 10 ng/mL after stimulation for 0 h, 3 h, 6 h, and 9 h. *TNFα*, *VCAM1,* and *IL6* expression were induced in a time-dependent fashion by TNFα compared to the negative control in each corresponding time point. Greater fold change was observed for *TNFα* (~2000x), compared to *IL6* (~3x) (Figure 5 a/c). *VCAM1*, on the other hand, had a significant decrease between 6 h and 9 h under TNFα stimulation (Figure 5b). *RAGE* expression showed a tendency towards an increased expression, but with no significant difference between both experimental conditions (with and without TNFα) (Figure 5d/e) was observed. These results suggest that the differences in expression of *TNFα*, *VCAM1*, and *IL6* were independent of *RAGE* expression variations.

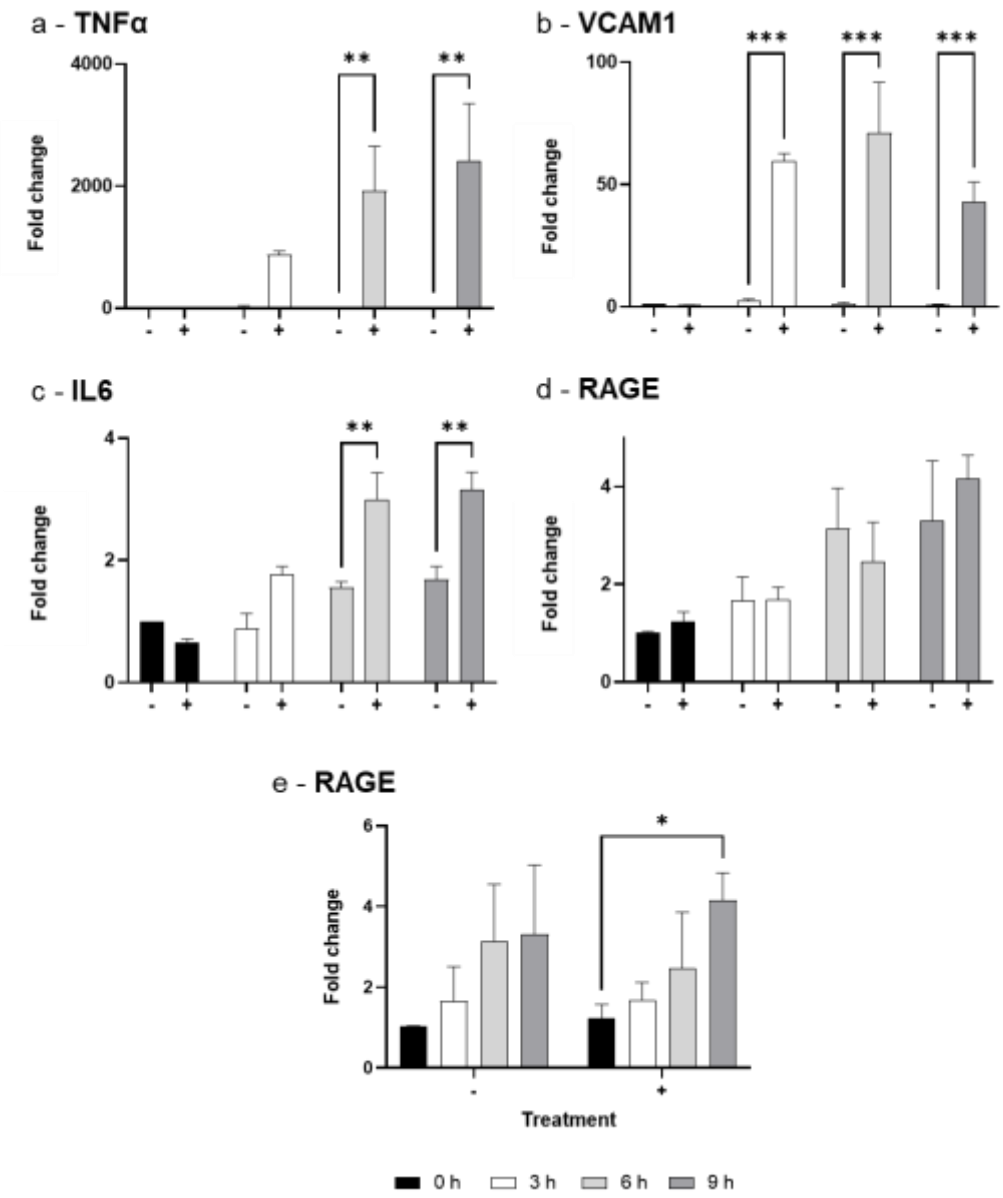

Figure 5. Relative expression of (a) TNFα, (b) VCAM1, (c) IL6, and (d) RAGE along 0 h, 3h, 6 h, and 9 h of stimulation with (+) TNFα (10 ng/mL) and (-) without TNFα. Actin-β was used as endogenous control. Non-treated cells (0 h) were used as reference samples within each gene. The bars indicate the mean percentages followed by their standard error of mean (n=3). The significance of differences in fold changes within each time point was verified by Tukey's post-hoc test (α = 0.05). p<0.001(***); p<0.002 (**); p<0,05 (*).

With the interest in understanding, if the modulation of RAGE expression would be associated with medium starvation rather than TNFα only, a fourth assay was independently performed with cell exposition to TNFα (10 ng/mL) during 0 h, 3 h, 6 h, 9 h, and 24 h. Results are shown in Figure 6. No statistical variation on *RAGE* relative expression was observed neither between the different time-points nor between treatments with or without TNFα. *VCAM1* had a faster and significant ($p<0.001$) expression increase within the first 3 h, which might be related to biological variations (*e.g.* cell donor genetics). On the other hand, *TNFα* and *IL6* respected the same expression profile during the first 9 hours as presented in Figure 5. Both *TNFα* and *VCAM1* expressions decreased between the 9 h and 24 h of treatment. On the other hand, *IL6* expression was increased by almost 3x between 9 h and 24 h of exposition to TNFα. Which reinforces the late participation of IL6 in late inflammation responses.

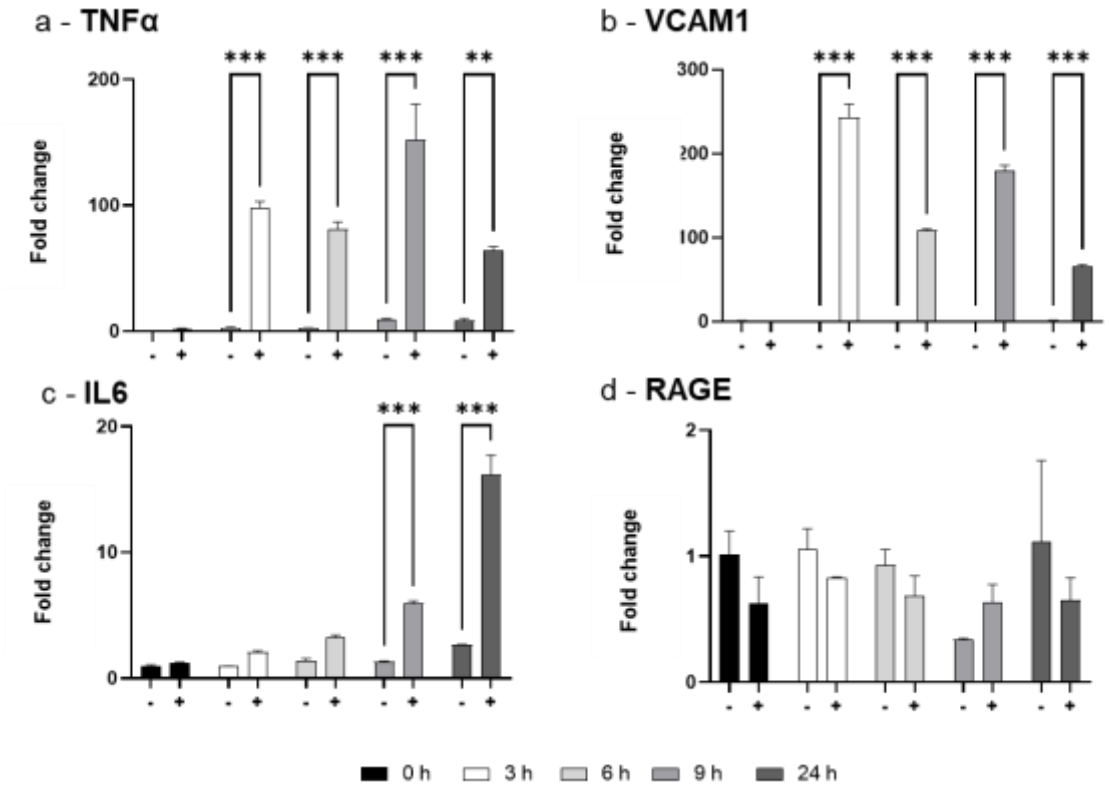

Figure 6. Relative expression of (a) TNFα, (b) VCAM1, (c) IL6, and (d) RAGE along 0 h, 3h, 6 h, 9 h, and 24 h of stimulation with (+) TNFα (10 ng/mL) and (-) without TNFα. Actin-β was used as endogenous control. Non-treated cells (0 h) were used as reference samples within each gene. The bars indicate the mean percentages followed by their standard error of mean (n=1). The significance of differences in fold changes within each time point was verified by Tukey's post-hoc test (α = 0.05). p<0.001(***); p<0.002 (**); p<0,05 (*).

Altogether, TNFα had a noticeable effect on inflammation markers activation in HUVEC culture, but no variation in *RAGE* transcript levels was observed. The expression of both cytokines respected what has already been classically described in the literature. TNFα is a cytokine that acts in the acute phases of inflammation, while IL6 is a late-stage cytokine.(DIDION, 2017; KUNG et al., 2020; SAWA et al., 2007; UCIECHOWSKI; DEMPKE, 2020), which explains the expression of *TNFα* in the first hours of stimulation. These results suggest that inflammation triggers may result from RAGE/TNFα interaction, but it does not require increasing *RAGE* transcription for signal amplification.

The observed expression of *RAGE* had been demonstrated by different authors (MUKHERJEE; MUKHOPADHYAY; HOIDAL, 2005; TANAKA et al., 2000). Human lung microvascular endothelial cells (HMVEC) were responsive to TNFα (100 ng/mL) towards an increase in both *RAGE* gene transcripts and protein levels (TANAKA et al., 2000). However, comparing mRNA stability between controls and treated cells along the first 4 hours of experiments no difference was observed between the two experimental groups (non-treated cells: $t_{1/2}$=2.2; treated cells: $t_{1/2}$=2.1) which suggests the same expression profile observed between treated and non-treated cells in our results. Therefore, we

estimate that although cell stimulation participates in RAGE temporal expression increase, other culture conditions (*e.g.* cell confluence, medium starvation) may contribute to the same profile in non-treated cells and deserve better understanding.

An immunostaining experiment was proceeded to test RAGE expression on the protein level (Figure 7). In contrast to the presented rt-PCR data, RAGE protein did not show a progression in fluorescence within time progression. Immunoreactive RAGE signal was only observed on cell surface immediately after TNFα addition (time 0). On the protein level, such a phenomenon could be associated with the enzymatic cut of RAGE by metalloproteinases such as ADAM10 which participates in soluble RAGE formation (RAUCCI et al., 2008).

NF-κB, on the other hand, was translocated to the nucleus up to 3h with TNFα treatment and after 6 h without the addition of the stimulator. NF-κB is part of several stress-related paths and has been demonstrated to result from the RAGE activation cascade (ROUHIAINEN et al., 2013). These results showed that TNFα induced the translocation of NF-κB in a shorter period compared to non-treated cells. However, noticing the same phenomenon on non-treated cells at 6h of culture suggests that a stress-related process was ongoing. However, these results corroborate the fact that the modulation of inflammatory markers as NF-κB does not depend on the concomitant increase of RAGE.

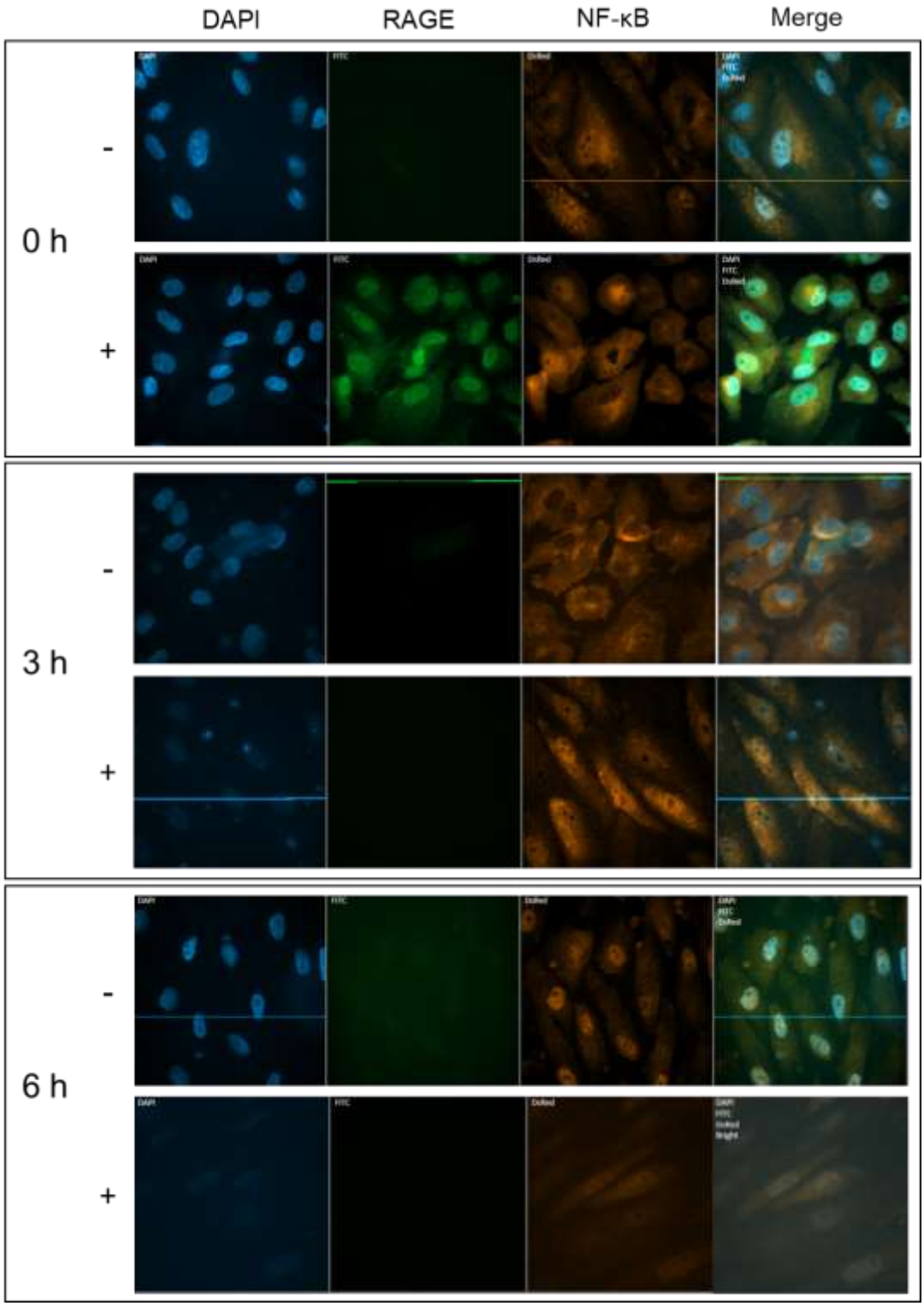

Figure 7. Immunofluorescence assay of HUVECs treated with or without TNFα (10 ng/mL) for 0 h (control), 0 h, 3 h, and 6 h. Cells were treated with anti- NF-κB antibody and RAGE antibody. The pictures presented here are the most

representative of duplicate wells. Obs.: lines crossing pictures are artifacts of the camera lenses.

The translocation of NF-κB to the nucleus was demonstrated to be a RAGE-dependent mechanism as evidenced by a comparative study between immune-blocked RAGE cells and non-treated cells. Together with NF-κB translocation, authors demonstrated an increase in 2-4x increase in RAGE protein expression after 12h to 48 h of stimulation of lipopolysaccharide (LPS) in HUVECs (WANG et al., 2017). Under the effects of TNFα (10 μg/mL), (CHENG et al., 2015) demonstrated a similar behavior for NF-κB, but RAGE was not addressed in this study.

5.2. HMGB1 effects over HUVEC cell culture

The second part of this study aimed to test whether *RAGE* expression could be modulated by a well-characterized RAGE ligand, HMGB1. Experiments proceeded as described before for TNFα along 0 h, 3 h, 6 h, and 9 h with the addition of HMGB1 at 1 μg/mL (Figure 8). Surprisingly, HMGB1 had no pro-inflammatory effect. *TNFα, VCAM1,* and *IL6* genes presented variations towards repression of expression (Figure 8a/b/c). *RAGE*, as previously noticed did not respond to the stimulation (Figure 8d/e). This last fact suggests that *RAGE* expression is independent of ligand (TNFα or HMGB1) and modulation on the transcript levels is not required to modulate other genes involved in endothelial inflammation activation.

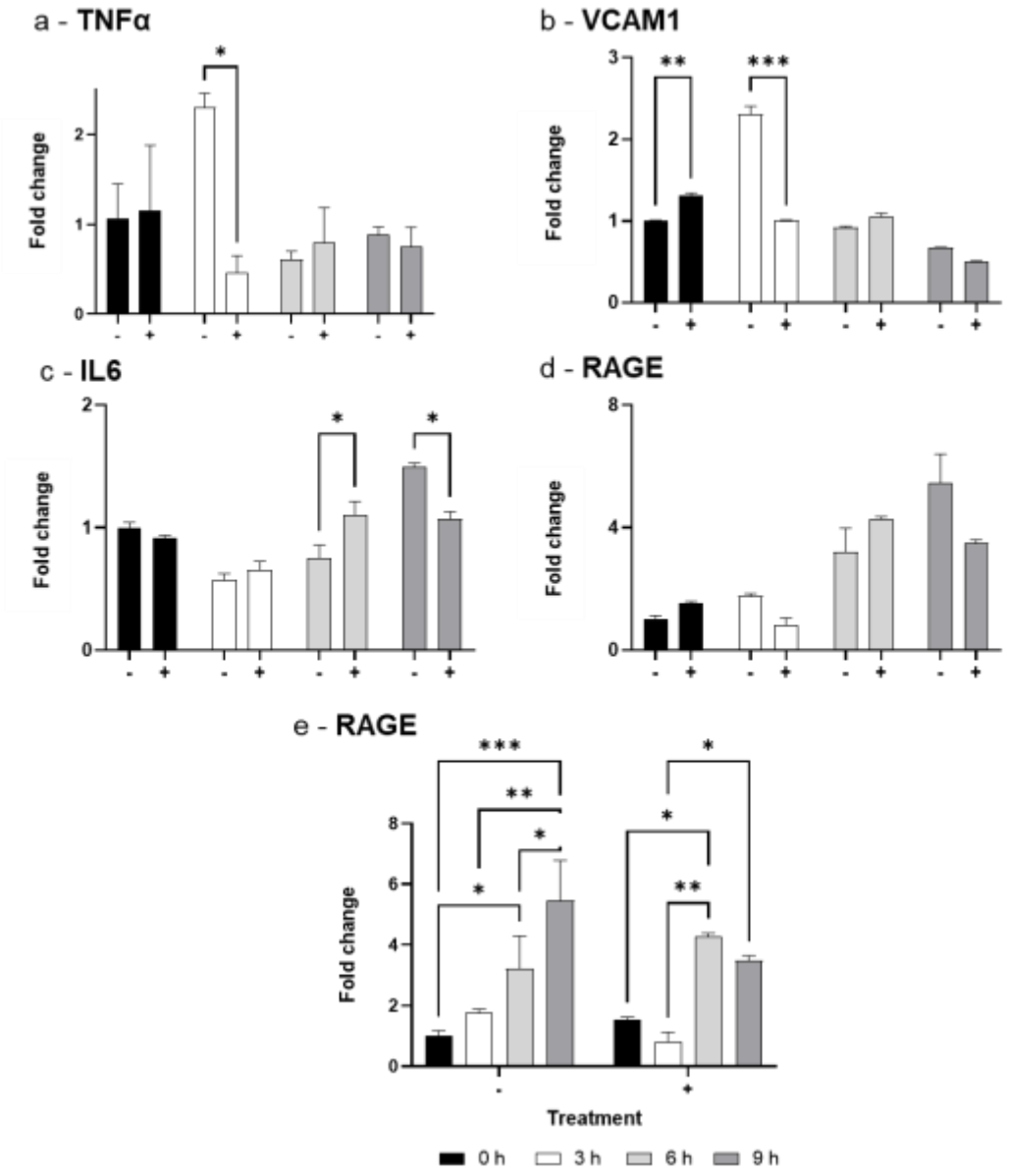

Figure 8. Relative expression of (a) TNFα, (b) VCAM1, (c) IL6, and (d) RAGE along 0 h, 3h, 6 h, and 9 h of stimulation with HMGB1 (1000 ng/mL). Actin-β was used as endogenous control. Non-treated cells (0 h) were used as reference samples within each gene. The bars indicate the mean percentages followed by their standard error of mean (n=1). The significance of differences in fold changes within each time point was verified by Tukey's post-hoc test (α = 0.05). p<0.001(***); p<0.002 (**); p<0,05 (*).

The absence of response of HUVECs to HMGB1 disagrees with publications from different groups in the same cellular model. HMGB1 was demonstrated to take part in *NF-κB* nuclear translocation mediated by RAGE once cells receiving anti-RAGE antibodies did not respond to the treatment. This finding was reported to be followed by the temporal increase of *RAGE* in response to HMGB1 (LUAN et al., 2010).

6. CONCLUSIONS AND PERSPECTIVES

Based on the results presented in this chapter, the research on the characterization of HUVECs as a functional model of inflammation analysis merits further investigation. Here we demonstrated to be able to induce and detect inflammation biomarkers on HUVEC culture, by the use of TNFα, but no effect was observed by using HMGB1. Evidence has been shed over *RAGE* expression within 24 h of stimulation, which did not vary among the experimental conditions here presented and further research should be done to properly attribute the effects exclusively related to RAGE (*e.g.* experiments on RAGE knockout cells, and the administration of RAGE antagonists).

Results observed from the immunofluorescence analysis revealed that RAGE was only detectable immediately after TNFα addition. Here, a complementary analysis should be done to identify if RAGE was not expressed on the protein level or if the activation of metalloproteinase pathways would contribute to the reduction of cell membrane RAGE. In this sense, it would be interesting to analyze the cell culture medium to quantify RAGE in the supernatant.

Studies have been published on the use of different endothelial cell types for the functional characterization of different compounds, but we have encountered several limitations to reproduce results in our laboratory. Such limitations could work as confounding facts if not better understood. Therefore, besides the limitations here expressed, we believe that good progress has been done in the process of validating HUVECs as an inflammation model which may corroborate the proposition of a functional methodology to screen diverse molecules including dietary neo-formed compounds in near future.

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FINAL REMARKS

Hibiscus was demonstrated to be a relevant food source with wide bioactivity, playing a role not only as an antioxidant, but also as a neuroprotective, and anti-glycation agent. Hibiscus varieties were shown to be important sources of phenolics and flavonoids. Such natural compounds contribute to the antioxidant performance of these extracts, but also play a role as neuroprotectors and affect other processes associated with oxidation such as protein glycation. Therefore, it can be inferred that hibiscus is a food with important bioactive properties that can contribute to the maintenance of human well-being by reducing the effects of free radicals, glycation, and the progression of chronic diseases. These discoveries are the first step to further screening on the effectiveness of hibiscus-based strategies to attenuate the progression of pathological processes. Research in this field would benefit from discoveries on extract digestibility, more prolific *in vivo* assays, and pre-clinical and clinical studies.

For years gamma radiation has been used in food processing and here, we demonstrated that this technique was efficient in reducing microbial contamination while maintaining hibiscus bioactivity while respecting international regulations. As presented, such a technique would fit in different steps of the hibiscus supply chain and would corroborate with higher efficiency in product distribution and longer shelf-life.

Finally, we understand that RAGE signaling activation is dependent on several biological mechanisms such as receptor conformation or ligand polymerization, which means that reproducing such conditions in functional models is a challenge. HUVECs have been used successfully as a microvascular

injury model and here we demonstrated to be able to induce endothelial inflammation in this cell model. At the same time, we have shown that RAGE transcription did not follow inflammation progression with TNFα as an inducer. Although RAGE is known to operate inflammation initiation on the interaction with HMGB1, we were not able to validate the HMGB1/RAGE axis, once no inflammatory engagement has been detected. That being so, the effort has been done to better characterize this interaction in laboratory conditions.

PERSPECTIVES

- Improve *in vivo* analyses on *D. melanogaster* to reduce analysis deviations and better estimate the biological effects.
- To evaluate the antioxidant effects and toxicity of extracts obtained on *C. elegans*.
- Contribute to the elaboration of hibiscus-based products which combine antioxidant and neuroprotective actions with greater efficiency.
- To propose technological applications for the use of hibiscus with nutraceutical capacity.
- Better understand the mechanisms regulating RAGE expression on HUVEC and optimize cell culture conditions.
- Prospect HUVECs on the characterization of the anti-inflammatory and cell-toxicity assays of hibiscus extracts.

CONTRIBUTIONS TO A SUSTAINABLE DEVELOPMENT

As an important commercial crop, hibiscus production has social and environmental implications. Sustainability *per se* defines the maintenance of natural resources in order to reduce the economic, environmental, and nutritional vulnerability of future generations in order to allow long-lasting development. In this scenery, food supply for the next 30 years is one of the greatest challenges

for a population-growing world since the global claim for food by 2050 must demand up to 50% production increase compared to current production. Strategies to reduce the risk of food production chains collapse in an environmentally and socially sustainable manner should provide a state of food security for the following generations resulting in access to sufficient, high-quality, and nutritious food for everyone, permitting a healthy and active lifestyle as disposed of by Food and Agriculture Organization (FAO).

Plenty of natural resources can be used in local and global scales to fulfill trade, environmental, and alimentary requirements around the world. The diversification of crops, use of native and endemic species adapted to local climate and soil conditions, and the integral use (stem, leaves, and flowers) of plant species used as food, for instance, must deal with current limitations faced by industrial agriculture. The susceptibility to several pests, climate changes, environmental degradation, use of chemicals, and desertification is mainly a consequence of biodiversity depletion derived from the commercial production of a limited number of genetically identical crops.

Plants are great sources of bioactive compounds, vastly used by the pharmaceutical, cosmetic, and fine chemistry industries as high-value molecules for treatment of diseases as Alzheimer, for use as antioxidants or enzyme inhibitors, and even as antibiotics. There is a clear link between secondary metabolism (and production of bioactive compounds) and genetic diversity. Therefore, biodiversity can supply macro and micronutrients, minerals, and vitamins as has been demonstrated for *Hibiscus sabdariffa*.

Hibiscus flowers are easy-growing and abundant in many countries, and, besides their decorative uses, scientific studies pointed this plant as an interesting source

of antioxidants, neuroprotective, and antiaging compounds. However, increase on positive economic impact of hibiscus depends on the knowledge on composition and activity of secondary metabolites present in its calyx. From our results we understand that great progress has been done at characterizing a great diversity of hibiscus plants with potential and real applications by different industrial sectors. Such effort aimed at popularizing hibiscus use in human feed. Together with studies from different group around the world, hibiscus role as a sustainable and affordable nutritional and therapeutic resource can be highlighted providing a low-cost asset to improve nutritional insecurity and to improve life quality of patients with neurodegenerative diseases.