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Effect of osmotic conditions and germination time on the content and profile of glucosinolates, carotenoids and phenolic compounds in kale, and their antioxidant and anti-inflammatory properties

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Dedication

“Only with faith in God dreams come true, battles are won, and miracles become possible”.

I dedicate this thesis to God.

And to my family, especially to my parents who are my world and have always been my greatest example of perseverance, kindness and love.

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"*Through the eyes of gratitude, everything is a miracle*"

- Maky Davis

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Effect of osmotic conditions and germination time on the content and profile of glucosinolates, carotenoids and phenolic compounds in kale, and their antioxidant and anti-inflammatory properties

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Abstract

In the past years, abiotic stresses such as saline soaking and the exogenous application of phytohormones such as methyl jasmonate (MeJA) have been studied as an effective tool to improve the accumulation of bioactive phytochemicals in fresh products. While the application of selenium (Se), sulfur (S), and methyl jasmonate (MeJA) has been studied in mature crops of Brassicaceae family such as kale, information about their effects on carotenoid and phenolic compound content in sprouts is scarce. Moreover, bioactivity studies of kale extracts treated with Se, S, and MeJA have not been performed. Therefore, the present thesis evaluated, the effect of Se, S, and MeJA on the accumulation of glucosinolates, carotenoids and phenolic compounds in kale sprouts. On the other hand, the individual and combined effects of Se, S, and MeJA stress over the anti-inflammatory and antioxidant activity in an *in vitro* model of Inflammatory bowel disease (IBD).

The present study was divided into three phases. In the first phase, the effects of Se, S, and MeJA on lutein, glucosinolate, and phenolic accumulation were assessed in kale sprouts. Red Russian and Dwarf Green kale were chamber-grown using different treatment concentrations of Se (10, 20, 40 mg/L), S (30, 60, 120 mg/L), and MeJA (25, 50, 100 µM). Sprouts were harvested every 24 h for 7 days to identify and quantify phytochemicals. The highest lutein accumulation occurred 7 days after S 120 mg/L (178%) and Se 40 mg/L (199%) treatments in Red Russian and Dwarf Green kale sprouts, respectively. MeJA treatment decreased the level of most phenolic levels, except for kaempferol and quercetin, where increases were higher than 70% for both varieties when treated with MeJA 25 µM. The most effective treatment for glucosinolate accumulation was S 120 mg/L in the Red Russian kale variety at 7 days of germination, increasing glucoraphanin (262.4%), glucoerucin (510.8%), 4-methoxy-glucobrassicin (430.7%), and glucoiberin (1150%). Results show that kales treated with Se, S, and MeJA could be used as a functional food for fresh consumption or as raw materials for different industrial applications.

In the second phase of this study, it was assessed the individual and combined effects of Se, S, and MeJA on the content of carotenoids, phenolic compounds, and glucosinolates present in undigested extracts and intestinal-digested fractions of 7-day-old Red Russian kale sprouts. Also, the effect of undigested extract and digested kale on cellular antioxidant

activity (CAA) and nitric oxide (NOx) production was evaluated. Kale sprouts treated with Se showed higher levels of total glucosinolates (53.2%) and lutein (149.3%) in the ethanolic extract, whereas phenolic content remained without significant changes. After *in vitro* digestion, a significant liberation of lutein was observed in all samples of kale, mainly the Se-enriched fraction (210.3%). On the other hand, phenolic compounds and glucosinolates suffered significant losses. Abiotic stress did not cause a significant change in phenolic content compared to control sprouts; however, Se treatment significantly preserved the concentration of glucoraphanin (70.8%%), glucobrassicin (58.2%), 4-methoxy-glucobrassicin (41.6%) and total glucosinolate content (63.2%) in kale compared with control sprouts. In correlation, the maximum percentage of CAA and NOx was achieved in the undigested extract and intestinal digested fraction from kale sprouts treated with selenium.

Due to the best results of phases 1 and 2 were obtained with Se (40 mg/L) and S (120 mg/L) treatments, they were selected for the third phase of this research. In this study, phytochemical extracts from 7-days-old Red Russian kale sprouts treated with Se and S were encapsulated with maltodextrin by spray-drying to evaluate its ability to protect phytochemicals from degradation during the digestion process. Particle morphology, encapsulation efficiency, and storage stability were characterized. Mouse macrophages (Raw 264.7) and human intestinal cells (Caco-2) were treated with the digested fraction to assess the cellular antioxidant capacity, the production of nitric oxide (NOx), and the levels of inflammatory biomarkers such as cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β, IL-2, IL-6, and IL-10. Se (89.4%) and S (85.8%) kale sprouts showed the highest encapsulation efficiency and spherical morphology. Digestion affected the content of compounds in both encapsulated and non-encapsulated extracts. However, encapsulation promoted the control of oxidative processes during storage, and the kale extracts germinated with S and Se showed less degradation of lutein (35.6%, 28.2%), phenolic compounds (20.3%, 25.7%) and glucosinolates (15.4%, 18.9%) compared to non-encapsulated samples, respectively. The encapsulate treated with S exerted the highest antioxidant capacity (94.2%) and anti-inflammatory activity by stimulating IL-10 by 88.9% and inhibiting COX-2 and NO production by 84.1% and 92.2%, respectively. Therefore, kale sprouts with enhanced levels of phytochemicals could be used as feedstock to produce processed foods or undergo further processing for extraction and purification of high-value health-promoting biomolecules.

Based on these results, general strategies to yield a high accumulation of specific phytochemicals are presented in this work. With this information, stressed kale with high concentrations of Se or S can be used as a plant model of high-value biomolecule for the treatment of inflammatory diseases.

Efecto de condiciones osmóticas y tiempo de germinación sobre el contenido y perfil de glucosinolatos, carotenoides y compuestos fenólicos en kale, y sus propiedades antioxidantes y antiinflamatorias

Por

Erika Ortega Hernández

Resumen

En los últimos años, los estreses abióticos como el remojo salino y la aplicación exógena de fitohormonas como el metil jasmonato (MeJA) se han estudiado como una herramienta eficaz para mejorar la acumulación de fitoquímicos bioactivos en productos frescos. Si bien se ha estudiado la aplicación de selenio (Se), azufre (S) y metil jasmonato (MeJA) en cultivos maduros de la familia Brassicaceae como el kale, la información sobre sus efectos en el contenido de carotenoides y compuestos fenólicos en el germinado es escasa. Además, no se han realizado estudios de bioactividad los extractos de kale tratados con Se, S y MeJA. Por lo tanto, la presente tesis evaluó el efecto del Se, S y MeJA sobre la acumulación de glucosinolatos, carotenoides y compuestos fenólicos en germinado de kale; y, por otro lado, se evaluaron los efectos separados y combinados del Se, S y MeJA sobre la actividad antiinflamatoria y antioxidante en un modelo *in vitro* de enfermedad inflamatoria intestinal (EII).

El presente estudio se dividió en tres fases. En la primera fase, se evaluaron los efectos del Se, S y MeJA sobre la concentración de luteína, glucosinolatos y compuestos fenólicos en germinado de kale. Se cultivaron las variedades de kale Red Russian y Dwarf Green en cámara usando diferentes concentraciones de Se (10, 20, 40 mg/L), S (30, 60, 120 mg/L) y MeJA (25, 50, 100 µM). Los brotes se recolectaron cada 24 h durante 7 días para identificar y cuantificar los fitoquímicos. La acumulación más alta de luteína ocurrió 7 días después de los tratamientos con S 120 mg/L (178%) y Se 40 mg/L (199 %) en brotes de kale Red Russian y Dwarf Green, respectivamente. El tratamiento con MeJA disminuyó el nivel de la mayoría de los compuestos fenólicos, excepto para el kaempferol y la quercetina, donde se incrementó más del 70 % para ambas variedades cuando se trataron con MeJA 25 µM. El tratamiento más eficaz para la acumulación de glucosinolatos fue S 120 mg/L en la variedad Red Russian a los 7 días de germinación, aumentando la glucorafanina (262.4%), la glucoerucina (510.8%), la 4-metoxiglucobrasicina (430.7%) y la glucoiberina (1150%). Los resultados muestran que el kale tratado con Se, S y MeJA podría utilizarse como un alimento funcional para consumo en fresco o como materia prima para diferentes aplicaciones industriales.

En la segunda fase, se evaluaron los efectos individuales y combinados de Se, S y MeJA sobre el contenido individual y las fracciones digeridas *in vitro* de carotenoides, compuestos fenólicos y glucosinolatos presentes en germinado de kale. Además, se evaluó el efecto de los extractos de kale y la fracción intestinal digerida sobre la actividad antioxidante celular (CAA) y la producción de óxido nítrico (NOx). El kale tratado con Se mostró niveles más altos de glucosinolatos totales (53.2%) y luteína (149.3%) en el extracto etanólico, mientras que el contenido fenólico se mantuvo sin cambios

significativos. Después de la digestión *in vitro*, se observó una liberación significativa de luteína en todas las muestras de kale, principalmente la fracción enriquecida con Se (210.3%). Por otro lado, los compuestos fenólicos y los glucosinolatos sufrieron pérdidas importantes. El estrés abiótico no provocó un cambio significativo en el contenido fenólico en comparación con el control; sin embargo, el tratamiento con Se preservó significativamente la concentración de glucorafanina (70.8 %), glucobrassicina (58.2 %), 4-metoxi-glucobrassicina (41.6 %) y contenido total de glucosinolatos (63.2%) en kale en comparación con el control. En correlación, el porcentaje máximo de actividad antioxidante celular y producción de óxido nítrico se logró en el extracto etanólico no digerido y la fracción digerida intestinal del germinado de kale tratado con selenio.

Debido a que los mejores resultados de las fases 1 y 2 se obtuvieron con los tratamientos Se (40 mg/L) y S (120 mg/L), se seleccionaron para la tercera fase de esta investigación. En este estudio, los extractos fitoquímicos de kale Ruso rojo de 7 días de germinación tratados con Se y S se encapsularon con maltodextrina mediante secado por aspersión para evaluar su capacidad para proteger los fitoquímicos de la degradación durante el proceso de digestión. Se caracterizó la morfología de las partículas, la eficiencia de encapsulación y la estabilidad de almacenamiento. Los macrófagos de ratón (Raw 264.7) y células intestinales humanas (Caco-2) se trataron con la fracción intestinal para evaluar la capacidad antioxidante y la producción de óxido nítrico (NO), de ciclooxigenasa (COX)-2, factor de necrosis tumoral (TNF)-α y las interleucinas (IL)-1β, IL-2, IL-6 e IL-10. Los extractos germinados con Se (89.4%) y S (85.8%) mostraron alta eficiencia de encapsulación y morfología esférica. La digestión afectó el contenido de compuestos tanto en extractos encapsulados como no encapsulados. Sin embargo, el encapsulado promovió el control de los procesos oxidativos durante el almacenamiento, los extractos germinados con S y S mostraron una menor degradación de luteína (35.6 %, 28.2 %), compuestos fenólicos (20.3 %, 25.7 %) y glucosinolatos (15.4 %, 18.9 %) en comparación con las muestras no encapsuladas, respectivamente. El encapsulado tratado con S ejerció la mayor capacidad antioxidante (94.2%) y actividad antiinflamatoria al estimular la IL-10 en un 88.9% e inhibir en un 84.1%, y 92.2 %, la producción de COX-2 y NO, respectivamente. El germinado de kale con niveles mejorados de fitoquímicos podría usarse como materia prima para producir alimentos procesados o someterse a un procesamiento posterior de extracción y purificación de biomoléculas de alto valor promotoras de la salud.

Con base en estos resultados, se presentan estrategias generales para producir una alta acumulación de fitoquímicos específicos. Con esta información, el kale estresado mediante altas concentraciones de Se o S se puede utilizar como un modelo vegetal de biomoléculas de alto valor para el tratamiento de enfermedades inflamatorias.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Motivation

In Mexico, as in the rest of the world, enormous social changes have occurred. Medicine has not been immune to these changes. There are factors that have had a profound influence on the general health conditions, such as education, urbanization, labor organization, economic growth, sanitation and nutrition. It can be said that all of them together have contributed to reducing the frequency of most communicable diseases, while other non-communicable, chronic diseases of multifactorial etiopathogenesis have become more common (WHO, 2018; Demmer & Barondess, 2018).

Chronic and degenerative diseases, within a demographic structure where almost 20 percent of the population is over 40 years of age (INEGI, 2020), are very common problems influenced by several immunogenetic factors and environmental factors of all kinds such as diet, habits, and exposure to physical, chemical and biological agents (WHO, 2018).

The most common chronic diseases are cardiovascular diseases, cancer, chronic pulmonary disease, and diabetes, accounting for almost 63% of deaths that occur in the world. It has been estimated that chronic diseases cause 38 million deaths a year, almost 70% of deaths worldwide, of which 80% corresponds to cardiovascular diseases and diabetes (Roth et al., 2015; WHO, 2018). The deterioration of the population's health is reflected in lower productivity, a decrease in the quality of life and an economic impact for the global health system (Aldridge & Kelley, 2015).

Metabolic syndrome, a common disorder that results from the rising prevalence of heart disease, diabetes, and obesity, has been related with a parallel outbreak of autoimmune diseases, including inflammatory bowel disease (IBD) (Rivero, 2016). IBD, defined as either Crohn’s disease (CD) or ulcerative colitis (UC), is a multifactorial condition which involves both genetic and environmental factors. The clinical development of IBD starts with a defective mucosa which lets the intestinal bacteria across the mucosal barrier and induce an immune response; consequently, a chronic inflammation occurs (Michalak, Mosińska, & Fichna, 2016). Approximately 15-40% of patients with IBD shows clinical characteristics of metabolic syndrome (Meligrana et al., 2019). Recent findings indicate similarities in pathophysiological features between metabolic syndrome and IBD, including adipose tissue dysregulation, inadequate immune response, inflammation, and increased cardiovascular disease risk (Estay et al., 2017).

IBD is characterized by its evolution with relapses, complications and limited medical therapeutic efficacy. Currently, its treatment is only symptomatic through the use of inflammation modulators, such as mesalazine or 5-aminose-licilic acid, steroids and

immunosuppressants, which are expensive and in many cases of unfavorable clinical evolution (Danese et al., 2014). For these reasons, the study of the mechanisms that participate in its pathogenesis is relevant and can guide the development of better treatment tools. In this regard, immunology is making its way as a powerful knowledge tool on the etiology of IBD (Hernández, Castañeda, & Sánchez, 2021).

It has been observed that in IBD there is a dense infiltrate of inflammatory cells, especially macrophages, neutrophils, and lymphocytes that secrete proinflammatory cytokines and reactive oxygen species (ROS), which are the effectors of the inflammatory cascade. The high production of ROS and the decrease in antioxidant enzymes in the intestinal mucosa of patients with IBD cause the appearance of oxidative stress and the consequent lipid peroxidation and inflammation (Russo, Luciani, De Cicco, Troncone, & Ciacci, 2012). Reactive nitrogen metabolites (RNM), such as nitric oxide (NOx), also play an important role in tissue damage. Nitric oxide synthase (iNOS) is present in the intestinal epithelium and in epithelial cells. iNOS is activated in the presence of proinflammatory factors such as certain cytokines that are commonly found in active IBD (Krzystek-Korpacka, Kempiński, Bromke, & Neubauer, 2020). High iNOS activity has been found in the intestinal mucosa of patients with IBD, as well as high levels of nitric oxide metabolites (nitrates and nitrites) in the colonic lumen (Moret et al., 2014).

Just as IBD has strong immune and anti-inflammatory components, there are various chronic-degenerative diseases that share these characteristics such as the metabolic syndrome. In addition to the current therapies used in the treatment of inflammatory diseases, the search for functional ingredients that can prevent them or mitigate their progression represent an effective alternative. The clinical benefits of antioxidant combinations may target different ROS/RNM pathways and reduces the implication of oxidative stress in the pathophysiology of the disease (Moret et al., 2014).

The development of a functional ingredient represents a highly attractive market for both consumers and investors. At a global level, it is estimated that the market for functional ingredients exceeds USD $25,000 million and has an annual growth significantly higher than that of the conventional food industry (FIA, 2017). Therefore, the motivation is to create a low-cost ingredient related to health and nutrition that leads to the development of more effective antioxidant and anti-inflammatory therapies, and that can serve as the basis for the formulation of ready-to-eat or daily consumption foods such as tortillas, bread, sausages, pasta, etc.

1.2. Problem Statement and Context

Since preventing the risk of developing chronic diseases is a better approach than their treatment, the scientific community has directed its efforts on the generation of functional foods. Functional foods refer to natural or processed food products that in addition to satisfying basic nutritional needs, provide health benefits or reduce the risk of disease (Vidal et al., 2018). Their beneficial characteristics are based on the deactivation of free radicals, inhibition of receptors, activation of enzymes, and variation of gene expression

(Correia, Borges, Medeiros, & Genovese, 2012). They are an emerging technology in food science and are projected to become one of the main market trends in the next 15 years (Kuesten & Hu, 2020).

A vegetable that is often on the list of the healthiest foods is kale (*Brassica oleracea var.* acephala*).* Originating from eastern Turkey, kale is one of the oldest leafy green vegetables. During the first millennium, it arrived in Europe, where it settled in various cultures, and only until the early 1980s did it become popular in America (Šamec, Urlić, & Salopek-Sondi, 2019). Due to its good tolerance to unfavorable weather conditions and inexpensive production, kale has become an important crop for the agriculture-based economy (USDA, 2017).

Kale has been widely used worldwide in traditional medicine for the prevention and treatment of different health disorders, including gastric ulcers, high levels of cholesterol, hyperglycemic, rheumatism, and hepatic diseases among others (Kuerban et al., 2017; Lemos, Santin, Júnior, Niero, & Andrade, 2011). Its health-related benefits have been attributed to a great combination of bioactive phytochemicals, including glucosinolates, carotenoids, and phenolic compounds (Abellán, Domínguez-Perles, Moreno, & García-Viguera, 2019; Šamec & Salopek-Sondi, 2019). The main glucosinolates found in kale are glucoerucin, glucoraphanin, progoitrin, gluconapin, glucoiberin, glucobrassicanapin, gluconasturtiin, glucobrassicin, 1-hydroxy-3-indoylmethyl, neoglucobrassicin, 4-hydroxy-glucobrassicin, and 4-methoxy-glucobrassicin. Phenolic compounds are secondary metabolites produced via the shikimate and phenylpropanoid pathways (Saltveit, 2017). It has been found that their antioxidant activity is mainly due to the hydroxycinnamic acids (up to 92.8%) and flavonoid glycosides present in kale, including quercetin, kaempferol, derivatives of caffeic, ferulic and sinapic acid (Akdaş & Bakkalbaşı, 2017; Šamec et al., 2019; Wang, Hu, Liu, Zhang, & He, 2017). Carotenoids are fat-soluble pigments showing antioxidant and immunomodulation activities, which may prevent degenerative diseases, such as cardiovascular diseases. Kale contains comparatively a higher amount of β-carotene than broccoli, cabbage, kale, cauliflower, and Brussels sprouts (USDA, 2017).

However, the intrinsic concentration of the health-related compounds in the available genotypes of plants is not always sufficient for the consumer's daily intake (Mattoo et al., 2010). Manipulation of these secondary metabolites can be used to control levels of desirable compounds and improve plant quality. It has been shown that it is possible to increase the concentration of phytochemicals in other vegetables, such as carrots and broccoli, through the application of abiotic stresses (Cisneros-Zevallos, 2003; Cisneros-Zevallos & Jacobo-Velázquez, 2020; Jacobo-Velázquez, González-Agüero, & Cisneros-Zevallos, 2015).

Since plants are sessile organisms, they need constant monitoring of environmental changes to modify and adjust development and metabolism in accordance with such changes. The response to these environmental stimuli requires an integrated mechanism where internal and external signals are detected and cause an appropriate reaction in the plant (Pandey, Iqbal, Pandey, & Sawant, 2017).

The perception of stress involves the amplification and transduction of a stimulus by the transduction machinery that is composed of protein kinases, phosphatases and binding proteins. Once amplified in the cytoplasm, the stress signal is transduced to the nucleus, which would stimulate the expression of genes implicated on the primary and secondary metabolism of the plant; as well as a late reaction related to an increase in the enzyme activity implied in the biosynthesis of secondary metabolites, and with the storage of secondary metabolites (Jacobo-Velázquez et al., 2015).

Through this document, a deep analysis of stress conditions is performed and applied in four areas: i) accumulation of health-related compounds in kale, ii) development of a delivery system that protects the assets from the digestion process, iii) evaluation of antioxidant and anti-inflammatory activities

1.3. Research Question

This thesis aims to elucidate the behavior of biosynthesis and accumulation of carotenoids, glucosinolates, and phenolic compounds in kale (*Brassica oleracea* L. var. Dwarf Green and *Brassica oleracea* L. var. Red Russian) due to the exposure to three different concentrations of Se, S, and MeJA during germination, as well as to evaluate its bioactive potential as a functional ingredient with antioxidant and anti-inflammatory activity through the modulation of biomarkers of inflammation and oxidative stress on cellular models of IBD.

Therefore, the following research questions were established: in

I. How do different concentrations of Se, S, and MeJA affect the accumulation of phytochemicals in kale during the first 7 days of germination?
II. Which abiotic stress treatment produces the highest phytochemical content and antioxidant activity?
III. How does the treatment of kale with Se, S, and MeJA affect its bioaccessibility?
IV. Does kale encapsulation improve its bioaccessibility and bioactivity for an *in vitro* IBD model?

1.4. Solution overview

To answer the stated research questions, the next solutions are proposed:

1. Identify the conditions of abiotic stress that induce the highest production of phytochemicals (carotenoid, glucosinolates, and phenolic compounds) in kale (*Brassica oleracea* L. var. Dwarf Green and *Brassica oleracea* L. var. Red Russian), by:

- Germination of kale under different concentrations of Se, S, and MeJA during the first 7 days.

• Analysis of the carbon flux in the biosynthesis of lutein, phenolic compounds and glucosinolates, through their identification and quantification by HPLC-DAD in extracts obtained from stressed tissues.

2. Select the kale sprout extract with highest content of phytochemicals and the highest antioxidant and anti-inflammatory activity.

• Evaluation of the free radical scavenging activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.
• Evaluation of the Fe^{2+} chelating activity of kale extract by spectrophotometry.
• Evaluation of nitric oxide production through the nitric oxide radical assay.

3. Evaluate the *in vitro* functionality of undigested extracts and intestinal-digested fractions of kale, by:

• Quantification and identification of carotenoid, phenolic compounds and glucosinolates of intestinal-digested fractions from the simulated gastrointestinal digestion process by HPLC-DAD.
• Determination of cellular antioxidant activity (CAA) in human colon (Caco-2) cells.
• Determination of anti-inflammatory activity through the nitric oxide production (NOx) in murine macrophage (Raw 264.7) cells.

4. Development of a functional ingredient through the encapsulation of kale sprouts and evaluation of its *in vitro* functionality, by:

• Assess the bioaccessibility of carotenoid, phenolic compounds, glucosinolates, and sulforaphane in encapsulated of kale after a simulated gastrointestinal digestion process.
• Evaluation of cell permeability of carotenoid, phenolic compounds, glucosinolates, and sulforaphane in encapsulated of kale after a simulated gastrointestinal digestion process using a monolayer cell model with human colon Caco-2 cells.
• Determination of cellular antioxidant activity (CAA) in Caco-2 cells.
• Determination of anti-inflammatory activity through the nitric oxide production (NOx) in Caco-2 and Raw 264.7 cells.
• Determination of anti-inflammatory activity through the biomarkers: cyclooxygenase 2 (COX-2), NF-α, interleukin-1 (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6) and interleukin-10 (IL-10).

1.5. Main contributions

This doctoral thesis contributes new knowledge and advances in two main areas: food technology and health. An alternative and economic process is presented to produce secondary metabolites of biotechnological interest through abiotic stress. In addition, the use of a natural crop of the Brassicaceae family of easy production and growth in

unfavorable climatic conditions is promoted. Furthermore, an immunomodulatory and anti-inflammatory potential ingredient is developed.

1.6. Dissertation organization

The present thesis is composed of 6 chapters. **Chapter 1** presents a general introduction of the topic addressed in this thesis. **Chapter 2** consists of a review of current literature regarding the biosynthesis and nutraceutical properties of the glucosinolates, carotenoids and phenolic compounds found in kale. This chapter also discusses the application of abiotic stresses as a tool to enhance phytochemical levels and the physiology of stress response in plants. Emphasis is made on the reported mechanisms and effects of Se, S and MeJA, on the biosynthesis of phytochemicals.

Chapter 3 presents the results of phase 1 of this study where the aim was to investigate the effect of three doses of Se, S or MeJA on the concentration of individual glucosinolate, carotenoids and phenolic compounds in kale sprouts. Likewise, the antioxidant activity of these phytochemicals on kale.

Chapter 4 presents a study of the effect of Se, S, and MeJA on the content of carotenoids, phenolic compounds and glucosinolates presents in 7-day-old kale sprouts, as well as on their bioaccessibility after a simulated digestion process. Also, the effect of intestinal-digested fractions and undigested extracts of Red Russian kale on cellular antioxidant activity (CAA) and nitric oxide (NOx) production.

Chapter 5 presents a study on the protector effect of encapsulation on the phytochemical content of kale treated with S and S during simulated gastrointestinal digestion, as well as its impact on the cellular antioxidant activity (CAA), nitric oxide (NOx) production and immunomodulatory activity.

Finally, based on the results presented herein, **Chapter 6** summarizes a series of general conclusions and recommendations for this and future studies.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Numerous studies have reported that diets high in vegetables are highly correlated with a reduced risk of developing common chronic diseases (Vidal et al., 2018). A vegetable that is often on the list of the healthiest foods is kale. Originating from eastern Turkey, kale is one of the oldest leafy green vegetables. During the first millennium, it arrived in Europe, where it settled in various cultures. It was not until the early 1980's that kale became popular in America (Šamec et al., 2019). Due to the adequate tolerance to unfavorable weather conditions and inexpensive production cost, kale has become an important crop for the agriculture-based economy ("Agricultural Research Service: Kale Raw," 2017).

Kale belongs to the Brassicaceae family, which also includes cauliflower, broccoli, arugula, and brussel sprouts. It is characterized by a sweet, slightly bitter taste and an appearance similar to a mix of lettuce and Swiss chard. Kale exhibits multiple varieties mainly differentiated by color shades, size, and leaf type. Different shapes of the plant are available, including tree kale, marrow kale, thousand-headed kale and collard. The varieties most commonly grown are the Scotch and Siberian kales (Šamec et al., 2019).

Kale has been widely used worldwide in traditional medicine to prevent and treat different health disorders, including gastric ulcers, high cholesterol levels, hyperglycemic, rheumatism, and hepatic diseases (Kuerban et al., 2017; Lemos et al., 2011). Its health-related benefits have been attributed to a great combination of bioactive phytochemicals, including glucosinolates, carotenoids, and phenolic compounds (Abellán et al., 2019; Šamec & Salopek-Sondi, 2019). Likewise, kale has a higher nutritional value compared to other foods. According to the USDA database, 100 grams of raw kale provide 2.9 g of protein, 4.4 g of carbohydrates, 4.1 g of fiber, and only 1.49 g of lipids. In addition, it offers more iron (1.6 mg/100 g) than meat, 2–3 times more calcium (254 mg/100 g) than milk, 3–4 times more folic acid (241 ug/100 g) than eggs, and two times more vitamin C (93.4 mg/100 g) than oranges ("Agricultural Research Service: Kale Raw," 2017).

The content of primary and secondary metabolites can be modified by diverse factors, including the development stage, harvest season, environmental conditions, postharvest handling, and variety (Saini & Keum, 2018). The manipulation of these metabolites can be used to control levels of desirable compounds and improve plant quality. It has been shown that it is possible to increase the concentration of phytochemicals in other vegetables, such as carrots and broccoli, through the application of abiotic stress (Cisneros-Zevallos, 2003; Jacobo-Velázquez et al., 2015).

2.2. Kale as a Novel Source of Nutraceuticals

In this section, the main nutraceuticals present in kale and their health benefits are presented. Nutraceuticals are chemical compounds present in foods that exert pharmacological activity, resulting in the prevention and treatment of chronic diseases (Dudeja & Gupta, 2017). Glucosinolates, carotenoids, and phenolic compounds from kale are health-related secondary metabolites associated with several beneficial characteristics, showing various pharmacological effects correlated to their antioxidant activity (Biegańska-Marecik, Radziejewska-Kubzdela, & Marecik, 2017; Johnson,

McElhenney, & Egnin, 2019). The main biological activities related to kale are antioxidant, anti-cancerogenic, and protective effects on the cardiovascular and gastrointestinal tract (Biegańska-Marecik et al., 2017; Lemos et al., 2011; Luang-In et al., 2020; Olsen, Grimmer, Aaby, Saha, & Borge, 2012).

The antioxidant properties of kale have been previously evaluated through *in vivo* studies. For instance, Sikora & Bodziarczyk (2013) reported lower lipid oxidation products (LOP) and malondialdehyde (MDA) in the blood serum of rats fed with a modified diet with raw and lyophilized kale. Likewise, Horst et al. (2010) found that kale extract supplementation in Wistar rats (4 ± 0.2 μg/g) showed protection against H2O2-induced DNA damage. Moreover, Chung et al. (2005) described that treatment with kale juice powder for 8 weeks improved serum lipid profiles by increasing the HDL level and decreasing the triglycerides level in Sprague Dawley rats previously fed with a high cholesterol diet.

The neuroprotective potential of kale has been shown on neuroinflammation mechanisms. The evidence indicates that an extract from Tuscan black kale sprouts reduces inflammatory key-markers (p-selectin, GFAP, Iba-1, ERK1/2, and TNF-α) during cerebral ischemia and reperfusion in rats (Giacoppo et al., 2015). On the other hand, kale is also able to block the inflammatory response in the digestive system. Lima de Albuquerque et al. (2010) proved that administration of lyophilized kale (500 mg/kg) modulates the colonic microbiota in rats with colitis induced by Trinitrobenzenesulfonic (TNBS) acid. In addition, kale showed intestinal anti-inflammatory effects by decreasing the production of the TNF-α and IL-1β, and the MPO activity.

Previous clinical studies focused on evaluating the nutraceutical potential of kale have reported that the consumption of its powder for 8 weeks restored blood pressure and glucose levels within the normal range in subjects with potential metabolic syndrome (Ide et al., 2016). Similarly, Kondo et al. (2016) showed that intake of kale-containing food at a dose of 7 g and 14 g decreased postprandial plasma glucose levels in healthy Japanese subjects. In addition, the supplementation with kale juice improved serum lipid profiles and antioxidant systems in male subjects with hyperlipidemia (Kim, Yoon, Kwon, Park, & Lee-Kim, 2008).

2.2.1. Glucosinolates

Glucosinolates are sulfur- and nitrogen-containing thioglucosides derived from glucose and amino acids. They are found in certain families of dicotyledonous plants, including the *Moringaceae*, *Capparidaceae*, *Resedaceae*, and *Brassicaceae* (Possenti et al., 2017).

The basic chemical structure of glucosinolates consists of a β-D-thioglucose group linked to a sulfonated aldoxime moiety and a variable aglycone side derived from one of eight amino acids (**Figure 1**). They are currently classified into subgroups based on their precursor amino acid into aliphatic (alanine, leucine, isoleucine, methionine, or valine), aromatic (phenylalanine or tyrosine), and indole glucosinolates (tryptophan) (Ishida, Hara, Fukino, Kakizaki, & Morimitsu, 2014).

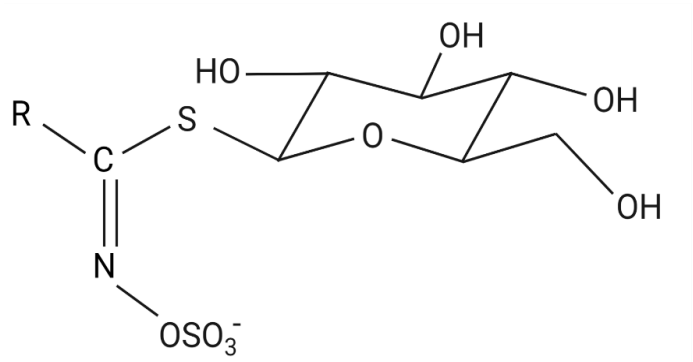

Figure 1. Basic chemical structure of glucosinolates. The basic structure of glucosinolates comprises a thioglucose residue and a sulfate group bound to a central carbon, along with a variable aglycone. Figure created with BioRender.com

Glucosinolates are hydrolyzed by the action of the endogenous enzyme myrosinase (β-thioglucosidase glucohydrolase, EC 3.2.3.1) when the plant tissue is disrupted. Then, the enzyme myrosinase binds with the substrate and releases a molecule of glucose, bisulfate, and the corresponding aglycone. As a result, biologically active compounds are obtained, including isothiocyanates (ITC), nitriles, and thiocyanates, depending on the nature of the aglycone and the physicochemical conditions of the medium (pH, cofactors, and specifier proteins) (Barba et al., 2016; Narbad & Rossiter, 2018). These molecules contribute to the defense of plants against insects and pathogens and have beneficial properties for human health due to their potential protective mechanisms, including xenobiotic detoxification by induction of phase II enzymes, decreased carcinogen activation by inhibition of phase I enzymes and slowed tumor growth, and induction of apoptosis (Chhajed et al., 2020; Keck & Finley, 2004; Šamec & Salopek-Sondi, 2019).

Today, the chemical structure of around 200 different glucosinolates has been elucidated. The qualitative and quantitative glucosinolate profile varies from one species to another, and it is directly dependent on the type of plant tissue. Kale contains glucosinolates in a concentration of 2.25–93.90 μmol/g dry weight (DW basis), but the ratio of indole and aliphatic glucosinolates differs in samples depending on multiple factors, including plant variety, origin tissue, maturity stage, growing conditions, and method of analysis (Cartea, Francisco, Soengas, & Velasco, 2010).

The predominant glucosinolates found in kale are: glucoerucin, glucoraphanin, progoitrin, gluconapin, glucoiberin, glucobrassicanapin (aliphatics); gluconasturtiin (aromatic); glucobrassicin, 1-hydroxy-3-indoylmethyl, neoglucobrassicin, 4-hydroxy-glucobrassicin, and 4-methoxy-glucobrassicin (indolics) (Bhandari, Jo, & Lee, 2015; Ishida et al., 2014; Sun et al., 2019). Hahn et al. (2016) evaluated the glucosinolate concentration and profile in 25 cultivars of 5-month-old kale. The gluconapin content was significantly higher (19.6 mg/100 g FW) in the American varieties (Georgia Southern, Champion, and Vates). In addition, the American kale had significantly more progoitrin than the others. Conversely, American varieties rarely contained glucoraphanin. It was mainly detected in the Italian Black Tuscany (68.69 mg/100 g FW). In most samples, gluconasturtiin was contained in very low amounts, ranging from 0.002 mg/100 g FW (several varieties) to 1.26 mg/100 g FW in Siberian cultivar. Finally, the glucosinolate found in all varieties was glucobrassicin. The highest total amount was detected in the German cultivar Neuefehn (195 mg/100 g FW).

2.2.1.1. Glucosinolates Biosynthesis

The initial step in the biosynthesis of glucosinolates proceeds by *N*-hydroxylation of a precursor amino acid, followed by decarboxylation to form an aldoxime (Fahey, Zalcmann, & Talalay, 2001). It is widely accepted that the formation of glucosinolates involves three independent stages: (i) chain elongation of selected precursor amino acids (only methionine and phenylalanine), (ii) formation of the core glucosinolate structure from the amino acid, and (iii) secondary modifications of the amino acid side chain (Sønderby, Geu-Flores, & Halkier, 2010).

For aliphatic glucosinolates derived from methionine, the amino acid chain elongation begins in the cytosol with the addition of methylene groups to the side chain. Then, deamination is carried out by a branched-chain amino acid aminotransferase (BCAT4) (Schuster, Knill, Reichelt, Gershenzon, & Binder, 2006). The result is the formation of a 2-oxo acid (2-oxo acid 4-methylthio-2-oxobutyrate, MTOB), which is transported into the chloroplast by the bile acid transporter 5 (BAT5) (Kakizaki & Ishida, 2017). This molecule undergoes further modifications by the incorporation of a methylene group in a three-step cycle, consisting of repeated condensations with acetyl-CoA (Sønderby et al., 2010)). In this cycle, an initial condensation of the 2-oxo acid with acetyl-CoA yields a 2-alkylmalic acid; the step is catalyzed by methylthioalkylmalate synthase (MAM) (Schuster et al., 2006). Subsequently, the 2-alkylmalic acid is isomerized by isopropylamate isomerase (IPMI) to form 3-alkylmalic acid (Gao, Yu, Ma, & Li, 2014). A final decarboxylation step is then catalyzed by an isopropylmalate dehydrogenase (IPMDH), generating an elongated 2-oxo acid that may enter the cycle again or may be transaminated by a different aminotransferase (i.e. BCAT3), to be further transferred to the cytosol to enter the glucosinolate core structure biosynthetic pathway (Seo, Jin, Sohn, & Kim, 2017).

Once in the cytosol, the amino acid precursor is converted into an aldoxime by cytochromes P450 of the CYP79 family. Particularly, CYP79F1 and CYP79F2 metabolize methionine derivatives in the aliphatic pathway. While CYP79F1 is able to convert all methionine derivatives, CYP79F2 can convert only long-chained methionine derivatives, specifically pentahomo- and hexahomo-methionine (Yang, Liu, Li, & Yu, 2018). The aldoxime is then oxidized by cytochromes P450 of the CYP83 family (CYP83A1 and CYP83B1 for the aliphatic and indole routes, respectively) to form nitrile oxides or aci-nitro compounds, which then conjugate with cysteine (present in glutathione), as it functions as thiol donor (Kakizaki & Ishida, 2017). This reaction is catalyzed by a glutathione-*S*-transferase (GSTF). This step yields a glutathione conjugate that is further processed by a γ-glutamyl peptidase (GGP1) (Petersen et al., 2019) to form a *S*-alkylthiolhydroxymate. The latter intermediate may be cleaved by a *C*-*S* lyase superroot 1 (SUR1) to yield the thiohydroximate (Chhajed et al., 2020). Afterwards, the thiohydroximate is *S*-glycosylated by soluble UDPG:thiohydroximate glucosyltransferases from the UGT74 family (UGT74B1 in both aliphatic and indole branches, and UGT74C1 only in the aliphatic route) to produce a desulfoglucosinolate. In the aliphatic branch, desulfoglucosinolates are further sulfated by one of two

desulfoglucosinolate sulfotransferases (SOT17 and SOT18) to form different methylthioalkyl glucosinolates. For instance, glucoerucin (4-methylthiobutyl), glucoibervirin (3-methylthiopropil) and/or glucoberteroin (5-methylthiopenthyl) are within the parent glucosinolates initially formed in the aliphatic pathway (Chhajed et al., 2020)

After the glucosinolate core structure biosynthesis is completed, the third, and final stage involves the modification of their side chain. These secondary modifications, including oxygenation, hydroxylation, methoxylation, desaturation, sulfation, benzoylation and glycosylation reactions, give rise to the structural diversity of glucosinolates (Lu, Snowdon, & Li, 2018). Five flavin monooxygenases ($FMO_{GS\text{-}OH1\text{-}5}$) are involved in the *S*-oxygenation of the newly synthesized methylthioalkyl glucosinolates, resulting in the formation of the methylsulfinylalkyl glucosinolates glucoraphanin (4-methylsulfinylbutyl), glucoiberin (3-methylsulfinypropyl), and glucoalyssin (5-methylsulfinylpentyl), when glucoerucin, glucoibervirin and glucoberteroin are used as precursors, respectively (Cang, Sheng, Evivie, Kong, & Li, 2018).

Next, two 2-oxoglutarate-dependent dioxygenases (AOP2 and AOP3) catalyze the oxidation of aliphatic glucosinolates. AOP2 catalyzes the conversion of *S*-oxygenated glucosinolates to alkenyl glucosinolates. Thus, AOP2 converts glucoraphanin, glucoiberin and glucoalyssin to the alkenyl glucosinolates gluconapin (3-butenyl), sinigrin (2-propenyl) and glucobrassicanapin (4-pentenyl), respectively. On the other hand, the *AOP3* gene product catalyzes the conversion to hydroxyalkyl glucosinolates (e.g. 3-hydroxypropyl and 4-hydroxybutyl) (Chhajed et al., 2020; Seo et al., 2017). Following the activity of AOP2, the 2-oxo acid dependent dioxygenase (GS-OH) is then responsible for the hydroxylation of newly synthetized alkenyl glucosinolates. gluconapin, glucobrassicanapin to produce the hydroxyalkenyl glucosinolates progoitrin (2-hydroxy-3-butenyl) and gluconapoleiferin (2-hydroxy-4-pentenyl), respectively. Furthermore, hydroxyalkyl glucosinolates produced by AOP3 may undergo condensation with sinapoyl-CoA to yield sinapoylated glucosinolates or with benzoyl-CoA to form benzoylated glucosinolates, which can also be formed by condensation with progoitrin (Chhajed et al., 2020; Yi et al., 2015).

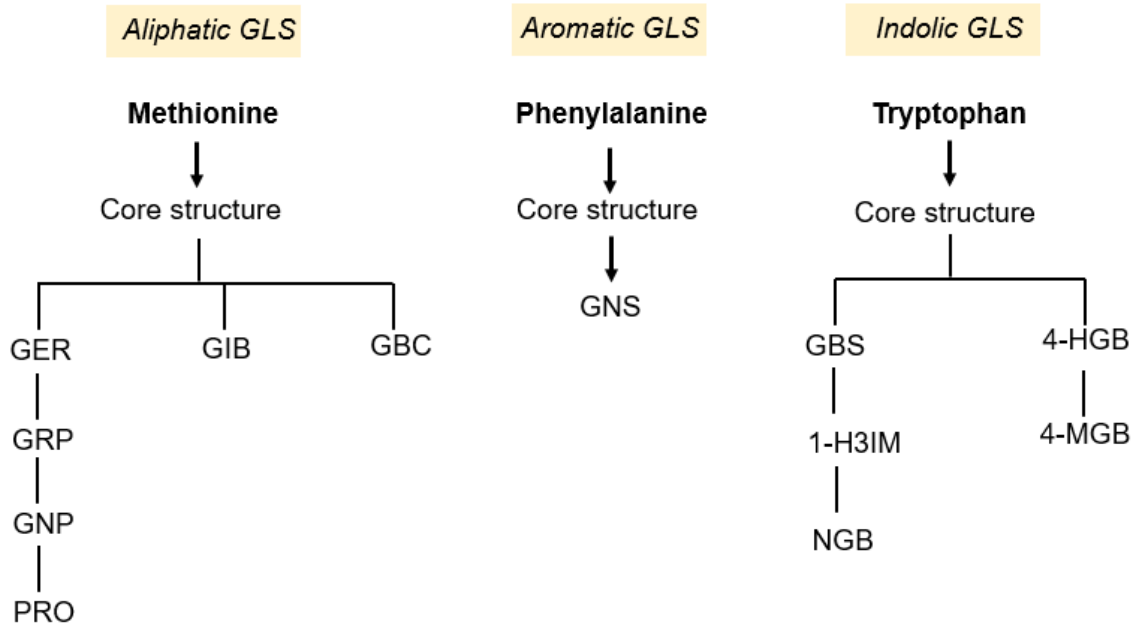

Figure 2. Biosynthesis pathway of glucosinolates in kale (*Brassica oleracea* var. acephala): (1) amino acid chain elongation; (2) core structure of glucosinolates; (3) side chain modification. Glucoiberin (GIB); Progoitrin (PRO); Glucoraphanin (GRP); 1-hydroxy-3-indoylmethyl (1-H3IM); Gluconapin (GNP); Gluconasturtiin (GNS); 4-hydroxy-glucobrassicin (4-HGB); Glucobrassicanapin (GBC); Glucoerucin (GER); Glucobrassicin (GBS); 4-methoxy-glucobrassicin(4-MGB); Neoglucobrassicin (NGB).

On the other hand, biosynthesis of indole glucosinolates starts with conversion of the precursor amino acid tryptophan to the corresponding aldoxime (indole-3-acetaldoxime) by cytochrome P450 monooxygenase CYP79B2 or CYP79B3 (Han Yang et al., 2018). The aldoxime enters the glucosinolate core biosynthesis to form the indol-3-methyl-desulfoglucosinolate and is finally sulfated by the sulfotransferase SOT16 to form the first indolyl glucosinolate, indol-3-ylmethyl (glucobrassicin). Modification of the built indol-3-ylmethyl glucosinolate by members of the cytochrome P450 monooxygenase family CYP81F leads to formation of 1-hydroxy-indol-3-ylmethyl and 4-hydroxy-indol-3-ylmethyl (4-hydroxy-glucobrassicin) glucosinolates that could be respectively methylated to 1-methoxy-indol-3-ylmethyl (neoglucobrassicin) and 4-methoxy-indol-3-ylmethyl (4-methoxy-glucobrassicin) glucosinolates by a specific class of plant family 2-*O*-methyltransferases (IGMT1 and IGMT2) (Chhajed et al., 2020; Pfalz et al., 2011).

2.2.1.2. Glucosinolates as Bioactive Compounds

The beneficial effects of Brassica vegetables have been attributed to the physiological properties of the glucosinolate breakdown products, ITC. The hydrolysis products of glucosinolates present in kale are associated with numerous therapeutic benefits, summarized in **Table 1** and include the potential to reduce the risk of various types of cancers, diabetes, atherosclerosis, and inflammatory and cardiovascular diseases (Lopez-Rodriguez, Gaytán-Martínez, de la Luz Reyes-Vega, & Loarca-Piña, 2020; Mazumder, Dwivedi, & du Plessis, 2016).

Table 1. Predominant glucosinolates in kale and their related isothiocyanates (ITCs).

Glucosinolates		
Side Chain Name	**Trivial Name**	**Isothiocyanate**
Methylthiobutyl	Glucoerucin	Erucin
3-Methylsulfinylpropyl	Glucoiberin	Iberin
3-Methylsulfinylbutyl	Glucoraphanin	Sulforaphane
2-Phenylethyl	Gluconasturtiin	Phenylethyl ITC
3-Indolylmethyl	Glucobrassicin	indol-3-carbinol
2-Hydroxyl-3-butenyl	Progoitrin	2-hydroxyalkenyl

ITCs are potent stimulators of enzymes involved in carcinogen detoxification, such as glutathione-S-transferase, which helps neutralize potential carcinogens by turning them into water-soluble compounds and excreting them through urine (Dufour, Stahl, & Baysse, 2015).

Stimulation of transcription of phase II enzyme genes by isothiocyanates is achieved through the Keap1-Nrf2-ARE pathway, in which isothiocyanates first bind to thiol residues of the Kelch-like ECH-associated protein 1 (Keap1), leading to conformational changes and eliminating its ability to target NF-E2-related factor 2 (Nrf2) for ubiquitination and degradation. Consequently, Nrf2 translocates into the nucleus, where it forms heterodimers with small Maf transcription factors, binds to antioxidant response elements (AREs) in the upstream promoter region of genes encoding for phase II enzymes, and then accelerates their transcription (**Figure 3**) (Kołodziejski et al., 2019; Yin, Wang, Qing, Lin, & Wu, 2016).

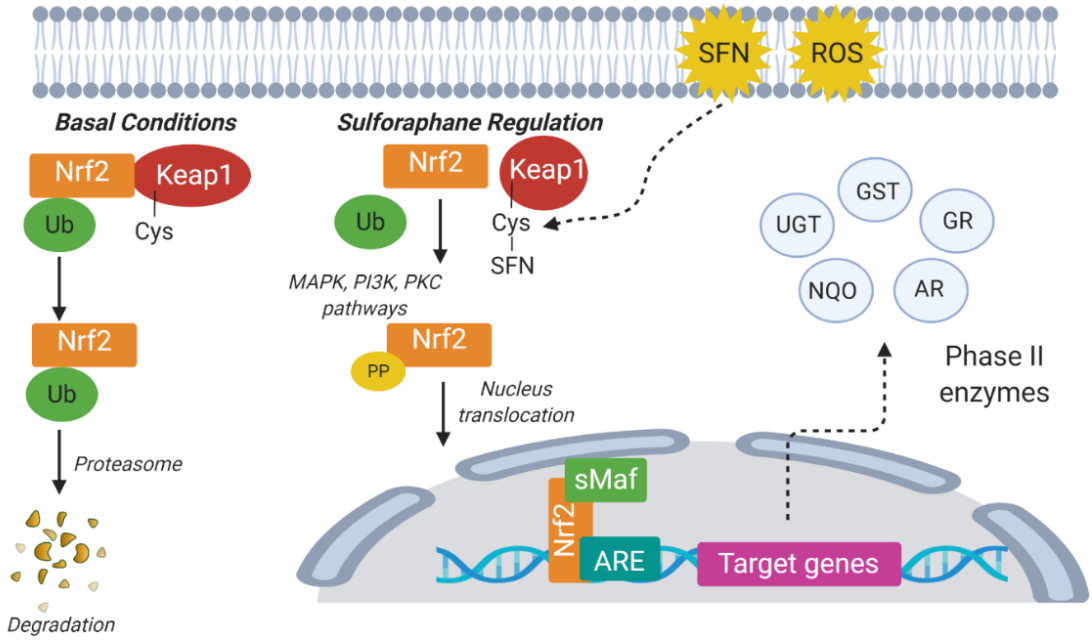

Figure 3. Regulatory mechanism of sulforaphane Keap1-Nrf2-ARE signaling pathway and induction phase Ⅱ metabolic enzyme expression. Under normal circumstances, Nrf2 binds to Keap1 in the cytoplasm and undergoes ubiquitin-mediated degradation. Under SFN treatment or ROS attack, Nrf2 is activated though the MAPK, PIK3, and PKC signaling pathways, and translocated to the nucleus, where it binds to the promoter ARE region of the target genes and activates the expression of phase II metabolic enzymes. ARE: Antioxidant response element; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; PKC: Protein kinase C; ROS: reactive oxygen species;

SFN: Sulforaphane; sMaf: small Maf transcription factors; GST: Glutathione S-transferase; GR: Glutathione reductase; AR: Aldehyde reductase; UGT: Uridine 5′-diphospho (UDP)-glucuronosyltransferase; NQO: NAD[P]H:quinone oxidoreductase (Yin et al., 2016). Figure created with BioRender.com.

Additionally, ITCs can inhibit phase I enzymes, such as cytochrome P450, which are involved in the metabolic activation of most carcinogens in humans (Dufour et al., 2015). ITCs also effectively induce cell cycle arrest and apoptosis of cancer cells, suggesting their use in chemopreventive treatment (Jaafaru et al., 2018; Mantso et al., 2019).

Numerous *in vitro* and *in vivo* studies suggest that sulforaphane (SFN), indol-3-carbinol (I3C), and iberin may help to reduce the risk of estrogen-sensitive cancer as well as other types of cancer, such as prostate, liver, colorectal, melanoma, and pancreas (Esteve, 2020; Georgikou et al., 2020; Lin, Xu, Zhao, & Qiu, 2020; Mitsiogianni et al., 2021; Pocasap, Weerapreeyakul, & Thumanu, 2019; Serini, Guarino, Ottes Vasconcelos, Celleno, & Calviello, 2020). Likewise, Nrf2 is recognized as a significant regulator in oxidative stress and inflammation processes triggered during obesity. All elements of metabolic syndrome are related to the decontrolling of the PI3K/AKT/mTOR, MAPK/EKR/JNK, and Nrf2 signaling pathways (Esteve, 2020).

Previous reports have shown that supplementation with glucoraphanin can moderate weight gain, reduce fat storage, and improve glucose tolerance and insulin sensitivity through the activation of the Nrf2 pathway in mice (Nagata et al., 2017). Similarly, it has been reported that isothiocyanates and indoles, such as sulforaphane and brassinin, suppressed inflammation and inhibited adipocyte differentiation in 3T3-L1 cells and mice through the activation of apoptosis via the ERK pathway (Chae et al., 2015; Chang et al., 2011; Jing Chen et al., 2018; Hee Yang et al., 2017; Yao et al., 2015).

Choi et al. (2014) described that a treatment with sulforaphane reduced the level of leptin and cholesterol and increased the level of adiponectin in plasma through inhibition of C/EBPα and PPARγ, and activation of the AMPKa pathway. Chuang et al. (2019) found that benzyl isothiocyanate and phenethyl isothiocyanate had similar effects diminishing adipogenesis and preventing body weight gain through the inhibition of transcription factors PPARγ and LXRα, and cell cycle arrest at the G0/G1 phase in 3T3-L1 cells. Alike, results for allyl isothiocyanate in literature have showed decrease of body weight gain, diminished fat storing in liver, and reduced inflammation involving the activation of Sirt/AMPKa signaling, the upregulation of PPARα, and the decrease of TNF-α, IL1β, and IL6 levels (Li et al., 2019). Besides, benzyl isothiocyanate, phenethyl isothiocyanate, and allyl isothiocyanate reduced hyperglycemia improving insulin sensibility.

Clinical studies directed to evaluate the effect of ITCs and indoles on metabolic syndrome are limited. Bahadoran et al. (2012) reported a significant decrease in serum insulin concentration with improved insulin resistance in diabetic patients treated with 10 g/d of broccoli sprouts powder for 4 weeks. Likewise, administration of 30 g/d of broccoli sprouts to healthy overweight individuals for 10 weeks, showed a positive effect on inflammatory parameters with a significant decrease in IL-6 levels (López-Chillón et al., 2019). Finally, Kikuchi et al. (2015) have reported that healthy men treated with 10 g of nasturtium leaf showed significantly increased levels of peptide YY (PYY), a satiety gut hormone, after intake during 6 h.

Therefore, this evidence supports that the use of glucosinolates and their derivatives from cruciferous vegetables might be considered for the treatment of metabolic syndrome.

Human studies have found no significant side effects derived from ITC consumption (Shapiro et al., 2006). **Table 2** summarizes the main health benefits of ITC found in kale.

Table 2. Health benefits of isothiocyanates (ITC) found in kale, determined through *in vivo* and *in vitro* studies.

Compound	Metabolic Effect	Main Findings	Reference
Sulforaphane (SFN)	Nrf2 Mitigate insulin resistance	C57BL/6JSlc mice were supplemented with 0.3% glucoraphanin for 14 weeks. Results showed diminished weight gain, reduced hepatic steatosis, and improved insulin sensitivity.	(Nagata et al., 2017)
	C/EBPα Adipocyte differentiation	3T3-L1 pre-adipocytes cell line treated with SFN inhibited the elevation of triglycerides in the adipocytes by activation of PPARγ and (C/EBP) α, and inhibition of (C/EBP) β. SFN arrested the cell cycle at the G0/G1 phase.	(Jing Chen et al., 2018)
	C/EBPα	3T3-L1 pre-adipocytes cell line treated with SFN (10μM) showed significant inhibition of adipocyte differentiation and lipid accumulation by degradation of CCAAT/enhancer-binding protein (C/EBP) β.	(Hee Yang et al., 2017)
Benzyl isothiocyanate Phenethyl isothiocyanate	Adipocyte differentiation	C57BL/6J mice were fed with 1g/Kg of benzyl isothiocyanate or phenethyl isothiocyanate for 18 weeks. Results showed that these isothiocyanates have the potential to prevent body weight gain.	(Chuang et al., 2019)
Allyl isothiocyanate	Sirt1/AMPK NF-κB	AML-12 mouse hepatocyte cell line was treated with allyl isothiocyanate (20 μmol/L) for 24 h. Allyl isothiocyanate reduced lipid accumulation and inflammation *in vitro* through the Sirt1/AMPK and NF-κB signaling pathways.	(Li et al., 2019)
Phenethyl isothiocyanate	Hormone regulation	C57BL/6J mice were fed with 25 mg/Kg phenethyl isothiocyanate for 18 weeks. Results showed that phenethyl isothiocyanate has the potential to stimulate hypothalamic leptin signaling.	(Yagi et al., 2018)
Indol-3-carbinol (I3C)	Liver enzymes	C57BL/6J mice were fed with 40 mg/Kg I3C for 35 days. Treatment modulated glucose tolerance and insulin sensibility. Also, supplementation with I3C increased SOD, CAT, GPx levels.	(Jayakumar, Pugalendi, & Sankaran, 2014)
	Apoptosis	I3C inhibited the phosphorylation and following activation of enzyme Akt kinase in PC-3 cell line. Akt kinase is involved in apoptosis and cell cycle regulation.	(Chinni & Sarkar, 2002)
Indol-3-carbinol (I3C)	Apoptosis	*In vitro*, I3C caused DNA strand breaks in three cervical cancer cell lines. (LD50) = 200 μmol/L I3C.	(Chen, Qi, Auborn, & Carter, 2001)
	Adipogenesis	Mice fed with a I3C-supplemented diet (1 g/kg diet) for 10 weeks showed significantly decreased expression levels of key adipogenic transcription factor PPARγ2, and its target genes, such as leptin and adipocyte protein 2.	(Choi, Kim, Park, Lee, & Park, 2012)
	Anti-carcinogenic	MCF-7 cell line treated with I3C (10–125 μm/L) showed a significant inhibition of the ER-alpha signaling and the	(Meng et al., 2000)

	expression of the estrogen-responsive genes, *pS2*, and *cathepsin-D*. On the other hand, breast cancer susceptibility gene 1 (*BRCA1*) expression was upregulated.	
Anti-carcinogenic	Rats treated intraperitoneally with I3C showed significant inhibition in the development and metastases of prostate cancer and overall survival advantage.	(Garikapaty et al., 2005)

Abbreviations: C/EBP) β: CCAAT enhancer binding protein beta; NF-κB: Nuclear factor kappa B; AML-12: alpha mouse liver 12 cell line; Sirt1/AMPK: histone/protein deacetylase/ AMP-activated protein kinase; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; I3C: Indol-3-carbinol; PC-3: Caucasian prostate adenocarcinoma; *BRCA1*: breast cancer 1; MCF-7: Michigan Cancer Foundation-*7 (human breast cancer cell line)*; ER-alpha: Estrogen receptor alpha.

2.2.2. Phenolic Compounds

Phenolics, the most abundant secondary metabolites in plants, are characterized by a common chemical structure, having an aromatic ring with at least one hydroxyl substituent. Based on their chemical structures, phenolic compounds can be classified as flavonoids, phenolic acids, tannins, stilbenes, and lignans (Ayad & Akkal, 2019).

In nature, most phenolic compounds are found glycosylated, although they can also be found esterified or as polymers. Glycosylation of phenolic compounds increases their solubility and stability in water, protecting them from oxidation (Lu et al., 2015). Once ingested, the absorption rate, the nature of the circulating metabolites, and their elimination will be determined by their chemical structure (Hussain et al., 2019). Phenolic compounds are highly relevant in the dietary supplement and pharmacological industries due to their physiological action and health-protective mechanisms (Botelho, Canas, & Lameiras, 2017).

The main phenolic compounds found in kale are hydroxycinnamic acids and flavonoid glycosides, including quercetin, kaempferol, derivatives of caffeic, ferulic, and sinapic acids (Akdaş & Bakkalbaşı, 2017; Šamec & Salopek-Sondi, 2019; Wang et al., 2017). Few comparative studies have been carried out to characterize the main phenolics in kale from different cultivars and origins. So far, anthocyanins have been identified in the red variety of curly kale, predominating cyanidin glycosides (Jeon et al., 2018). Ferioli et al. (2013) compared the phenolic content of kale populations from Italy, Portugal, and Turkey. Portuguese kales showed the highest amount, followed by Turkish and Italian samples. Flavonols were more abundant than hydroxycinnamic acids, accounting for over 80% of phenolics in all samples.

2.2.2.1. Phenolic compounds biosynthesis

The shikimic acid route is of extreme importance in the production of phenolics in higher plants, being responsible for the synthesis of most phenolic compounds (Saltveit, 2017). It takes place in two different places of the plant; first, the synthesis of aromatic amino acids in the plastids and, secondly, the synthesis of secondary metabolites in the cytosol (Parthasarathy et al., 2018).

Phenolic biosynthesis starts in the cytosol of plant cells where sucrose is cleaved to produce glucose 6-P and fructose 6-P. Part of the hexose-P pool is transported to the plastid and converted to erythrose 4-P by the oxidative pentose phosphate pathway (OPPP). The other fraction of the hexose- P pool is converted to glycerone-P in the

cytosol by glycolysis. Glycerone-P is transported to the plastid and converted to phosphoenolpyruvate (PEP). Erythrose 4-P and PEP are the substrates for the shikimate pathway occurring in the plastid. This shikimate pathway begins with the condensation of phosphoenol pyruvate to erythrose-4-P, producing a sequence of reactions that leads to the synthesis of chorismate and derived from it, aromatic amino acids: phenylalanine, tryptophan, and tyrosine (Santos-Sánchez et al., 2019). Most phenolics derive from phenylalanine and are known as phenylpropanoids.

After the production of phenylalanine, it suffers a deamination to generate the *trans*-cinnamic acid, due to the enzyme phenylalanine ammonia lyase (PAL). Cinnamic acid 4-hydroxylase (C4H) catalyzes the introduction of a hydroxyl group at the *para* position of the phenyl ring of cinnamic acid, producing *p*-coumaric acid, the precursor of the hydroxycinnamic acids (e.g., caffeic, ferulic and sinapic acids) (Saltveit, 2017).

Hydroxylation of *p*-coumaric acid by *p*-coumaroyl quinate 3'-hydroxylase (C3H) generates caffeic acid, which receives a methyl group from the enzyme caffeic acid 3-Omethyltransferase (COMT) to form ferulic acid. Subsequently, a hydroxylation step by ferulate-5-hydroxylase (F5H) followed by a methylation step by COMT, results in the synthesis of sinapic acid. Thereafter, sinapic acid may be further metabolized into sinapate esters, which are intended for lignin and lignin formation (Shigeto, Ueda, Sasaki, Fujita, & Tsutsumi, 2017).

On the other hand, the carboxyl group of *p*-coumaric acid can be activated by formation of a thioester bond with CoA to produce *p*-coumaroyl CoA, a process catalyzed by *p*-coumaroyl: CoA ligase (4CL) (Di et al., 2012). Esterification of p-coumaroyl CoA with caffeic, ferulic, *p*-coumaric or quinic acid, results in the biosynthesis of caffeoylquinic acids (chlorogenic acids) (Fraser & Chapple, 2011).

In addition, *p*-coumaroyl CoA is also condensed with 3 molecules of malonyl CoA, that with the action of stilbene synthase, forms resveratrol (stilbene) or with chalcone synthase forms chalcone naringenin, the precursor of flavonoids (Cheynier, Comte, Davies, Lattanzio, & Martens, 2013). The latter molecule is converted into naringenin (flavanone) by the enzyme chalcone isomerase (CHI). An isoflavone synthase (IFS) then produces the isoflavone genistein from naringenin. Naringenin chalcone may also yield the isoliquiritigenin chalcone by a chalcone reductase (CHR), followed by a CHI to form liquiritigenin, and an IFS to yield the isoflavone daidzein (Artigot, Daydé, & Berger, 2013). Alternatively, a flavanone 3-hydroxylase (F3H) may produce dihydrokaempferol from naringenin (Cheynier et al., 2013).

2.2.2.2. Phenolic Compounds as Bioactive Compounds

Phenolic compounds are efficient antioxidants. Proven biological effects of phenolics include inhibiting oxidative effects on proteins, DNA, and lipids by stabilizing free radicals (Serçe et al., 2016). In this tenor, phenolic compounds exhibit a wide range of beneficial properties for human health, such as anti-allergenic, anti-inflammatory, antimicrobial,

anti-carcinogenic, protective effects against cardiovascular and neurological diseases, and have shown the ability to induce vasodilatory effects (Ayaz et al., 2008; Ha et al., 2003; Hämäläinen et al., 2011; Perez et al., 2014; Shimojo, Ozawa, Toda, Igami, & Shimizu, 2018; Hee Yang et al., 2020; Zhang et al., 2015). **Table 3** summarizes the proven health benefits of the main phenolic compounds found in kale.

The neuroprotective potential of flavonoids has been demonstrated through two main mechanisms: oxidative stress and neuroinflammation. The evidence indicates that flavonoids can maintain the integrity and functionality of neurons, and prevent the increase in the production of reactive oxygen species (ROS) and lipid peroxidation in the hippocampus of rats (Cuevas et al., 2009). Improvement on learning and memory in mice, and inhibition of lipid peroxidation and scavenging radicals in neuronal cells due to antioxidant activity of quercetin was reported by Li et al. (2017). In addition, it has been reported that quercetin supplementation in cortical cells culture inhibited up to 61% of the neurotoxicity produced by the addition of N-methyl-D-aspartate (NMDA) neutralizing free radicals in brain injury (Ha et al., 2003). Flavonoids can also block the inflammatory response related to Alzheimer's disease by inhibiting microglia, the activity of astrocytes, and pro-inflammatory molecules, such as IL-1β and TNF-α (Rubio-Perez & Morillas-Ruiz, 2012). Flavonoids are steroidogenesis modulators in hormone-dependent cancer, as these compounds can bind to estrogen receptors and DNA (Panche, Diwan, & Chandra, 2016). They can also chelate metal ions, such as Fe^{3+}, Cu^{2+}, Zn^{2+}, catalyze the transport of electrons, and neutralize free radicals (Lozano-Sepúlveda, Rincón-Sanchez, & Rivas-Estilla, 2019).

Likewise, it has been shown that phenolics protect vitamin E from photooxidation in the cell membrane and inhibit the oxidation of low-density lipoproteins (LDL), thus preventing the formation of atheroma and reducing the cytotoxicity of LDL (Rufino, Costa, Carvalho, & Fernandes, 2021). Steffen et al. (2008) provided evidence that a large variety of flavonoids and their metabolites can protect vascular endothelial cells against $O^{2\cdot-}$, mainly through the inhibition of NADPH oxidase (Zhang et al., 2015). Also, Perez et al. (2014) demonstrated the vasodilator effects of quercetin in healthy volunteers treated with 200–400 mg for 3 weeks. An increase in brachial arterial diameter was correlated with an increase of plasma and urinary levels of glutathione.

Phenolic compounds also have anti-inflammatory and anti-carcinogenic effects related to their radical scavenging activity and their ability to inhibit lipid peroxidation. It has been reported that kaempferol and quercetin have the anti-inflammatory capacity to inhibit the lipopolysaccharide (LPS)-induced PGE2 production, LPS-induced COX-2 expression, and mPGES-1 expression in activated macrophages (Hämäläinen et al., 2011). Also, kaempferol and quercetin display therapeutic potential as an anticancer drug. Zhang et al. (2015) reported that kaempferol and quercetin (50 and–100 μM) act as topoisomerase inhibitors and interrupt the process of DNA replication in HepG2 cells.

Sinapic acid and its derivatives can inhibit NF-κB, which regulates inflammatory status and plays a key role in the immune response to infection. Via NF-κB inactivation, sinapic acid suppresses the expression of proinflammatory mediators, such as inducible nitric oxide synthase, COX-2, TNF-α, and IL-1β. Sinapic acid and derivatives have shown antiproliferative action on breast cancer cell lines. Due to its metal-chelating ability, sinapic acid has exerted a protective effect against arsenic-induced toxicity in rats (Nićiforović & Abramovič, 2014).

Ferulic acid and its derivatives are other hydroxycinnamic acids found in kale. These compounds possess several health-promoting properties, most of them related to the treatment of metabolic syndrome, including antioxidant, anti-inflammatory, anti-lipidemic, antidiabetic, antihypertensive, and antimicrobial activity. Ferulic acid also exhibit anti-viral properties as, for instance, Ayaz et al. (2008) showed that kale extract rich in ferulic acid inhibits the replication of Gram-positive (*S. aureus*, *E. faecalis*, *B. subtilis*), Gram-negative (*M. catarrhalis*) bacteria, and two yeast-like fungi (*C. tropicali* and *C. albicans*).

Table 3. Health benefits of the main phenolic compounds found in kale, determined through *in vivo* and *in vitro* studies.

Compound	Metabolic Effect	Main Findings	Reference
Quercetin	Antioxidant activity Neuroprotective	Cortical cells treated with quercetin (100 μM) inhibited up to 61% of the neurotoxicity produced by adding NMDA (n-methyl-d-aspartate) and kainate. In addition, quercetin showed a significant decrease in free radicals in brain injury caused by exposure to chemical agents.	(Ha et al., 2003)
	Vasodilator Normotensive, Normocholesteroleic	Healthy volunteers treated with quercetin (200–400 mg) showed an increase in brachial arterial diameter, demonstrating its vasodilator effects. That effect was correlated with an increase in plasma and urinary levels of glutathione.	(Perez et al., 2014)
Kaempferol and quercetin	Anti-inflammatory	The capacity of flavanoids wa proved to effectively inhibit the lipopolysaccharide (LPS)-induced PGE2 production, LPS-induced COX-2 expression, and mPGES-1 expression in activated macrophages.	(Hämäläin et al., 2011)
	Antiproliferative	HepG2 cells exposed to a *Ginkgo biloba* leaf extract, kaempferol, and quercetin (50 and–100 μM) showed DNA damage and topoisomerase II inhibition.	(Zhang et al., 2015)
Kaempferol- glucoside	Synergistic effect with *Lactobacillus paracasei* A221	The oral administration of kaempferol-rich kale extract to Sod1-deficient mice improved various pathologies, including skin thinning, fatty liver, and anemia.	(Shimojo et al., 2018)
Gallic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, caffeic, ferulic and sinapic acid	Antioxidant and anti-bacterial activities	Phenolic extracts of kale showed anti-bacterial effect on Gram-positive (*S. aureus, E. faecalis, B. subtilis*), Gram-negative (*M. catarrhalis*) bacteria, and two yeast-like fungi (*C. tropicali* and *C. albicans*).	(Ayaz et al., 2008)

2.2.3. Carotenoids

Carotenoids represent a group of more than 600 fat-soluble pigments; they are responsible for the yellow, orange, and red coloration in fruits, roots, flowers, fish, invertebrates, birds, algae, bacteria, and yeasts. Their coloration is due to the high number of conjugated double bonds present in their chemical structure (Maoka, 2020). Carotenoids are generally divided into two classes: carotenes, which are unsaturated C40 hydrocarbons, and xanthophylls, which are oxygenated derivatives of carotenes (Maoka, 2020).

In their natural state, carotenoids are bound non-covalently to protein or esterified with saturated fatty acids. Once carotenoids are released from the dietary matrix, they are

circulated in the gastrointestinal tract with the help of dietary lipids and bile salts and are associated with lipoproteins in the form of micelles (Canene-Adams & Erdman, 2009). Xanthophyll esters are hydrolyzed by lipase or esterase and absorbed (Wingerath, Sies, & Stahl, 1998). A part of provitamin A carotenoids are converted into retinal in the mucous of the small intestine by *β*-carotene-15,15′-dioxygenase. Absorbed carotenoids are incorporated into chylomicrons and then transported to the liver and various organs through the blood. All three major lipoproteins: very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), are involved in the transport of carotenoids (Maoka, 2020).

Kale is an excellent source of β-carotene, α-carotene, and lutein (Becerra-Moreno, Alanís-Garza, Mora-Nieves, Mora-Mora, & Jacobo-Velázquez, 2014; Muzhingi, Yeum, Bermudez, Tang, & Siwela, 2017). Previous reports have shown that kale contains a comparatively higher amount of β-carotene than broccoli, cabbage, cauliflower, and brussel sprouts ("Agricultural Research Service: Kale Raw," 2017). The highest levels of β-carotene and lutein content in kale are reached between the first and third week of growth (Lefsrud, Kopsell, Wenzel, & Sheehan, 2007).

Kim et al. (2017) evaluated the carotenoid concentration and profile in eight common kale cultivars at commercial maturity: Starbor, Beira, Scarlet, Premier, Olympic Red, Toscano, Dwarf Siberian, and Red Russian. Toscano kale was the highest in total carotenoid content due to the high levels of neoxanthin (177.4 μg/g), lutein (712.2 μg/g), and *β*-carotene (958.9 μg/g).

2.2.3.1. Carotenoid biosynthesis

The first step in carotenoid synthesis is the conversion of GGPP to phytoene (Srinivasan, Babu, & Gothandam, 2017). IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate) are produced in the plastid compartment through the metabolic pathway methylerythrole phosphate (MEP) (Sun et al., 2018).

The condensation of three IPP molecules with one DMAPP molecule catalyzed by the enzyme geranylgeranyl diphosphate synthase (GGDS) provides GGPP (Srinivasan et al., 2017). The condensation of two GGPP molecules catalyzed by the enzyme phytoene synthase (PSY), forms phytoene. Two structurally and functionally similar enzymes, phytoene desaturase and δ-carotene desaturase, convert phytoene to lycopene via δ-carotene (Ma, Zhao, Xie, Ho, & Chen, 2019).

Cyclization of lycopene is a key step in generating carotenoid diversity because it marks a branch point to two major cyclic carotenoid groups. One branch forms carotenoid with two β-rings, while the other introduces both β- and ε- rings to lycopene to form α-carotene, which is then converted to lutein (Cazzonelli, Roberts, Carmody, & Pogson, 2010).

2.2.3.2. Carotenoids as Bioactive Compounds

In plants, carotenoids evolve to serve in photoprotection, oxidative stress, and developmental regulations. In human health, β-carotene and lutein have shown antioxidant and immunomodulation activities, which may prevent degenerative diseases, such as cardiovascular diseases, UV-induced skin damage, cataracts and macular degeneration, diabetes, and several types of cancer, especially prostate and digestive

tract tumors (Binawade & Jagtap, 2013; Boccardi et al., 2020; Levy, Zaltsberg, Ben-Amotz, Kanter, & Aviram, 2000; Ma et al., 2009; Qiu et al., 2015; Rafi, Kanakasabai, Gokarn, Krueger, & Bright, 2015; Sowmya Shree et al., 2017; Sun et al., 2014).

The distribution of carotenoids in human organs shows specificity. Lutein is found on the surface of the skin and subcutaneous tissue in an esterified form and acts as a UV absorber and quencher of singlet oxygen (Wingerath et al., 1998). In the eye, lutein and retiral derived from β-carotene are present as macular pigments, acting as light screening and playing an essential role in photoprotection (Canene-Adams & Erdman, 2009). Rafi et al. (2015) reported that lutein supplementation (6 mg/d or 12 mg/d) to healthy subjects for 12 weeks improved visual function, particularly in contrast sensitivity. Arnold et al. (2013) evaluated the use of an oil-based kale extract to improve the vision of AMD-patients. The concentrations of the xanthophylls in plasma and the optical density of the macular pigment increased significantly in the kale group after 4 weeks of intervention.

In addition, carotenoids show cancer-preventive effects through multiple mechanisms. It has been reported that carotenoids cause cell cycle arrest and induce apoptosis and differentiation of cells (Shi, Ho, & Shahidi, 2010). It was reported that the culture of PC-3 cells treated with lutein extract decreased in proliferation, modulating the expression of growth genes associated with prostate cancer cells. Besides, results showed a synergic between lutein- and drug-induced effects with cell cycle arrest and apoptosis in prostate cancer (Rafi et al., 2015). Shree et al. (2017) found that β-carotene induced apoptosis in MCF cells by caspase-3 activity and inhibited the expression of the anti-apoptotic proteins, Bcl-2 and PARP.

In addition, carotenoids have excellent quenching activity for singlet oxygen and lipid peroxidation. The mechanism for quenching of singlet oxygen is a physical reaction. Carotenoids take up thermal energy from singlet oxygen and release it by polyene vibration (Shi et al., 2010). Levy et al. (2000) demonstrated that supplementation with 60 mg/d of β-carotene to patients for 3 weeks resulted in a reduction in LDL susceptibility to oxidation, exhibiting a decrease in malondialdehyde (MDA) and lipid peroxides (PD) generation by 25 and 40%, respectively. As well, carotenoid intake may play a role in protecting telomeres by oxidative stress reduction. In a cross-sectional observational study, Boccardi et al. (2020) associated the presence of β-carotene in plasma with telomerase activity in Alzheimer disease (AD) patients, since subjects affected by AD had significantly lower plasmatic levels of β-carotene (448 ± 66 mg/ml), as compared with healthy controls.

In animal models, the effect of lutein as a neuroprotector and modulator of oxidative stress has been reported. Qiu et al. (2015) reported that rats supplemented with lutein (0, 12.5, 25, or 50 mg/kg) for 45 days significantly improved body weight, total cholesterol and triglycerides accumulation, and insulin sensitivity. Similarly, Binawade et al. (2013) demonstrated that rats supplemented with lutein (50 -100 mg/kg) for 14 days improved fat loss and improved hind-limb impairment, motor coordination, and memory alterations. Also, the levels of lipid peroxidation, nitrite concentration, and glutathione in the rat brain were reduced.

Main health benefits of carotenoids found in kale are summarizes in **Table 4**. Human studies have found no significant side effects associated with the intake of 20 mg/d of β-carotene and lutein (Cao et al., 2014; Pagliaro, Santolamazza, Simonelli, & Rubattu, 2015; Pessarakli, 2014; Ranard et al., 2017).

Table 4. Health benefits of carotenoids found in kale, determined through *in vivo* and *in vitro* studies.

Compound	Metabolic Effect	Main Findings	Reference
Lutein	Antiproliferative	Lutein induced a decrease in the proliferation of PC-3 cells (*in vitro*), modulating the expression of growth genes associated with prostate cancer cells.	(Rafi et al., 2015)
	Antioxidant activity	Healthy subjects supplemented with 12 mg/d of lutein for 12 weeks showed improved visual function, particularly in contrast sensitivity.	(Ma et al., 2009)
	Neuroprotective	Rats supplemented with lutein (30, 15, and 7.5 mg/kg) significantly elevated the activities of superoxide dismutase, glutathione peroxidase, and catalase in brain and decreased the neurological deficit scores.	(Sun et al., 2014)
	Neuroprotective	Rats supplemented with lutein (50–100 mg/kg) for 14 days showed a fat loss, reduced neurobehavioral alterations, and reduced oxidative stress.	(Binawade & Jagtap, 2013)
	Antidiabetic and obesity control	Rats supplemented with lutein significantly improved body weight, hepatic levels of lipid accumulation, and insulin sensitivity.	(Qiu et al., 2015)
β-carotene	Apoptotic	β-carotene (1 μM) induced apoptosis in MCF cells by caspase-3 activity and inhibited the expression of the anti-apoptotic proteins, Bcl-2 and PARP.	(Sowmya Shree et al., 2017)
	Antioxidant activity	Patients supplemented with 60 mg/d of β-carotene for 3 weeks showed a reduction in LDL susceptibility to oxidation.	(Levy et al., 2000)
	Antioxidant activity	The content of β-carotene in plasma was significantly and positively correlated with telomerase activity of Alzheimer disease patients, independent of gender.	(Boccardi et al., 2020)

2.3. Application of Controlled Abiotic Stresses as a Tool to Induce an Increase in the Content of Bioactive Compounds in Kale

Traditionally, genetic engineering has been applied to increase the expression level of genes and consequently the production of metabolites of interest in plants. However, this technology is complex and has been proposed as a potential biological hazard, limiting its commercial use for a few crops (Jacobo-Velázquez & Cisneros-Zevallos, 2012). The application of abiotic stresses (i.e., wounding, modified atmospheres, temperature, soil composition, and phytohormones application) in fruits and vegetables has received great attention because they allow the accumulation of bioactive compounds with health-promoting properties (Cisneros-Zevallos, 2003; Cisneros-Zevallos & Jacobo-Velázquez, 2020).

Plant stress has been defined as a state where the plant is growing in non-ideal conditions, which induce an adaptive process and plant responses to these external factors or stressors that, in turn, cause a mixture of eustress and distress in the plant (Hideg, Jansen, & Strid, 2013). The eustress is a beneficial and reversible plant stress induced by a low or moderate exposition to a stressor. This process can modulate plant

metabolism inducing the synthesis and enhancing the accumulation of beneficial secondary metabolites, improving the plant defense system (Carillo et al., 2021). The application of stress or eustress needs to be a controlled process to avoid over-activation of the defense system that could have an adverse effect on plant growth (Rouphael et al., 2019; Vargas-Hernandez et al., 2017).

Since plants are sessile organisms, they need constant monitoring of environmental changes to modify and adjust parameters associated with development and metabolism. The response to these environmental stimuli requires an integrated mechanism, where internal and external signals are detected and cause an appropriate reaction in the plant (Pandey et al., 2017). The perception of stress involves the amplification of a stimulus by the transduction machinery composed of protein kinases, phosphatases, and binding proteins. Once amplified in the cytoplasm, the stress signal is transduced to the nucleus, where it would stimulate the expression of genes implicated in the primary and secondary metabolism of the plant, as well as a late reaction related to an increase in the enzyme activity implied in the biosynthesis and accumulation of secondary metabolites (Jacobo-Velázquez & Cisneros-Zevallos, 2012; Jacobo-Velázquez et al., 2015).

The responses of plants to abiotic stresses can be divided into an immediate and a late response. The immediate response is associated with the production of stress signaling molecules [i.e., reactive oxygen species (ROS), ethylene, jasmonic acid, methyl jasmonate (MeJA), etc.] that activate the expression of genes involved in the primary and secondary metabolism of the plant. On the other hand, the late response is associated with an increase in enzymes involved in the biosynthesis of secondary metabolites and the accumulation of secondary metabolites (Jacobo-Velázquez et al., 2015; Pessarakli, 2014). In the following sections, strategies to enhance the phytochemical composition of kale using pre-harvest and post-harvest controlled abiotic stresses, as described (**Table 5**).

2.3.1. Saline Stress Conditions

2.3.1.1. Sulfur as an Abiotic Stressor

Sulfur (S) is a key element that plays a pivotal role in plant growth and development. The management of sulfur in crop plant nutrition is essential due to its crucial role in fundamental processes, such as homeostasis, electron transport, catalysis, and regulation (Cao et al., 2014).

Similarly, to other macronutrients, S is taken up by the plant through the root as sulfate (SO_4^{2-}). To be incorporated in the metabolic pathways, sulfate is first activated by ATP sulfurylase to yield adenosine-5′-phosphosulfate (APS), which is then reduced to sulfite (SO_3^{2-}) by APS reductase. Finally, sulfite reductase converts the sulfite into sulfide that reacts with O-acetylserine in the presence of O-acetylserine lyase (OAS-TL) to produce Cys. From Cys, GSH is produced by two-step ATP-dependent reactions, where Cys is converted to γ-glutamylcysteine by γ-glutamylcysteine synthetase (also known as glutamate-cysteine ligase, GCL), and the subsequent reaction is catalyzed by glutathione synthetase. Cysteine also serves as a precursor of Met. Homocysteine is produced from cysteine and *O*-phosphohomoserine by the action of cystathionine γ-synthase (CGS) and cysta-thionine β-lyase (CBL). Homocysteine is then converted into Met by methionine synthase (MS). Methionine is the primary precursor of glucosinolate synthesis pathway by initiating the side chain elongation reaction to Met (Hirani et al., 2012).

Table 5. Effect of different abiotic stress conditions on the accumulation of health-promoting compounds in kale.

Abiotic Stress	Treatment	Main Findings on the Biosynthesis of Phytochemicals	References
Saline stress	6-week-old kale seedlings were supplemented with sulphur (S) solution (0.0, 0.5, 1.0-, and 2.0-mM) for 28 days.	Maximum levels of total GLSs and glucobrassicin were found in the leaves supplemented with 2 mM S. Aliphatic glucosinolates, and total glucosinolates increased by 67% and 35%, respectively. Glucobrassicin was the main glucosinolate accumulated.	(Park et al., 2018)
	2-week-old kale sprouts (Winterbor, Redbor, and Toscano) were treated with sulphur (S) solution (4, 8, 16, 32, and 64 mg/L) for 45 days.	There was a significant increase in the glucoiberin, glucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin content in the leaves supplemented with 32 and 64 mg of S/L. Glucobrassicin was the most abundant glucosinolate. There was no significant change in carotenoid accumulation by S treatment.	(Kopsell et al., 2003)
	6-week-old kale seedlings (*Brassica oleracea* var. *sabellica*) were exposed to Na_2SeO_3 (2 mg/L), NaCl (80 mM), or a combination for 14 days.	After seven days of treatment, gluconasturtiin concentration increased by 15%, 19%, and 27% with NaCl, Na_2SeO_3, or both, respectively. ITC concentration increased 30% with the combination of both treatments after 14 days.	(Sun Young Kim et al., 2018a)
MeJA Application	Thirty-day-old chinese kale seeds (*Brassica oleracea* var. *alboglabra*) were sprayed with a MeJA solution (100 µM) 6 days before sampling.	Glucobrassicin (520%), neoglucobrassicin (1420%), and total indole glucosinolates (230%) showed a significant increase after 1 d of treatment. MeJA treatment did not exert a significant effect on the content of vitamin C, carotenoids or phenolics.	(Sun, Yan, Zhang, & Wang, 2012)
	Kale cultivars Red Winter and Dwarf Blue Curled Vates were sprayed with a MeJA solution (250 µM) 4 days before harvest at commercial maturity.	MeJA treatments significantly increased total phenolics in Dwarf Blue Curled (2298 mg GAE/ 100 g) and Red Winter (2070 mg GAE/ 100 g) cultivar by 24% and 41%, respectively. In addition, antioxidant activity also increased by 31% in both kale cultivars.	(Ku & Juvik, 2013)
	Four-month-old kale plants were treated with a MeJA solution (250µM) 4 days before sample collection.	Glucoraphanin (735%), glucobrassicin (1708%), and neoglucobrassicin (1800%) increase significantly. Higher expression of *ST5a* (Bol026200), *CYP81F1* (Bol028913, Bol028914), and *CYP81F4* genes were associated with this accumulation.	(Yi et al., 2016)

Abbreviations: glucosinolates (GLS), isotyociantes (ITC), methyl jasmonate (MeJA).

Glucosinolates are sulfur-rich anionic secondary metabolites, and therefore their concentrations in vegetables are influenced by the addition of S fertilizer (Groenbaek et al., 2016). The influence of S on glucosinolates content in the *Brassicaceae* family has been widely studied. Park et al. (2018) performed a study to evaluate if sulfur positively affects the glucosinolate concentration in kale to enhance its health-promoting properties. The research required the germination of the kale seeds, 'TBC.' From 40 days after sowing, leaves were supplemented every 2 days with a sulfur (S) solution (0.0, 0.5, 1.0- and 2.0-mM) for 28 days. Individual and total glucosinolate content increased directly proportional to the S concentration. The maximum levels of total GLSs (26.8 mmol/g DW) and glucobrassicin (9.98 mmol/g DW) were found in the leaves supplemented with 2 mM S. Aliphatic glucosinolates, and total glucosinolates increased by 67% and 35%, respectively. Glucobrassicin was the main glucosinolate accumulated. Therefore, Park et

al. (2018) concluded that sulphur fertilizers constitute a method to enhance the anticancer phytochemical yields in young plants when soil nutrients are limiting.

Similarly, Kopsell et al. (2003) evaluated the effect of sulfur supplementation of three kale cultivars, Winterbor, Redbor, and Toscano, to consider the variability previously reported for glucosinolates and carotenoid accumulation. During the study, the 2-week-old plants were treated with a solution supplemented with 4, 8, 16, 32, and 64 mg of S/L for 45 days. The contents of glucoiberin, glucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin increased significantly in all three cultivars, mainly in the leaves supplemented with 16, 32, and 64 mg of S/L. Glucobrassicin was the most abundant glucosinolate, showing an increase of 505% (274.3 mg/g DW), 746% (335.2 mg/g DW), and 362% (261.5 mg/g DW) in the Winterbor, Redbor, and Toscano cultivar, respectively, when compared with the 4 mg S/L treatment. However, there was no significant change in carotenoid accumulation by S treatment. Kopsell et al. (2003) suggest that glucosinolate accumulation appears to be determined by S availability, cultivar, and their growing individual characteristics.

2.3.1.2. Selenium as an Abiotic Stressor

Selenium (Se) is a trace mineral element essential in human and animal nutrition because it acts as a cofactor of selenoenzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR). These enzymes reduce reactive oxygen species (ROS) levels and maintain the redox balance in the human body (Jaganjac, Milkovic, Sunjic, & Zarkovic, 2020). Selenium has been classified as a chemopreventive agent due to its ability to reduce the risk, delay the progression, and avoid the cancer recurrence (Brodowska, 2016; Guardado-Félix et al., 2019). Despite its importance, Se deficiency in food is a common nutritional problem in various parts of the world (Cox & Bastiaans, 2007).

Germination in the presence of Se condition could trigger sulfur (S) metabolic pathways due to their chemical similarity. Selenium forms (elemental, selenide, selenate, selenite, organic) present in nature determine its solubility and bioavailability in plants. Both selenate and selenite are the chemical forms predominantly absorbed by plants; however, selenate is the most mobile form within the plant. Selenate is probably absorbed in the root by sulfate transporters, located on the plasma membrane of the cell via S channels (SULTR1;2, SULTR1;1, and SULTR3;1). Apparently, it can enter leaf mesophyll cells by SULTR1;1 or SULTR1;2 and enters chloroplasts via SULTR3;1. Due to the similarity with S, selenate could enter the S metabolic pathways where would be converted in Se-amino acids. Once the inorganic Se enters the plastid, it must be converted by ATP sulfurylase (ATPS) in phosphoselenate (APSe). Then, the APSe is further reduced to selenite by the activity of APS reductase (APR). The conversion of selenite to selenide (Se^{-2}) is reduced by sulfite reductase (SiR) or by the interaction with reduced adenosine phosphosulfate (GSH). In the last case, selenite and GSH are converted nonenzymatically to selenodiglutatione (GSSeSG), which is then transformed to selenopersulfide (GSSeH) and finally to selenide through glutathione reductase (GR). Thereafter, Se^{-2} is incorporated into SeCys via the cysteine synthase (CS) complex, which consists of the enzyme serine acetyltransferase (SAT), its product, *O*-acetylserine (OAS), and the *O*-acetylserine thiol lyase (OASTL) enzyme (Schiavon & Pilon-Smits, 2017).

Likewise, the amino acid SeCys can be transformed to SeMet in three enzymatic steps. Briefly, SeCys is converted to selenocystathione (Se-cystathionine) through *O*-

phosphohomoserine (OPH) and SeCys, which is catalyzed by cystathione-c-synthase (CGS) (Schiavon & Pilon-Smits, 2017). Then, Se-cystathionine may be converted to selenohomocysteine (Se-homocysteine) by cystathione beta-lyase (CBL) (Schiavon & Pilon-Smits, 2017). Finally, methionine synthase (Met synthase) uses methyl-tetrahydrofolate as a carbon donor to convert Se-homocysteine into SeMet. Once more, the plant can volatize Se from SeMet by methylation to form methyl-selenometionina (SeMet) via S-adenosyl-L-Met:Met-S-methyltransferase (MMT), and then the conversion to DMSe by methylmethionine hydrolase (Schiavon & Pilon-Smits, 2017). A schematic representation of selenium metabolism in plants is shown in **Figure 4**. Se also can be incorporated into (seleno)glutathione, glucosinolates, and iron (Fe)-Se clusters. Se supplementation has been shown to up-regulate the secondary metabolism of plants that involves enzymatic and non-enzymatic antioxidants (Guardado-Félix, Serna-Saldivar, Cuevas-Rodríguez, Jacobo-Velázquez, & Gutiérrez-Uribe, 2017).

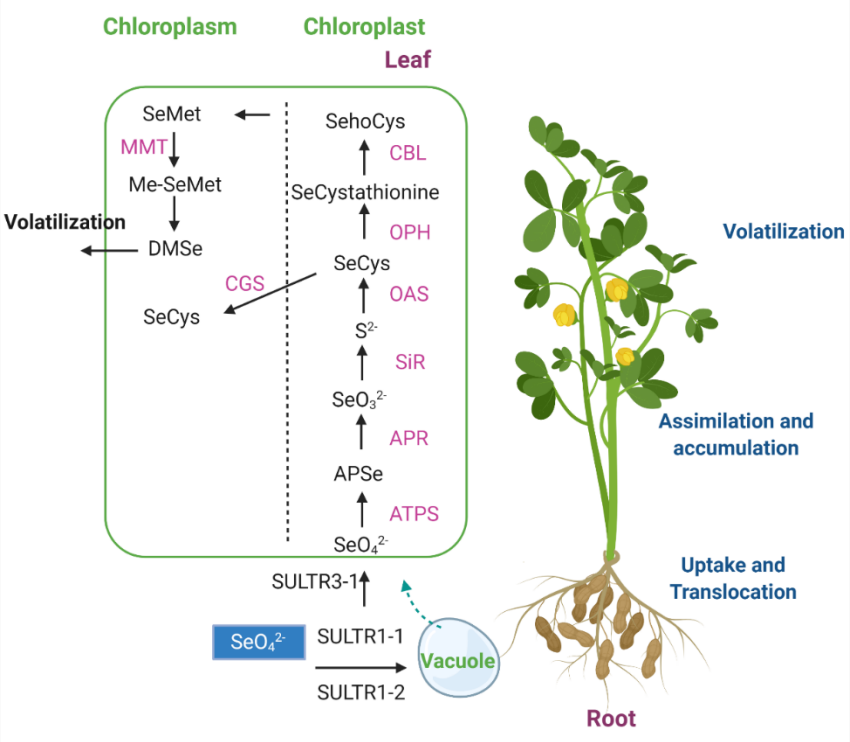

Figure 4. Scheme of main steps in selenium (Se) metabolism. SULTR: sulfate transporter; ATPS: ATP sulfurylase; APR: APS reductase; SiR: sulfite reductase; OAS: O-Acetyl Serine. OPH: *O*-phosphohomoserine; CBL: cystathione beta-lyase; MMT: S-adenosyl-L-Met:Met-S-methyltransferase; CGS: cystathione-c-synthase. Figure created with BioRender.com.

In adequate concentrations, the role of Se consists of soil nutrient enrichment, regulation of ROS, translocation of heavy metal, and restoring the cell membrane and chloroplast structures in plants. However, an excess of Se triggers the accumulation of ROS (Feng, Wei, & Tu, 2013).

The production of ROS at high Se levels may be partially related to an imbalance in the levels of GSH, thiols (-SH), and NADPH, which can play a vital role in the oxidizing cell environment. All these signaling molecules lead to the overexpression of stress-related genes, normally induced by defense signaling pathways (e.g., *PR-1*, *PR-2*, *PR-5*, and the defense gene *PDF1.2*) (Brosché & Strid, 2003). The high level of ROS upregulates the expression of several genes involved in phenolic biosynthesis (e.g., the maize

transcription factor *ZmP* and *MYB12,* and their target genes *CHS* and *CHI* –chalcone isomerase).

Plants of the *Brassicaceae* family can store Se at concentrations of up to 10–15 mg Se/ g DW in their shoots while growing on soils containing only 0.2–10 mg Se/kg. The non-specific integration of Se into the S assimilation pathway enables the plant to metabolize selenoamino acids, selenocysteine, and selenomethionine into proteins (Barickman, Kopsell, & Sams, 2013).

There are few reports of the effect of selenium supplementation on the concentration of bioactive compounds present in kale plants. Kim et al. (2018) evaluated the effects of sodium selenite on glucosinolates and ITC content in kale (*Brassica oleracea* var. *sabellica*). Six-week-old kale plants were exposed to 2 mg/L Na_2SeO_3, 80 mM NaCl, or a combination for 2 weeks. The results showed that kale roots accumulated higher levels of gluconasturtiin with NaCl (15%, 9.1 µmol/g DW), Na_2SeO_3 (19%, 11.8 µmol/g DW), or both (27%, 16.4% µmol/g DW) after 7 days of treatment; however, there was no statistically significant difference in the glucoraphanin content between the control and treated kale plant. In addition, the ITC concentration was increased 30% (6 µmol/g DW) by a 2-week treatment with the combination of both compounds. Kim et al. (2018) suggest that kale is a plant with a wide range of salt tolerance, and the elicitation of their individual glucosinolates responds differentially to it.

2.3.2. Methyl Jasmonate as an Abiotic Stressor

Phytohormones, such as MeJA, are another class of abiotic stressors that trigger cascades of physiological and molecular responses, resulting in the synthesis and accumulation of secondary metabolites (Zhao, Davis, & Verpoorte, 2005). These responses often involve activation of the antioxidant system (superoxide anion radical, peroxidase, and NADPH-oxidase), accumulation of amino acids (isoleucine and methionine), and soluble sugars, and regulation of stomatal opening and closing. In addition, the expression of genes implicated in secondary metabolism, cell-wall formation, and defense-related are upregulated (Park et al., 2013). Thus, exogenous phytohormones can be applied to plants as an approach to enhance their phytochemical content. A summary of the mechanisms of MeJA in abiotic stress tolerance is shown in **Figure 5**.

The effects of MeJA on the phytochemical profile in plants have been focused on the accumulation of glucosinolates. For instance, it is known that there are several transcription factors identified, which regulate the biosynthesis of glucosinolates in Arabidopsis and other related Brassica species, including *OBP2*, also called *AtDof1.1* (DNA- binding-with-one-finger). *OBP2* is a positive regulator of the network controlling indole glucosinolate biosynthesis in Arabidopsis (Augustine & Bisht, 2017). In Arabidopsis plants, the expression of OBP2 is stimulated by wounding, MeJA treatment, and in response to insect feeding, leading to an induced expression of glucosinolate biosynthetic genes and a subsequent accumulation of glucosinolates. In addition, the overexpression of OBP2 in transgenic plants resulted in the upregulation of *CYP79B2/B3* and *CYP83B1* genes involved in the glucosinolate biosynthetic pathway and an increase in indole glucosinolates (Skirycz et al., 2006). Moreover, it has been reported that MeJA treatment induces the accumulation of all types of indolyl glucosinolates, as it can induce other transcription factors involved in the indole glucosinolate pathway (i.e., *MYB51* and *MYB34*), subsequently altering the expression levels of *CYP79B2/B3* and *SOT16*, and finally resulting in enhanced indolyl glucosinolate levels (Mikkelsen et al., 2003).

Regarding other types of glucosinolates, MeJA can only induce the aliphatic transcription factor *MYB28*, while it has no response to other aliphatic regulators, such as *MYB29* and *MYB76* (Baskar, Gururani, Yu, & Park, 2012).

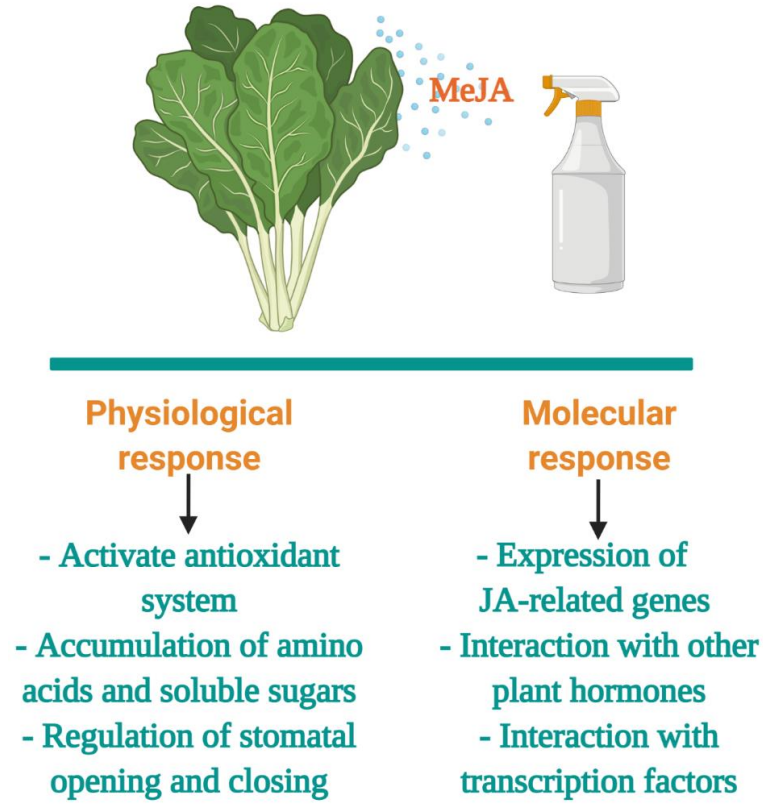

Figure 5. General mechanisms and functions in methyl jasmonate (MeJA) stress tolerance of plants. Figure created with BioRender.com.

Sun et al. (2012) performed a study to evaluate whether MeJA induces an effect in the content of glucosinolates in kale. MeJA (100 µM) was applied by vapor fumigation in thirty-day-old kale plants. The MeJA treatment induced a significant increase in the concentrations of glucobrassicin (520%, 4.25 µmol/ g DW), neoglucobrassicin (1420%, 3.16 µmol/ g DW), and total indole glucosinolates (230%, 13.2 µmol/ g DW), when compared with control at 1 d after treatment.

Yi et al. (2016) obtained similar results in four-month-old leaf kale sprayed with 250 µM MeJA. The application of the MeJA treatment induced an increase of indolyl and aliphatic glucosinolates in kale, including glucoraphanin (735%, 1.67 µmol/g DW) glucobrassicin (1708%, 4.52 µmol/g DW), and neoglucobrassicin (1800%, 0.38 µmol/g DW). In addition, gene expression levels of *ST5a (Bol026200), CYP81F1* (*Bol028913, Bol028914*), and *CYP81F4* were significantly upregulated, which suggests that the metabolic changes promoted by MeJA application share common activation mechanisms with the insect herbivory response (Chiu, Juvik, & Ku, 2018; Yi et al., 2016).

Few studies have been conducted regarding the elicitor effects of MeJA on the accumulation of phenolic compounds and carotenoids in kale and other plant species. For instance, Ku & Juvik (2013) observed that the exogenous application of MeJA (250 µM) in two kale cultivars, Red Winter (*Brassica napus* var. *pabularia)* and Dwarf Blue

Curled Vates (*Brassica oleracea* var. *acephala*), significantly increased total phenolics by 27% at commercial maturity.

2.4. Drawbacks of Using Abiotic Stresses at a Large Scale to Increase the Content of Bioactive Compounds in Kale

The application of abiotic stressors at larger scales remains labor intensive, especially in larger field areas and dense crop plantations. In addition, the dose-optimization requires variety-specific studies (Srivastava, Kumar, & Suprasanna, 2021). Further, under field conditions, various stresses occur in combinations that magnify stress severity. According to the National Climate Assessment -USDA, abiotic stresses can generate high losses in global crop production (~50%) (Garfin et al., 2014).

In addition, it is necessary to consider the possible accumulation of anti-nutritional factors present in kale due to exposure to abiotic stress. Kale has been reported as a source of oxalates, nitrates, tannin, and phytate. These compounds have a strong binding affinity to minerals, such as calcium, magnesium, iron, copper, and zinc, making them unavailable for absorption in the intestines (Satheesh & Workneh Fanta, 2020). Quality attributes of the crop, which are relevant for consumers, could also be affected by the application of pre- and post-harvest abiotic stresses (Jacobo-Velázquez & Benavides, 2021). Thus, the use of non-thermal technologies has been recently proposed as an effective tool to increase the content of health-promoting compounds in vegetables, while retaining quality attributes (Jacobo-Velázquez & Benavides, 2021).

2.5. Gastrointestinal digestion: Bioconversion of glucosinolates, phenolic compounds and carotenoids

Glucosinolates, phenolics and carotenoids in kale may contribute to health benefits, but it is important to understand the processes that are activated in the body in their presence.

2.5.1. Glucosinolates

Glucosinolates are chemically stable metabolites under normal conditions; however, when the plant tissue containing glucosinolates is disrupted (i.e., by cutting or chewing processes), the endogenous enzyme myrosinase is released from the cytoplasm and hydrolysis occurs (Barba et al., 2016).

Numerous works suggest that when active myrosinase is present in the vegetables, rapid break down of glucosinolates into their ITC metabolites occurs in the small intestine (Cramer & Jeffery, 2011). However, when myrosinase is inactivated (e.g., by cooking), intact glucosinolates transit to the colon and are hydrolyzed by microbial thio-glucosidase activity (Barba et al., 2016).

Once ITC have been absorbed, they are conjugated with glutathione (GSH) intracellularly and are metabolized via the mercapturic route in the liver. In this pathway, the dimeric enzyme glutathione transferase (GST) play an important role catalyzing the conjugation of glutathione (GSH) with ITC and endogenous compounds, thus facilitating their metabolism and excretion (Juge, Mithen, & Traka, 2007).

In the mercapturic acid pathway, the ITC glutathione conjugate is sequentially degraded to form conjugates with cysteinyl glycine, cysteine in the kidney, and N-acetylcysteine in the upper intestinal lumen (Vermeulen, Klöpping-Ketelaars, van den Berg, & Vaes, 2008). Finally, the conjugates are recirculated to the liver where they are acetylated.

After ITC have been metabolized via the mercapturic route, they are excreted as N-acetyl cysteine (NAC) conjugates in the urine (Hauder et al., 2011). In addition to the main pathway involving mercapturic acid, ITC can be excreted by other minor routes as exhalation, defecation, and perspiration (Barba et al., 2016).

Consumption of raw brassicas results in earlier absorption, higher bioavailability, and increased plasma amount of ITC, compared to cooked plants (Vermeulen et al., 2008).

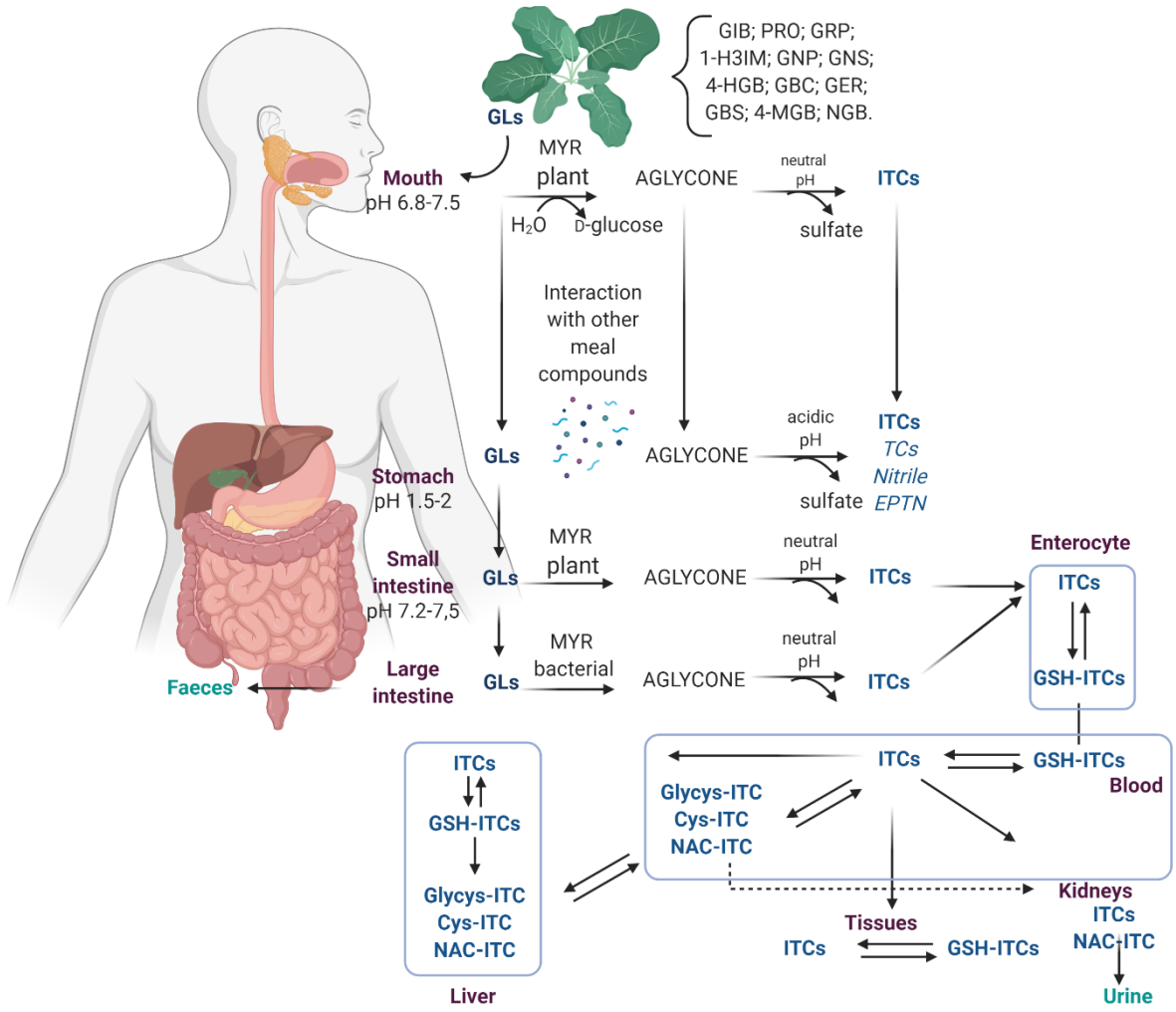

Figure 6. Overview of the fate of glucosinolates and their breakdown products in the human gut. Abbreviations: MYR, myrosinase; GSH-ITC, glutathione conjugate of ITC; Nac-ITC, N-acetylcysteine conjugate of ITC; glycys-ITC, glycine-cysteine conjugate of ITC; cys-ITC, cysteine conjugate of ITC; Glucoiberin (GIB); Progoitrin (PRO); Glucoraphanin (GRP); 1-hydroxy-3-indoylmethyl (1-H3IM); Gluconapin (GNP); Gluconasturtiin (GNS); 4-hydroxy-glucobrassicin (4-HGB); Glucobrassicanapin (GBC);

Glucoerucin (GER); Glucobrassicin (GBS); 4-methoxy-glucobrassicin(4-MGB); Neoglucobrassicin (NGB) (Oliviero, Verkerk, & Dekker, 2018).

2.5.2. Phenolics

In plants, most phenolic compounds are glycosylated, although they can also be found esterified or as polymers. Glycosylation of phenolic compound is considered a useful way to increase their solubility and stability in water, protecting them from oxidation (Lu et al., 2015). Once ingested, the absorption rate, the nature of the circulating metabolites and their elimination will be determined by their chemical structure (Hussain et al., 2019).

Glycosylated phenolics are considered non-absorbable, only aglycone forms can pass through the small intestine. Their deglycolysation start in the oral cavity, due to the activity of ß-glucosidase enzymes in saliva occurs or the oral microbiota; however, most of unmetabolized glycosylated phenolics (90-95%) directly reach the large intestine, where they are hydrolyzed by the gut microbiota (Gutiérrez-Grijalva, Ambriz-Pere, Leyva-Lopez, Castillo-Lopez, & Heiedia, 2016).

In the small intestine, the absorption is mediated by the lactase phlorizin hydrolase (LPH). LPH exhibits broad substrate specificity for flavonoid-O-β-glucosides, and the released aglycone may then enter the epithelial cells by passive diffusion. Some monoglycosides, such as quercetin-3-O-glycoside, can be hydrolyzed in the intestinal epithelium by the enzyme LPH, releasing the aglycon, which can enter the enterocyte by passive diffusion as a result off its increased lipophilicity and its proximity to the cellular membrane (Hussain et al., 2019).

The rapid transference into the enterocyte of the released aglycone is due to an efficient transport mechanism in parallel with the cytosolic β-glucosidase (CBG) activity. In order to CBC-mediated hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter (SGLT1) and glucose transport facilitating proteins (GLUT2) (Murota, Nakamura, & Uehara, 2018).

Prior to passage into the bloodstream, the aglycones undergo metabolism, forming sulphate, glucuronide and methylated metabolites through the phase II enzymes: catechol-O-methyltransferases (COMT), uridin-5'-diphosphate glucuronosyltransferases (UGTs) and sulfotransferases (SULT) (Del Rio et al., 2013). The metabolites may be efflux back into the intestinal lumen or delivered to the portal blood (Hussain et al., 2019).

After delivery to the portal blood, metabolites are able to enter into hepatocytes and are further metabolized before entry into circulation or elimination in bile. The unabsorbed phenolics along with those actively excreted by small intestine and by bile will reach the distal portion of the intestine where they become available for metabolism by gut microbiota or enterohepatic recirculation (Del Rio et al., 2013).

In colon, the phylogenetic composition of the intestinal microbiota is considered specific and stable over time for each individual, varying greatly between each person (Dueñas et al., 2015). Consequently, in addition to qualitative and quantitative variations in the daily intake of phenolic compounds, interindividual differences in the composition of the intestinal microbiota can lead to differences in the bioavailability of phenolic compounds and their metabolites (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013).

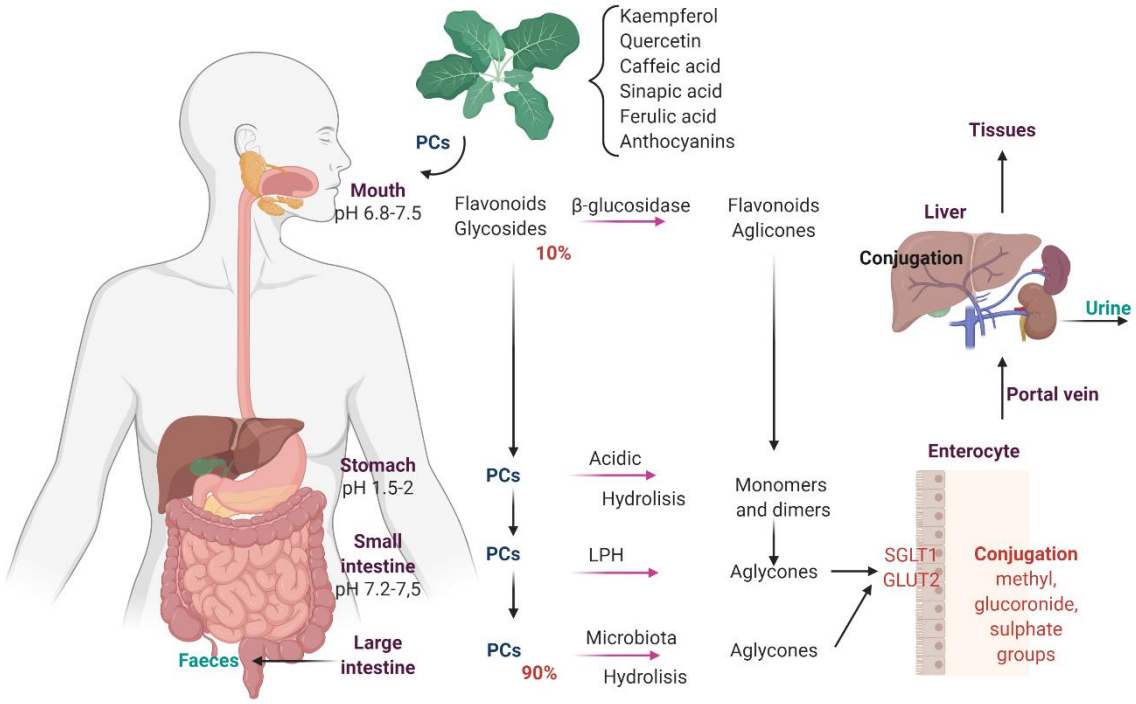

Figure 7. Overview of the steps involved in the human metabolism of phenolic compounds presents in kale. PCs: Phenolic compounds; SGLT1: Sodium-glucose cotransporter 1; GLUT2: Glucose Transporter 2.

The predominant catabolites generated by the microbiota from phenolic compounds are aromatic and phenolic acids with up to three aromatic hydroxyls, or their mono- or di-methoxylated analogs, which have a side chain of one to five carbons (Williamson & Clifford, 2017).

Metabolites of phenolic compounds are excreted via the biliary or the urinary route (Hussain et al., 2019).

2.5.3. Carotenoids

In their natural state, carotenoids are bound non-covalently to protein or esterified as mono- or diester with saturated fatty acids (Brotosudarmo, Limantara, Dwi Chandra & Heriyanto, 2018). Once carotenoids are released from the dietary matrix, they are circulated in the gastrointestinal tract with the help of dietary lipids and bile salts and are associated with lipoproteins in the form of micelles consisting of phospholipids, free fatty

acids and monoacylglycerols (Xavier & Mercadante, 2019). In general, carotenes such as β-carotene are not solubilized as easily as xanthophylls such as lutein (Huo, Ferruzzi, Schwartz, & Failla, 2007).

Then, carotenoids are transported by passive diffusion across the plasma membrane of enterocytes in the small intestine (Reboul, 2019). This process can be affected by soluble dietary fiber as well as other factors that interfere with the contact between the micelle and the mucosa intestinal. Similarly, it has been found that carotenoids can be mutually competitive during absorption, for example, high doses of canthaxanthin or lycopene have shown to reduce the absorption of β-carotene (Alemzadeh & Feehan, 2004).

After their absorption, carotenoids are integrated by intestinal mucosal cells into chylomicrons, which are circulated into the systemic circulation. Chylomicrons are digested very rapidly by the enzyme lipoprotein lipase within the lymphatic system. Chylomicron remnants are removed by the liver and carotenoids are incorporated into very low-density lipoproteins (VLDL) and released in the plasma (Reboul, 2019). Carotenoids also appear in low density lipoproteins (LDL) and high-density lipoproteins (HDL). In addition to plasma, carotenoids are found mainly in the adrenal gland, the retina, the corpus luteum and adipose tissues.

Various products of lutein and carotenes metabolism, such as 3'-epilutein, 3-hydroxy-β,ε-caro-ten-3'-one, 3'-hydroxy-ε,ε-caroten-3-one, and ε,ε-carotene-3,3'-dione, have been detected and identified in plasma and human tissues. It has been proposed that these metabolites are produced by repeated oxidation, reduction, and double bond migration. However, no enzyme involved in the above metabolic conversions has yet been characterized except for vitamin A formation (Arunkumar, Gorusupudi, & Bernstein, 2020). The responsible enzyme of the cleavage of carotenoids with a β-ionone ring to retinol is called beta-caroteno 15,15'-dioxygenase (Ansari & Emami, 2016).

Carotenoids are excreted in the feces; however, some polar products of carotenoids metabolism are most likely excreted in the urine (Bohn, 2018).

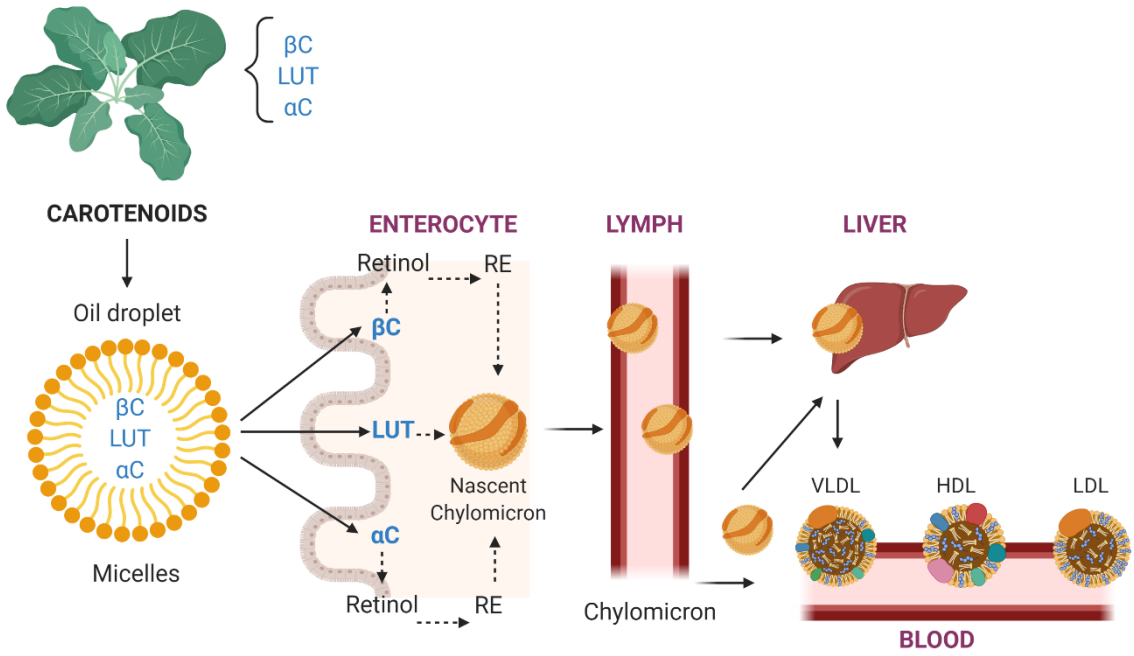

Figure 8. Summary of digestion and absorption of carotenoids from kale. Abbreviations: αC, α-carotene; βC, β-carotene; LUT, lutein; RE, retinyl esters.

Therefore, since kale sprouts are an exceptionally rich source of phenolic compounds, carotenoids and glucosinolates, but still underestimated as a therapeutic agent, the application of abiotic stress (e.g., selenium, sulphur and methyl jasmonate application) may be an interesting option to induce their accumulation, making them more attractive for food markets and industrial pharmaceuticals.

2.6. Inflammatory bowel disease (IBD): ulcerative colitis

The term Inflammatory Bowel Diseases (IBD) includes the entities ulcerative colitis (UC) and Crohn's disease (CD). They contribute considerably to gastrointestinal pathology due to their chronicity and exacerbating of the quality of life of patients, presenting a high associated medical and surgical morbidity. The precise cause of IBD is unknown, but individuals with genetic susceptibility seem to have an altered immune system associated with a defective mucosa for the normal commensal microbiota of the colon (Danese et al., 2014).

UC is an idiopathic chronic inflammatory disease of the colorectal mucosa, which does not present granulomas on histopathological analysis. It involves the rectum and generally extends to the proximal colon segments continuously and may involve the entire colon (Silverberg et al., 2005). It typically presents in the third or fourth decade of life and is characterized by intestinal disorders such as bloody diarrhea, urgency, and abdominal pain. Epidemiology UC has a bimodal pattern of incidence, the highest incidence is between 15 and 30 years, and the second highest incidence is between 50 and 70 years (Michalak et al., 2016). There are no studies that show preference according to gender (Bernstein, Rawsthorne, Cheang, & Blanchard, 2006). Developed countries such as the

United States (USA) and northern European countries have the highest prevalence and incidence of UC worldwide.

The incidence has increased in countries that have adopted an industrialized lifestyle, which suggests that environmental factors may influence the triggering of the onset of the disease, where improved health systems reduce gastrointestinal infections during childhood by restricting the maturation of the immune system associated with the intestinal mucosa (Moum & Ekbom, 2002).

Different factors define the pathophysiological evolution of IBD, including the following:

2.6.1. Genetic Factors

Family history of IBD is the most important independent risk factor, being higher in affected first-degree relatives. 5.7% -15.5% of patients with UC have a history of first-degree relatives with the same disease (Zárate & Chahuan, 2013). The most prominent risk genes include Nucleotide-Binding Oligomerization Domain Containing 2 (NOD2) for CD, certain human leukocyte antigens (HLA) for UC and interleukin 23 receptor for IBD (Michalak et al., 2016).

2.6.2. Environmental Factors

2.6.2.1. Gastrointestinal infections

Previous gastrointestinal infections caused by *Escherichia coli, Salmonella, Shigella, Campylobacter* multiply the risk of subsequently producing UC, which would indicate that acute gastrointestinal infections would alter the colonic microbiota, triggering the disease in genetically predisposed people (García Rodríguez, Ruigómez, & Panés, 2006).

2.6.2.2. High-Fat Diets and Dysbiosis

Diet, known to cause dysbiosis, is one of the environmental triggers for the onset of IBD, metabolic syndrome and associated diseases. This dysbiosis is characterized by a significant reduction in bacterial diversity, being rare to find bacteria of the *Bacteroidetes* and *Firmicutes* genera, as well as *Clostridium coccoides* and *Clostridium leptum,* with good representation under normal conditions (Rivero Gutiérrez, 2016).

A high-fat diet induced colonic inflammation and increased nitric oxide synthase (iNOS) expression during LPS-induced inflammation via toll like receptor (TLR)-4 signaling (Festi et al., 2014; Weisberg et al., 2003). A similar increase in TLR-4 has been reported for intestinal and colonic samples of UC and CD patients (Xu et al., 2003).

The activation of TLR's triggers innate and adaptive immune responses that lead to the activation of the nuclear transcription factor kappa beta (NF-Kb), which regulates pro-inflammatory functions and cell survival in macrophages and T cells (Spehlmann & Eckmann, 2009).

2.6.2.3. Stress

The physiological basis underlying this association remains in an increase in discharges of a cholinergic nature in the enteric nervous system, which translates into an increase in intestinal motility and hydroelectrolytic secretion, and in an increase in the levels of corticotropin-releasing hormone (CRH) and therefore cortisol (Rivero Gutiérrez, 2016). Elevated levels of systemic CRH and cortisol exert immunomodulatory effects that can alter intestinal homeostasis. It should be noted that CRH acting on mast cells produces the release of multiple soluble mediators that compromise intestinal barrier function (IBF) by significantly increasing transcellular permeability (Wallon et al., 2008).

2.6.2.4. Obesity

Since there is a high frequency of obese patients with IBD, an attempt has been made to link obesity in the pathogenesis of this disease. Common mechanisms include increased production of TNF-α, IL-6 and reactive oxygen species (ROS) (Estay et al., 2017).

In metabolic syndrome and IBD the inflammation affects adipose tissue and, consequently, disturbs the adipokine secretion. Both, CD and UC are associated with high resistinemia, visfatinemia and low leptinemia (Waluga, Hartleb, Boryczka, Kukla, & Żwirska-Korczala, 2014).

2.6.3. Immunoregulatory Defect

A central element in the pathogenesis of IBD is an aberrant function of the mucosal immune system. Defects in the innate and adaptive immune systems have been identified.

Innate immunity is the first defense against pathogens in the intestine and is composed in the first place of a series of physical and chemical barriers that ensure that microorganisms potentially harmful to the body are kept isolated to avoid contact with reactive immune cells of the lamina propria of the intestine (Silva, Gatica, & Pavez, 2019).

The first line of defense against microorganisms and antigens is a double layer of mucus that covers the entire intestinal epithelium. It is produced by the polymerization of gel-forming mucins (secreted by goblet cells) which are a highly hydrophilic type of glycoprotein that can bind complex carbohydrates from the glycocalyx on the surface of epithelial cells (Kelsall, 2008). It is divided into an outer layer in which commensal bacteria, mucins and antimicrobial peptides are found, and then an inner layer, which is normally sterile (Maloy & Powrie, 2011; Silva, Rodrigues, Ayrizono, & Leal, 2016). This mucus layer plays a crucial role in intestinal homeostasis since a low level of mucin-producing cells are an indicator of the disease.

For the maintenance of immune homeostasis in the intestine, it is also necessary to maintain an intact epithelial barrier that constitutes the second line of defense. This has been proven by various studies in which the oral administration of DSS (dextran sodium

sulfate) to mice implied an alteration in permeability leading to a large entry of antigens to the lamina propria. It activated innate immunity mechanisms in the first place and subsequently, with prolonged administration of DSS, adaptive response mechanisms resulting in chronic inflammation (Gassull, Gomollón, Obrador & Hinojosa, 2007). DSS is a sulfated polymer with cytotoxic capacity on intestinal epithelial cells and macrophages that also favors the increase of anaerobic Gram-negative bacteria, which together with the erosive potential on the intestinal barrier and the inappropriate response of macrophages favors the appearance of intestinal lesions (Almero, 2007).

The passage of small water-soluble molecules through the intestinal epithelium is carried out by the tight junctions that seal the spaces between the epithelial cells. They are the most apical intercellular junctions, and their function is essential in maintaining the barrier by regulating the passage of ions and proteins and limiting the translocation of luminal antigens. They are composed of multiprotein complexes made up of different families of transmembrane proteins: claudin, occludin, and tricellulin (Salvo-Romero, Alonso-Cotoner, Pardo-Camacho, Casado-Bedmar, & Vicario, 2015). Defects in tight junctions have been identified in patients with IBD related to changes in the regulation of the proteins due to the action of inflammatory factors such as IL-13, IFNγ and TNF-α and changes in the microbiota (Gassull, Gomollón, Obrador & Hinojosa, 2007). TNF-α increases the expression of the protein that facilitates pore formation, claudin-2, which directly increases permeability (Kelsall, 2008).

The secretion of defensins, proteins with bactericidal properties, is induced as part of the innate immune response. In the intestine, they are secreted mainly by epithelial cells and Paneth cells. Due to their properties, defensins can generate conductance pores in epithelial cells and activate IL-8 secretion, which recruits leukocytes to the site of infection (Yamamoto, 2010). Different classes of defensins exist, including α and β-defensins, which differ in their distribution, as well as in their regulation. The α-defensins are produced mainly by Paneth cells within the crypts of the small intestine, whereas the β-defensins are produced more extensively by the epithelial cells of the large intestine (Kelsall, 2008). Several studies have shown that in IBD there is a decrease in the ability of Paneth cells to secrete defensins, thus favoring intestinal colonization by pathogenic bacteria and producing an imbalance of cytokines. Likewise, Paneth cells express NOD2, whose polymorphisms represent the strongest genetic association with IBD (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014).

On the other hand, pattern recognition receptors (PRRs), expressed in many cell types including epithelial cells, macrophages, dendritic cells and B cells, are responsible for recognizing a limited number of highly conserved structures in microorganisms called pathogen-associated molecular patterns (PAMP). Activation of PRRs first involves the activation of phagocytic activity by macrophages, seeking to reduce exposure to pathogens and the release of chemokines and pro-inflammatory cytokines by epithelial cells. Two families of PRRs have been described, the Toll-like receptors (TLRs), which are transmembrane proteins, and the NOD proteins (nucleotide oligomerization domains)

that are in the cytoplasm, thus recognizing intracellular bacterial products. Both the genetic variants of PRRs and the physiology of the response derived from their activation have shown to participate in the development and susceptibility to IBD (Yamamoto, 2010).

Mice deficient in TLRs have been shown to have increased susceptibility to DSS-induced colitis as they lacked repair tissue. The signals produced by the TLRs have protective effects on intestinal epithelial cells since they induce various proliferative and anti-apoptotic factors promoting epithelial replacement and strengthening intercellular junctions. Likewise, the intrinsic signals of TLRs on epithelial cells also play a central role by limiting bacterial colonization and translocation because they stimulate the production of antimicrobial peptides by these cells (Kelsall, 2008).

TLR activation results in a series of subsequent events culminating in nuclear translocation of factor NFκβ, with the subsequent production of pro-inflammatory cytokines and therefore TH-specific responses (Geremia et al., 2014).

Intestinal dendritic cells act as a bridge between the innate and adaptive immune systems and are responsible for determining whether tolerance or immune activation occurs and, if activation occurs, whether a Th1 or Th2 type response predominates. Intestinal dendritic cells are antigen presenting cells found below the epithelium in the lamina propria, in Peyer's patches and in mesenteric lymph nodes. Dendritic cells sample luminal contents directly by extending projections into the intestinal lumen between epithelial cells, or indirectly through M cells, which are specialized epithelial cells that carry luminal antigens to intestinal lymphoid follicles and plaques of Peyer. By sampling luminal content through their surface TLRs, dendritic cells then express potent cytokines of the Th1 or Th2 variety. Dendritic cells exhibit motility in response to different stimuli and are capable of migrating from the peripheral blood to the intestinal mucosa in response to particular antigens (Abreu & Sparrow, 2007).

Unlike the innate immune response, the ***adaptive immune response*** has a high specificity since it is the result of a complex maturation of immune cells, it also confers lasting protection thanks to the immune memory. The protagonists of this response are T cells. Th0 cells can become activated and differentiate into different types of effector (or helper) T cells or lymphocytes: Th1, Th2 or Th17. Th1 cells will be essential in the elimination of intracellular pathogens while Th2 cells are protective against parasites and mediate allergic reactions, finally Th17 cells contribute to the elimination of extracellular bacteria and fungi. In addition, there are regulatory T cells (Tregs), which are crucially involved in the maintenance of intestinal mucosal homeostasis by suppressing abnormal immune responses against common intestinal microorganisms. They will exert their function by producing anti-inflammatory cytokines, IL-10 and TGF-β, and preventing both the activation and the effector function of T lymphocytes that have escaped other tolerance mechanisms (Geremia et al., 2014).

Furthermore, an increase in activated B cells has been observed, although their clinical relevance is unknown.

In both EC and UC, there is an increase in the concentrations of pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, IL-15, IL-18, IFN-γ, TNF-α and the members of the IL-12 family (IL-12, IL-23, IL-27), among others. Anti-inflammatory mediators, TGF-β and IL-10, will also increase, but the balance between the two groups is what ultimately determines the course of the disease (León, Garrote, & Arranz, 2006) (**Figure 9**).

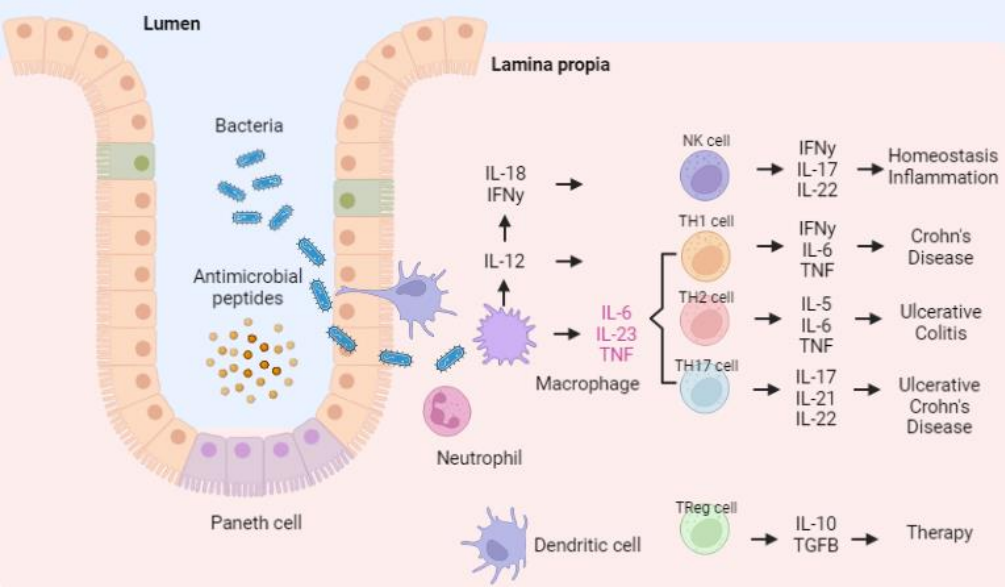

Figure 9. Conceptual framework cytokine production in pathogenesis of IBD

In summary, although the specific etiology of IBD remains unknown, current evidence indicates that the development of the disease leads to a loss of intestinal homeostasis and an imbalance in the patient's immune response towards the perpetuation of inflammatory processes. Inflammation, in turn, is a process closely linked to the production of reactive metabolites, such as ROS and nitrogen metabolites, which are produced in large quantities by activated immune cells that reach the mucosa in IBD (Moret et al., 2014).

For all these reasons, the inhibition of the development of harmful oxidative stress highlights the interest of exploring this pathway as a therapeutic target through the antioxidant compounds of kale. This is justified by the clinical improvement in the patients with IBD after treatment with drugs anti-TNFα, anti-NF-κB, etc (Russo et al., 2012).

CHAPTER 3. SELENIUM, SULFUR, AND METHYL JASMONATE TREATMENTS IMPROVE THE ACCUMULATION OF LUTEIN AND GLUCOSINOLATES IN KALE SPROUTS

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3.1. Introduction

Kale (*Brassica oleracea* var. acephala) is a vegetable native to eastern Turkey belonging to the Brassicaceae family (Balkaya & Yanmaz, 2005). This vegetable has been widely used worldwide in traditional medicine to prevent and treat different health disorders, including cancer, gastric ulcers, high cholesterol levels, hyperglycemia, rheumatism, and hepatic disease, among others (Kuerban et al., 2017; Lemos et al., 2011). Its biological properties have been attributed to different secondary metabolites such as glucosinolates, carotenoid, and phenolic compounds (Abellán et al., 2019; Ortega-Hernández, Antunes-Ricardo, & Jacobo-Velázquez, 2021). Their beneficial characteristics are based on the scavenging of free radicals, inhibition of receptors, activation of antioxidant enzymes, and variation of gene expression (Correia et al., 2012).

The accumulation of health-promoting bioactive compounds has been related to the application of biotic and abiotic stresses (i.e., wounding, UV radiation, and exogenous phytohormones) in fruits and vegetables through the generation of stress-signaling molecules, and the expression of genes implicated in the secondary metabolism of the plant (Cisneros-Zevallos & Jacobo-Velázquez, 2020). Abiotic stresses reported in kale to induce the biosynthesis of glucosinolates, as well as phenolic and carotenoid biosynthesis pathways, include UVA and UVB radiation (Alegre et al., 2019; Klopsch et al., 2019; Jai-Heo Lee & Myung, 2019; Yoon, Kim, & Son, 2020), drought (Barickman, Ku, & Sams, 2020; Yoon, Zhang, & Son, 2020), sulfur (S) (Kopsell et al., 2003; Park et al., 2018), selenium (Se) (Sun Young Kim et al., 2018a), sodium chloride (Kim et al., 2018; Linić et al., 2019; Wang et al., 2011), low and high temperatures (Hwang, Chun, & Kim, 2017; Jurkow, Wurst, Kalisz, Sękara, & Cebula, 2019; Jin-Hui Lee & Myung, 2015; Lee, Lim, Kim, & Myung, 2012), and methyl jasmonate (MeJA) (Ku & Juvik, 2013; B. Sun et al., 2012; Yi et al., 2016)

In plants, S and Se have positive effects, playing a key role in growth and development (Cao et al., 2014; Schiavon & Pilon-Smits, 2017). In tolerable concentrations, S and Se enrich soil nutrients, regulate reactive oxygen species (ROS) levels, and reestablish the cell membrane and chloroplast structures in plants. However, a high concentration of S or Se can trigger the accumulation of ROS (Feng et al., 2013), which upregulates the expression of several genes involved in phenolic biosynthesis (e.g., the transcription factors *ZmP* and *MYB12,* and their target genes *CHS* and *CHI* –chalcone isomerase) (Brosché & Strid, 2003). Moreover, S and Se addition influences the expression of genes involved in the biosynthesis and degradation of glucosinolates (*AT1G16400*) and carotenoids (*PSY* -phytoene synthase) in *Arabidopsis* (Sams, Panthee, Charron, Kopsell, & Yuan, 2011). Although the influence of S and Se in the Brassicaceae family has been previously reported, research has mainly focused on their effect on mature vegetables and on glucosinolate enhancement (Kim et al., 2018; Kopsell et al., 2003; Park et al.,

2018), and thus, there is scarce information on its application to enhancing carotenoid and phenolic compound content in kale sprouts.

On the other hand, phytohormones, such as MeJA are also abiotic stressors that activate physiological and signaling reactions that affect gene expression in plants and result in the production of secondary metabolites (Moreira-Rodríguez, Nair, Benavides, Cisneros-Zevallos, & Jacobo-Velázquez, 2017a; Zhao et al., 2005). In *Arabidopsis* plants, the expression of odorant binding protein (*OBP*) 2 has been shown to be stimulated by MeJA treatment, leading to an induced expression of glucosinolate biosynthetic genes and a subsequent accumulation of glucosinolates (Skirycz et al., 2006). Likewise, it has been confirmed that MeJA treatment can effectively improve the level of total phenolics by increasing phenylalanine ammonia-lyase (PAL) activity in broccoli (Guan et al., 2019). On the other hand, little or null change on carotenoid levels due to MeJA application in plants such as kale, broccoli, tomato and lettuce (Kim, Fonseca, Choi, & Kubota, 2007; Liu et al., 2012; Moreira-Rodríguez et al., 2017; Sun et al., 2012) has been reported. However, studies regarding abiotic stresses in kale sprouts with the aim of simultaneously inducing glucosinolate, carotenoid, and phenolic accumulation are very limited, if not non-existent.

The development of bioaccumulation strategies for nutraceutical compounds in plants, in any of their growth phases, has become a focus of attention in improving the health-benefits of foods and for the design and production of functional ingredients that can potentially be included in various food matrices or even some cosmetics. Therefore, the objective of this study was to determine the individual effects of Se, S and MeJA applied by spraying on the accumulation of glucosinolates, carotenoids, and phenolic compounds in two different varieties of seven-day-old kale sprouts (*Brassica oleracea* var. acephala).

3.2. Materials and Methods

3.2.1. Chemicals

Methyl jasmonate (MeJA); sulfatase (from *Helix pomatia*) ; diethylaminoethyl (DEAE)-sephadex A-25; sinigrin hydrate; sodium acetate; ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate); iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$); DPPH (1,1-diphenyl-2-picrylhydrazyl); sodium nitroprusside; acetonitrile (HPLC grade); methanol (HPLC grade); methyl tert-butyl ether (MTBE; HPLC grade); phosphoric acid; 4-*O*-caffeoylquinic acid; ferulic acid; sinapic acid; quercetin; kaempferol and lutein standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sulfanilamide and N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) solutions were acquired from Promega (G2930, Madison, WI, USA). Ethanol (HPLC grade), sodium selenite (Se; Na_2SeO_3), and potassium sulfate (S; K_2SO_4) were obtained from Desarrollo de Especialidades Quimicas, S.A. de C.V. (Monterrey, NL, Mexico). Desulfoglucoraphanin was acquired from Santa Cruz Biotechnology (Dallas, TX, USA).

3.2.2. Plant Material

Red Russian and Dwarf Green kale (*Brassica oleracea* var. acephala) seeds were provided by La Semilleria (Queretaro, Qro, Mexico). Kale seeds were sanitized for 15 min in sodium hypochlorite (1.5%, v/v) and rinsed with Milli-Q water.

3.2.3. MeJA, Se, and S Treatments

Kale seeds were soaked in Milli-Q water (control) or the treatment solutions for 5 h. The treatment solutions used were Se (10, 20, 40 mg/L), S (30, 60, 120 mg/L), and MeJA (25,

50, 100 µM). Afterwards, the soaking solutions were discarded, and the seeds were placed on germination trays in a dark chamber set at 25 °C and 85% of relative humidity for 7 days. Three replicates of each treatment were run concurrently. Each replica consisted of a tray containing 30 g of kale seeds sprayed with 5 mL of different treatment concentrations or control every 12 h throughout the experiment. Samples were collected every 24 h for 7 days, freeze-dried (Labconco, Kansas City, MO, USA), ground into a powder, and stored at −80 °C until further analysis of phytochemicals.

3.2.4. Phytochemical Analyses

3.2.4.1. Extraction of Secondary Metabolites

Extraction of glucosinolates and lutein from kale sprouts powder was carried out in a single process based on the protocol published by Moreira-Rodríguez et al. (2017). Briefly, kale samples (0.2 g) were mixed with 10 mL of ethanol/water (70:30,v/v), previously heated for 10 min at 70 °C to inactivation of myrosinase, followed by the addition of 50µL of sinigrin 3 mM as internal standard (I.S.).

Samples were immersed in a water bath (VWR, Radnor, PA, USA) at 70 °C and vortexed every 5 min for 30 min to inactivate myrosinase. Then, the extracts were left to cool at 25 °C and centrifuged (18,000× *g*, 10 min, 4 °C) (SL16R, Thermo Scientific, GER) to recover the supernatant. The supernatant obtained was used for the identification and quantification of individual glucosinolates, lutein, and phenolic compounds by HPLC-DAD (1260 Infinity, Agilent Technology, Santa Clara, CA, USA).

3.2.4.2. Identification and Quantification of Lutein

The identification and quantification of lutein were performed as described by Moreira-Rodríguez et al. (2017). Briefly, 25 µL of clarified ethanolic extracts, previously filtered using 0.45 µm nylon membranes (VWR, Radnor, PA, USA), were injected in the HPLC-DAD system (1260 Infinity, Agilent Technologies, Santa Clara, CA). Lutein was separated on a YMC C30 column (4.6 mm × 150 mm, 3 µm particle size) (YMC, Wilmington, NC, USA). The mobile phase consisted of (A) methanol, (B) MTBE, and (C) water. The elution system was isocratic: 50% A, 45% B, and 5% C for 30 min with a flow rate of 0.5 mL/min. Lutein was detected at 450 nm and identified by comparing its retention time and absorption spectra with a reference standard. Lutein was quantified using a calibration curve of lutein standard (0–12 ppm) and expressed as mg of lutein per kg of kale sprouts in dry weight (DW) basis.

3.2.4.3. Identification and Quantification of Phenolic Compounds

Phenolic compounds in kale extract were quantified using an HPLC system (Agilent Technologies 1260 series, Santa Clara, CA, USA) coupled with a diode array detector (DAD). The stationary phase was a C18 reverse phase column, 4.6 mm × 250 mm, 5 µm (Luna, Phenomenex, Torrance, CA, USA). Two mobile phases were used for chromatographic separation: (A) water adjusted to pH 2.4 with phosphoric acid and (B) methanol-water (60:40, *v*/*v*). Gradient elution consisted of 0/0, 3/30, 8/50, 35/70, 40/80, 45/100, 50/100 and 60/0 (min/% phase B) at a flow rate of 0.8 mL/min. The injection volume was 10 µL. The analysis was performed at 280, 320, and 360 nm and integrated by the OpenLAB CDS ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

Peak identification was based on retention time, UV-visible spectra and wavelengths of maximum absorption, as compared with the reported literature (Aguilar-Camacho, Welti-Chanes, & Jacobo-Velázquez, 2019; Moreira-Rodríguez et al., 2017a) and commercial

standards. For the quantification of phenolic compounds, standard curves of 4-*O*-caffeoylquinic acid (0.1–50), ferulic acid (0.4–40 ppm), sinapic acid (0.1–50 ppm), quercetin (0.1–12 ppm), and kaempferol (0.1–12 ppm) were prepared. Results were expressed as mg of each individual phenolic compound per 100 g of kale sprouts (mg/100 g) in dry weight (DW) basis.

3.2.4.4. Desulfation of Glucosinolates

After extraction, a desulfation step was carried out to purify glucosinolates and improve accuracy and identification from HPLC. DEAE-Sephadex A-25 resin was hydrated for at least 12 h in sodium acetate (0.02 M, pH 5). Polypropylene syringes were washed with 0.5 mL of water, and 0.5 mL of hydrated DEAE-sephadex A-25 was added, followed by an additional wash with 0.5 mL of water. Sephadex was left to pack and the excess medium removed after 20 min. Then, columns were loaded immediately with 3 mL of kale supernatant. After removing excess supernatant by elution, the columns were washed with 1 mL of water followed by 1 mL of sodium acetate (0.02 M). Purified sulfatase (75 µL) was added to each column and was left at 25 °C for 12 h. Desulfoglucosinolates were collected in vials by elution, slowly adding 1.25 mL of water.

3.2.4.5. Identification and Quantification of Glucosinolates

Glucosinolates were identified and quantified by HPLC-DAD. Separation was performed on a C18 reverse phase column, 4.6 mm × 250 mm, 5 µm (Luna, Phenomenex, Torrance, CA). Elution was conducted with (A) water and (B) acetonitrile. Separation was achieved with an initial A concentration of 100%, adjusting the A concentration to 80% at 28 min and to 100% at 35 min at a flow rate of 1.5 mL/min. The injection volume was 20 µL. Data were acquired at 227 nm and processed by the OpenLAB CDS ChemStation software (Agilent Technologies, Santa Clara, CA**,** USA).

Glucosinolate identification was based on retention time, UV-visible spectra and wavelengths of maximum absorption as compared with the reported literature (Aguilar-Camacho et al., 2019; Moreira-Rodríguez et al., 2017a) and commercial standards. For the quantification of glucosinolates, a standard curve of desulfoglucoraphanin (0–1250 ppm) was prepared. Results were expressed as mmol of desulfoglucoraphanin equivalents per Kg of kale sprouts (mM/Kg) (DW).

3.2.5. Antioxidant Activities

3.2.5.1. Free Radical Scavenging Activity

The free radical scavenging activity of ethanolic kale extracts was determined using the DPPH method proposed by Miceli et al. (2011). Briefly, an aliquot (20 µL) of extract of kale was added to 280 µL of daily prepared methanol DPPH solutions (0.1 mM). The solution was incubated for 30 min in the absence of light. Finally, the optical density change at 517 nm was measured. The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution. The results were reported as mean radical scavenging activity percentage (%) ± SD. The radical scavenging activity percentage (%) of the DPPH was calculated by **Equation (3.1):**

$$\%\ of\ scavenging\ activity\ = \left[\frac{Ac - As}{Ac}\right] x100$$

where *Ac* is the absorbance of the control and *As* is the absorbance in the presence of the sample.

3.2.5.2. Ferrous Ion (Fe^{2+})-Chelating Activity

The Fe^{2+}-chelating activity of the kale extracts was evaluated using the method described by Vavrusova et al. (2015). An aliquot (25 µL) of the kale extract diluted to 500 µg/mL was mixed with 100 µL of $FeSO_4$ (75 µM). The reaction was initiated by the addition of 100 µL of ferrozine (500 µM). Then the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–(Fe^{2+}) complex formation was calculated using the equation 3.1, where *Ac* is the absorbance of the control ($FeSO_4$ and ferrozine) and *As* the absorbance in the presence of the sample.

3.2.5.3. Nitric Oxide Radical Assay

The scavenging of nitric oxide radicals capacity of the kale sprout extracts was determined using the procedure described by Hazra et al. (2008). Sodium nitroprusside (100 µL, 10 mM) in standard phosphate buffer saline (pH 7.4) was incubated with 200 µL of kale extract (500 µg/mL) at 25° C for 2 h 30 min. After incubation, samples (50 µL) were diluted with 25 µL of sulfanilamide. The sample was incubated for 5 min at room temperature. Then, 25 µL of NED was added and the assay was left standing for 30 min at room temperature. The absorbance was observed at 540 nm on a spectrophotometer. The percentage of inhibition was calculated according to equation 3.1.

3.2.6. Statistical Analysis

Statistical analysis analyses were carried out using three replicates. Results were obtained as mean values and their standard error. Statistical analyses were performed using JMP software version 13.0 (SAS Institute Inc., Cary, NC). Data were analyzed by full factorial analyses of variance (ANOVA) followed by the least significant difference (LSD) test ($p < 0.05$).

3.3. Results

3.3.1. Carotenoids

In plants, carotenoids serve functions in photoprotection, oxidative stress, and developmental regulations, and for humans, they exert antioxidant activity (Ortega-Hernández et al., 2021). The main carotenoids reported for kale are lutein and carotene (Becerra-Moreno et al., 2014; Kim et al., 2017). The evaluation of the carotenoid profile of kale sprouts allowed for the identification and quantification of lutein (**Figure 10**). Values found for lutein were within previously reported ranges for chamber-grown Red Russian (49.8 mg/Kg DW) and Dwarf Green kale (29.0 mg/Kg DW) (Becerra-Moreno et al., 2014; Kim et al., 2017; Šamec, Ljubej, Redovniković, Fistanić, & Salopek-Sondi, 2022). Previous reports have also reported the presence of β-carotene, neoxanthin, and violaxanthin (Becerra-Moreno et al., 2014; Fiutak & Michalczyk, 2020; Kim et al., 2017). The difference in the carotenoid profile reported herein in comparison with previous reports could be attributed to the method of extraction, and the difference in the growing conditions of the other cultivars.

The daily concentration of lutein during germination (7 d) of kale sprouts treated and non-treated with MeJA, Se or S is shown in **Figure 10.** The application of MeJA, Se or S did not show an immediate significant change in the content of lutein in any variety of kale. The accumulation of lutein started around 3–4 d in both varieties, without significant differences between treatments. At 5 d of germination, samples showed a significant increase of lutein in both varieties of kale sprouts treated with MeJA 100 µM, Se 40 mg/L or S 120 mg/L. Thereafter, the concentration of lutein increased relatively constantly in

all treated samples until the end of germination. The highest increase of lutein was detected in Dwarf Green kale treated with Se 40 mg/L at 7 d of germination, being 199% higher than in the non-treated samples, followed by S 120 mg/L (174%) and MeJA 100 µM (109%). Likewise, lutein significantly increased in Red Russian kale treated with S 120 mg/L (178%), followed by MeJA 100 µM (89%) and Se 40 mg/L (86%).

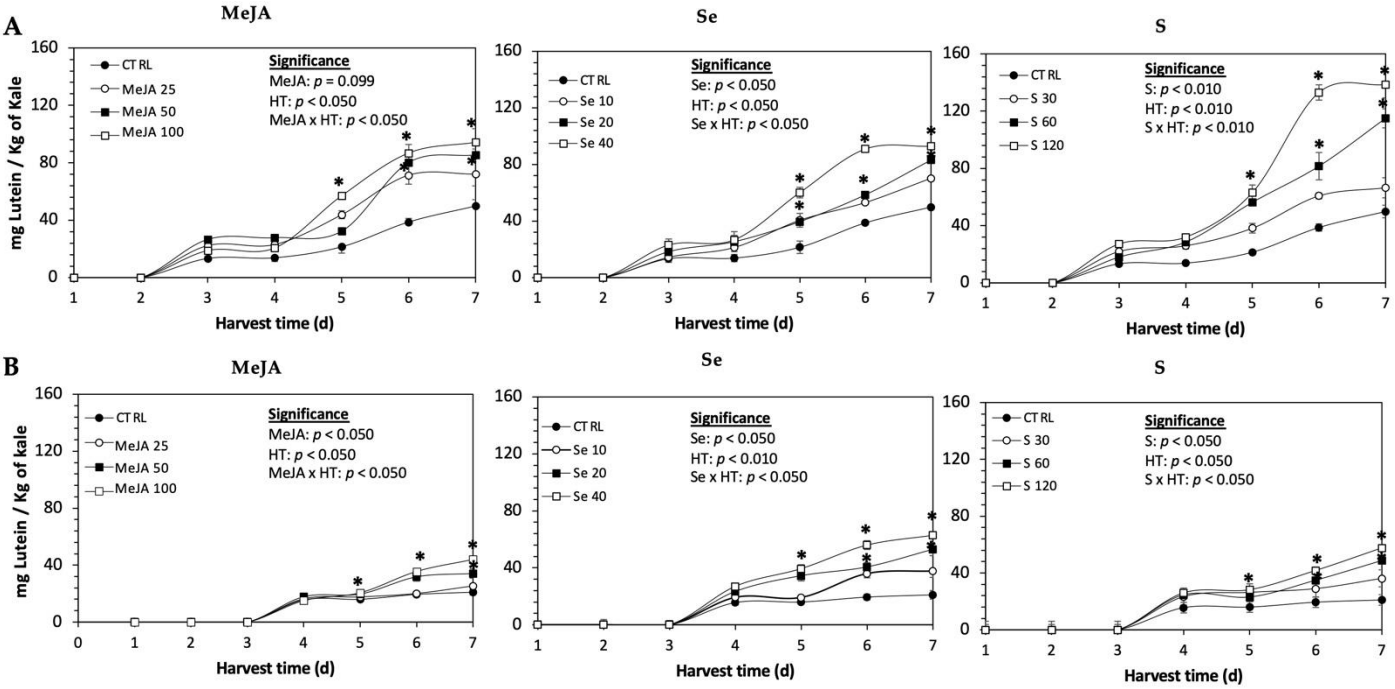


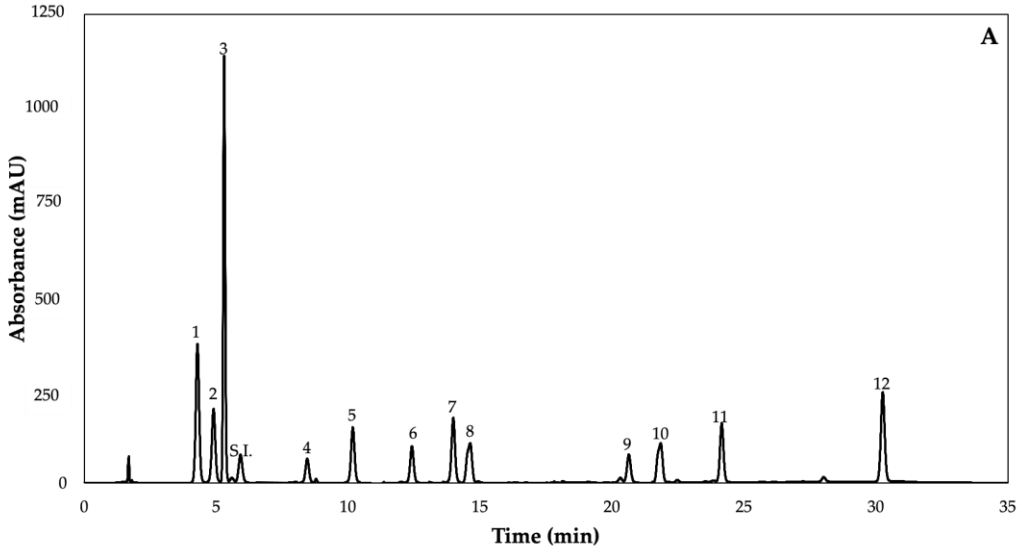
Figure 10. Concentration of lutein in untreated (A) Red Russian and (B) Dwarf Green kale sprouts and treated with methyl jasmonate, sulfur, and selenium during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se), and harvest time (HT). Results are expressed in dry weight basis.

3.3.2. Glucosinolates

Glucosinolates are sulfur- and nitrogen-containing thioglucosides biosynthesized from amino acids and glucose (Ortega-Hernández et al., 2021). The glucosinolate profile of kale sprouts is shown in **Figure 11.** Twelve glucosinolates were identified in kale sprouts in both control and treated samples. The glucosinolate profile included six aliphatic glucosinolates: glucoerucin, glucoraphanin, progoitrin, gluconapin, glucoiberin and glucobrassicanapin; one aromatic glucosinolate: gluconasturtiin; and five indolic glucosinolates: glucobrassicin, 1-hydroxy-3-indoylmethyl, neoglucobrassicin, 4-hydroxy-glucobrassicin and 4-methoxy-glucobrassicin. The glucosinolate profile of kale sprouts agrees with previous reports (Hahn et al., 2016; Kim et al., 2017). However, the concentration of 9 out of the 12 individual glucosinolates identified varied with the effect of the applied treatment and with harvest time (**Figures 12-17**).

The application of S MeJA 100 µM, Se 40 mg/L, and S 120 mg/L induced an immediate significant increase in the content of glucoiberin (52.9%, 120.3%, and 29.4%) and progoitrin (160%, 72.1%, and 28.5%), respectively, in Red Russian kale sprouts (**Figures 12-14**). Concentrations of glucoiberin and progoitrin in the treated Dwarf Green kale variety remained unaltered compared with control sprouts (**Figures 15-17**).

The glucosinolate 4-hydroxybrassicin reached its maximum accumulation around day 3–4 of germination in Red Russian and Dwarf green kale sprouts treated with S 120 mg/L (3200% and 3900%), Se 40 mg/L (6000% and 4200%), and MeJA 25 µM (7500% and 3950%), respectively, and its concentration began to decrease in the following days. Later, at 4–5 d of germination, a similar trend was observed in the concentration of the indolyl glucosinolate neoglucobrassicin with S, Se and MeJA treatments. There was a significant increase in neoglucobrassicin concentration by S (635.7% and 335.2%), Se (922.2% and 670.5%), and MeJA (777.7% and 352.6%) in Red Russian and Dwarf green kale sprouts, respectively, followed by a significant decrease. Likewise, the overproduction and subsequent decline of aliphatic gluconapin was also observed in the treated Red Russian kale variety at 4–5 d of germination, whereas in Dwarf green kale, it increased relatively constantly in all treated samples until the end of germination.



B

Peak number[a] (retention time)	λmax[b] (nm)	Tentative identification	Identification method[c]
1 (5.3)	222	Glucoiberin	a,b
2 (5.8)	224	Progoitrin	a,b
3 (6.3)	222	Glucoraphanin	a,b,c
4 (8.2)	226	1-hydroxy-3-indoylmethyl	a,b
5 (10.8)	225	Gluconapin	a,b
6 (12.5)	219	Gluconasturtiin	a,b
7 (13.6)	221, 226	4-hydroxy-glucobrassicin	a,b
8 (14.9)	243	Glucobrassicanapin	a,b
9 (20.6)	210	Glucoerucin	a,b
10 (22.3)	220, 280	Glucobrassicin	a,b
11 (24.3)	220, 268	4-methoxy-glucobrassicin	a,b
12 (30.3)	222, 290	Neoglucobrassicin	a,b

Figure 11. (A) HPLC-DAD chromatogram, shown at 227 nm of identified glucosinolates from ethanol/water (70:30*, v/v*) extracts of 7-day-old control (water) kale sprouts. (B) Tentative identification of individual glucosinolates in kale sprouts was obtained by HPLC-DAD. [a] Number of peak assigned according to the order of elution from the C_{18} reverse phase. [b] Wavelengths of maximum absorption in the UV/Vis spectra of each chromatographic peak. [c] Methods considered for identification of each chromatographic peak: (a) Identification by comparison of UV–Visible spectra and wavelengths of maximum absorbance reported in prior literature; (b) Identification by comparison with the order of chromatographic elution reported by previous authors (Jahns & Holzwarth, 2012; M. Lefsrud et al., 2007; Luo et al., 2020; Moreira-Rodríguez et al., 2017a; Pospíšil, 2016;

Sirhindi et al., 2020), (c) Identification by comparison with the retention time and UV–Visible spectra of authentical standard.

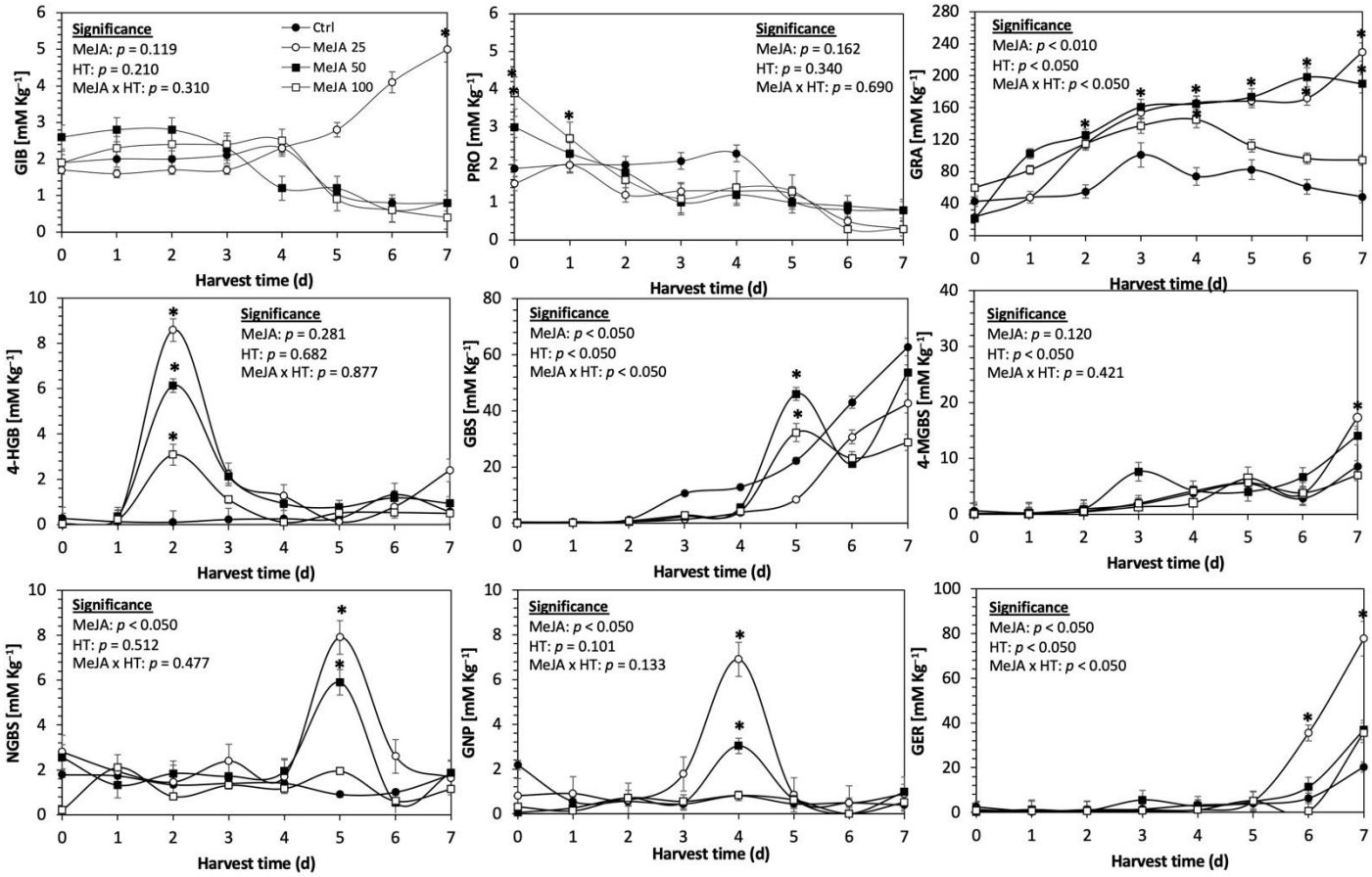


Figure 12. Concentration of individual glucosinolates in untreated Red Russian kale sprouts and treated with methyl jasmonate during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

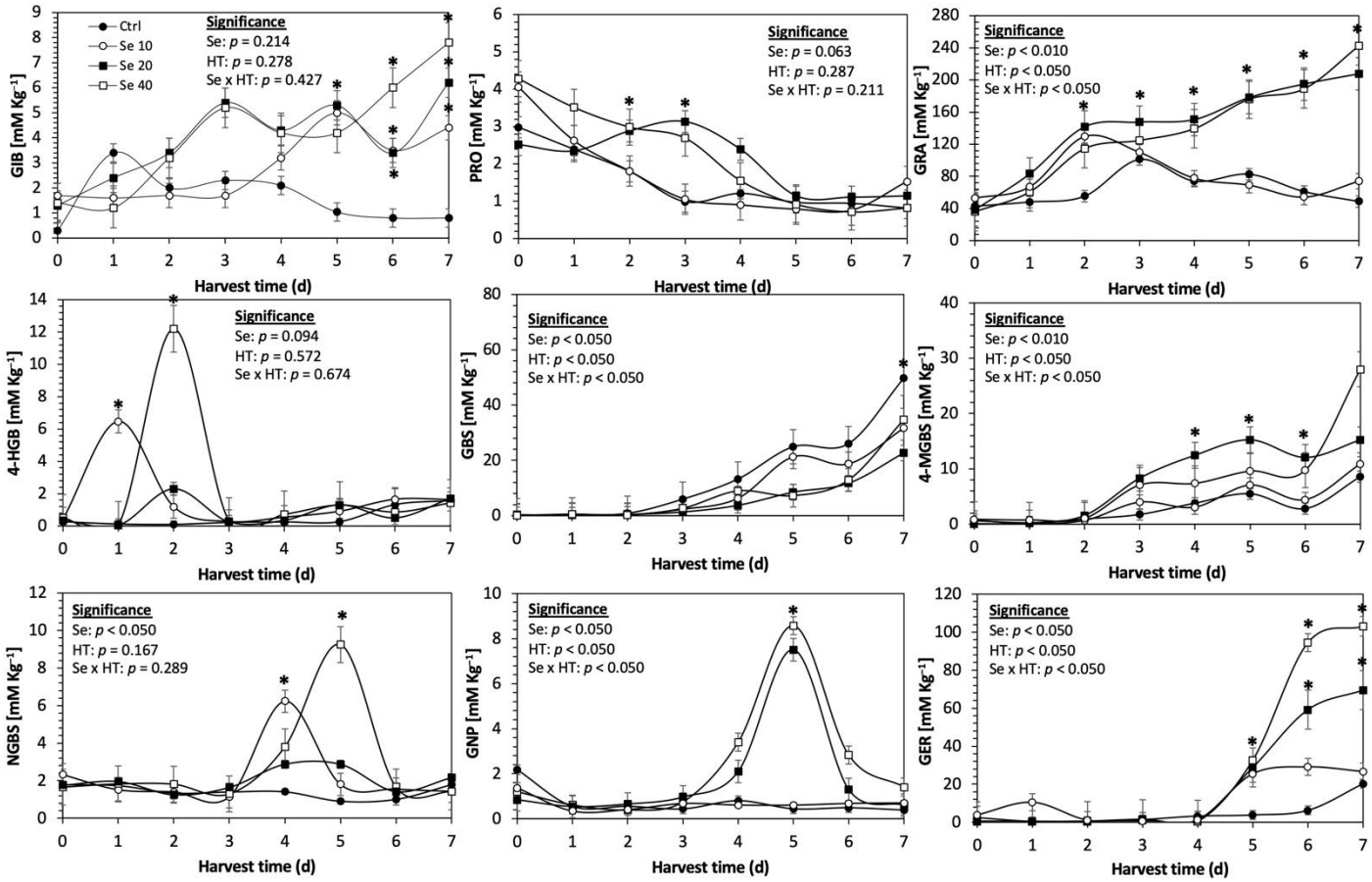


Figure 13. Concentration of individual glucosinolates in untreated Red Russian kale sprouts treated with selenium during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test (p < 0.050) between the control and treated samples. Abbreviations: selenium (Se), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

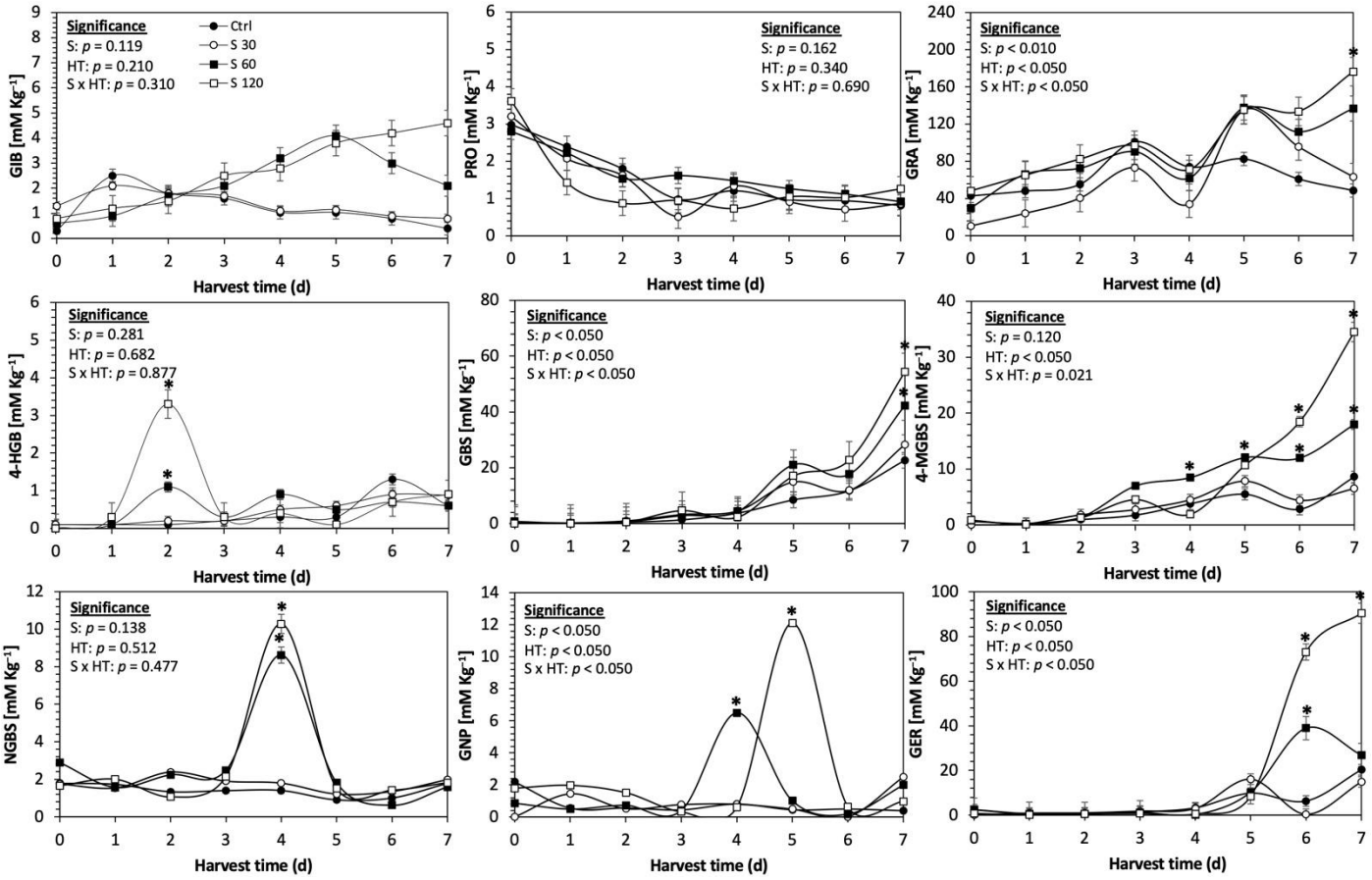


Figure 14. Concentration of individual glucosinolates in untreated Red Russian kale sprouts and treated with sulfur during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: sulfur (S), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

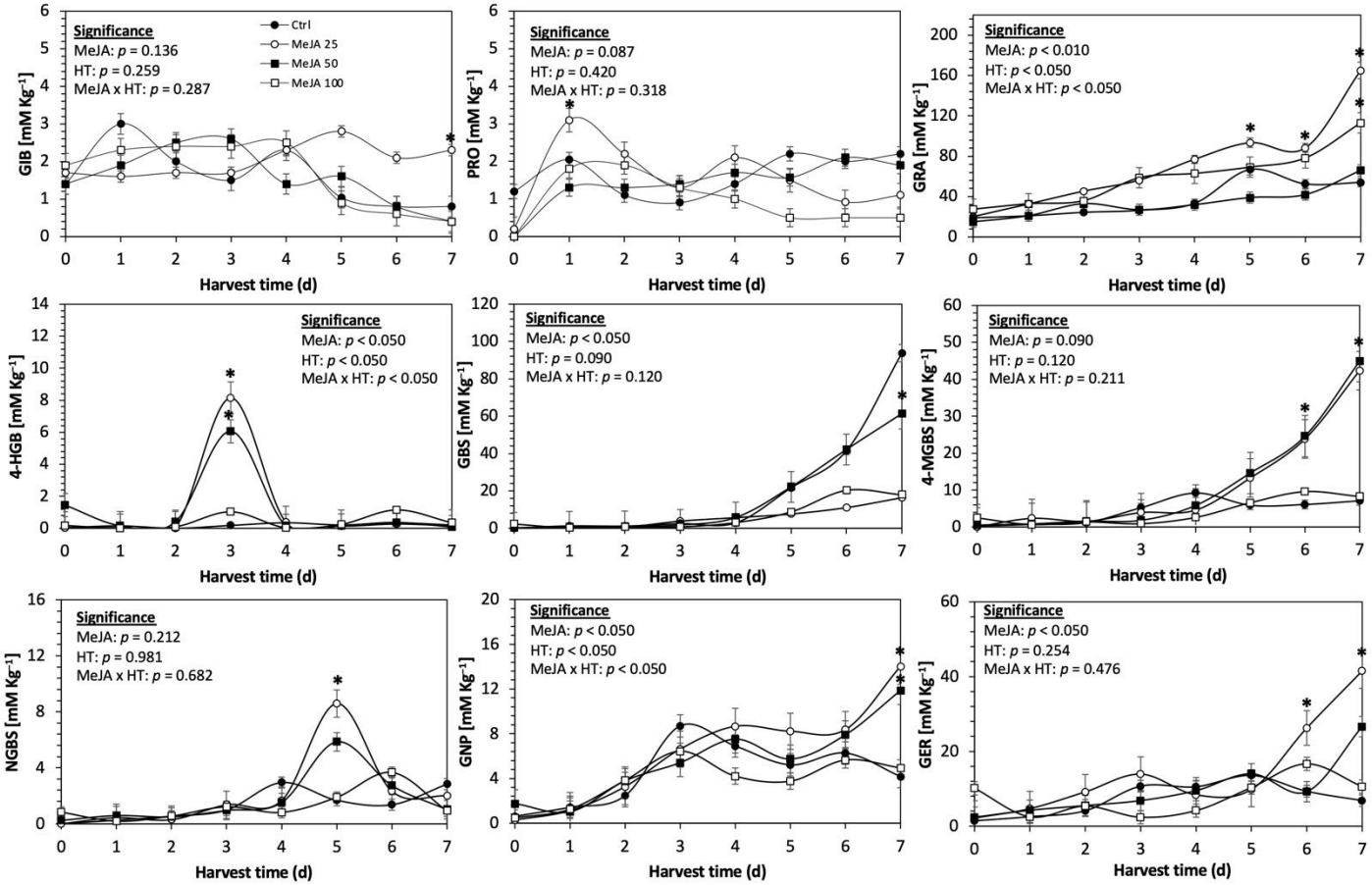


Figure 15. Concentration of individual glucosinolates in untreated Dwarf Green kale sprouts and treated with methyl jasmonate during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

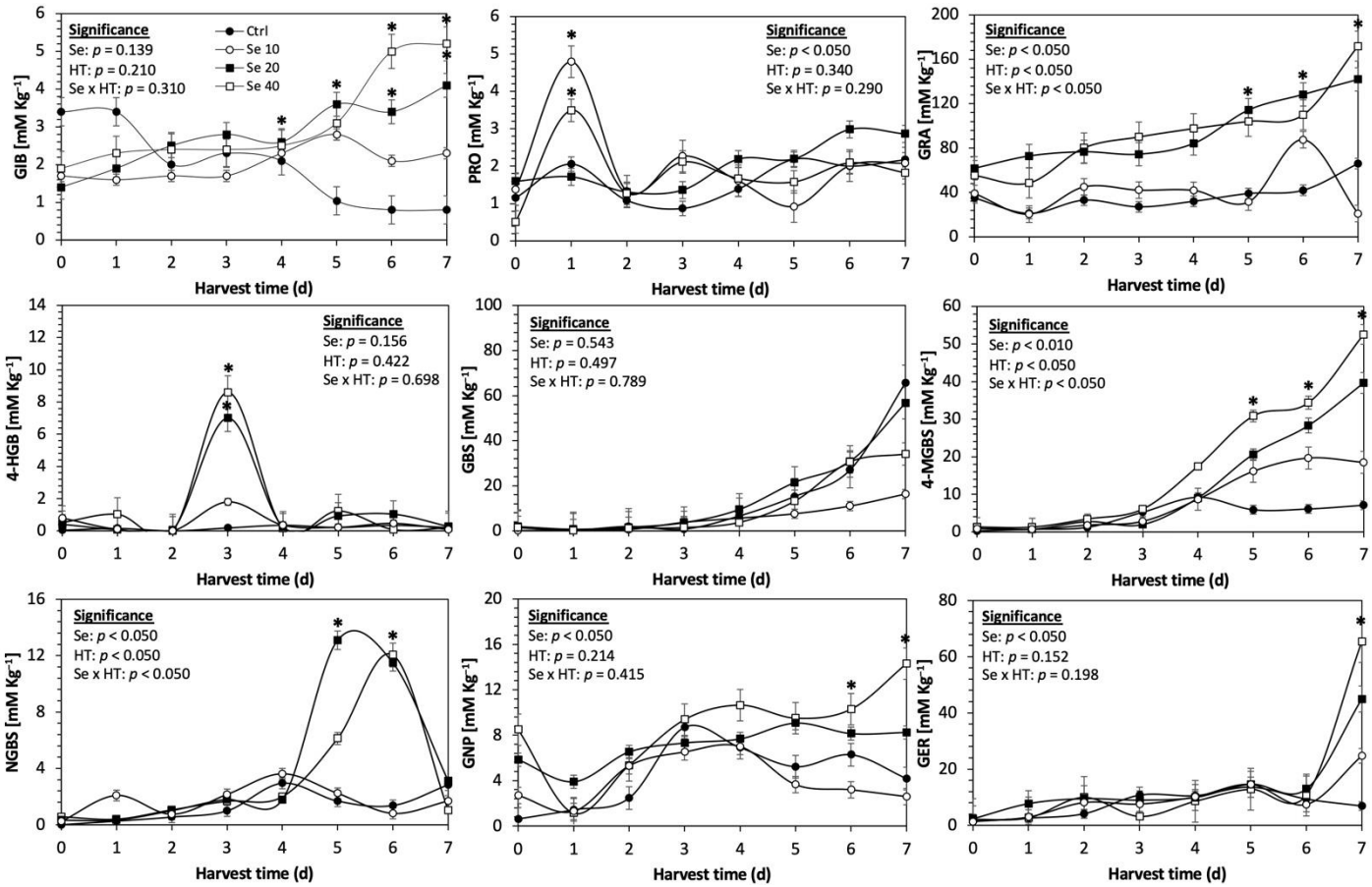


Figure 16. Concentration of individual glucosinolates in untreated Dwarf Green kale sprouts and treated with selenium during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: selenium (Se), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

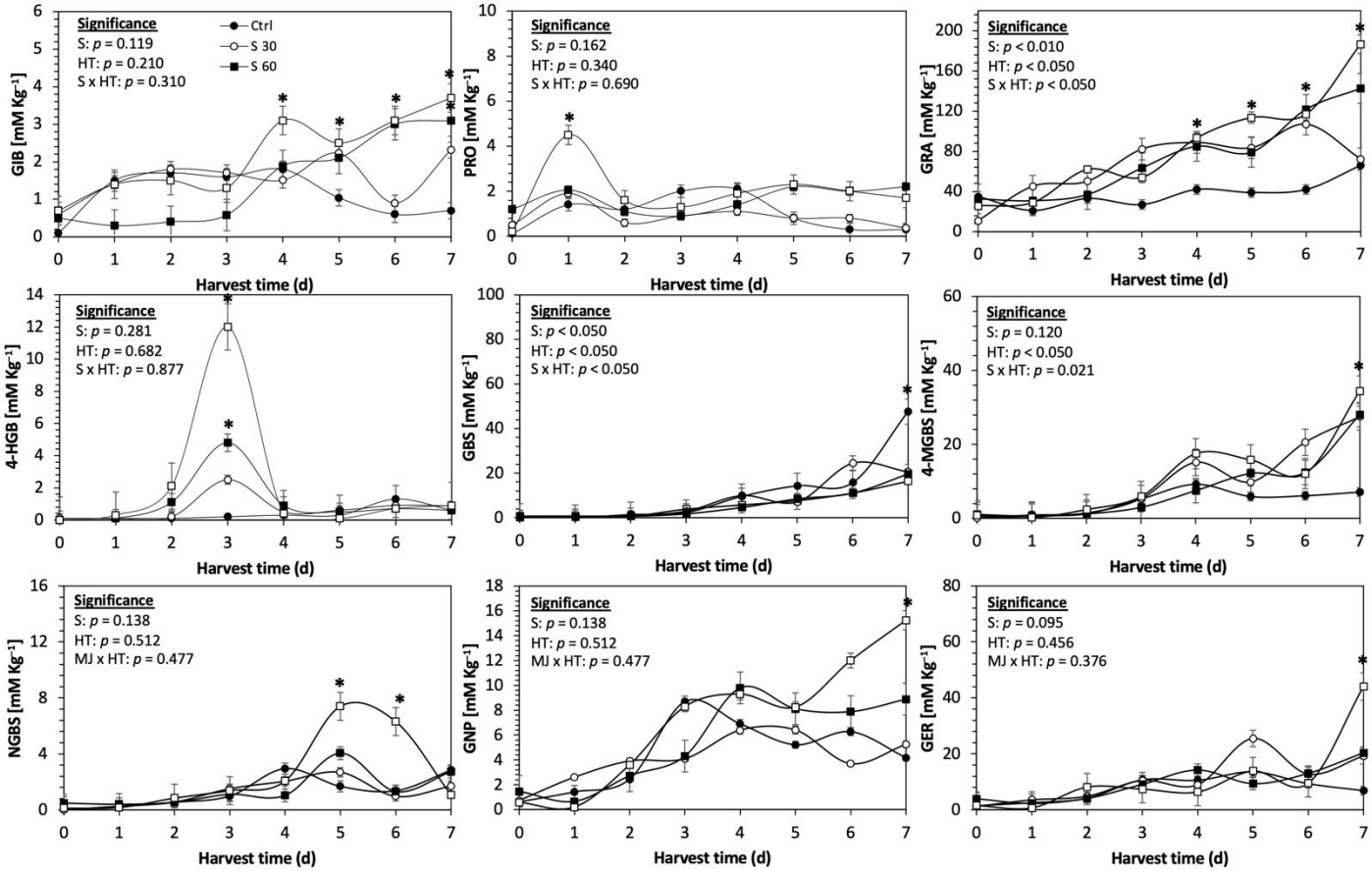
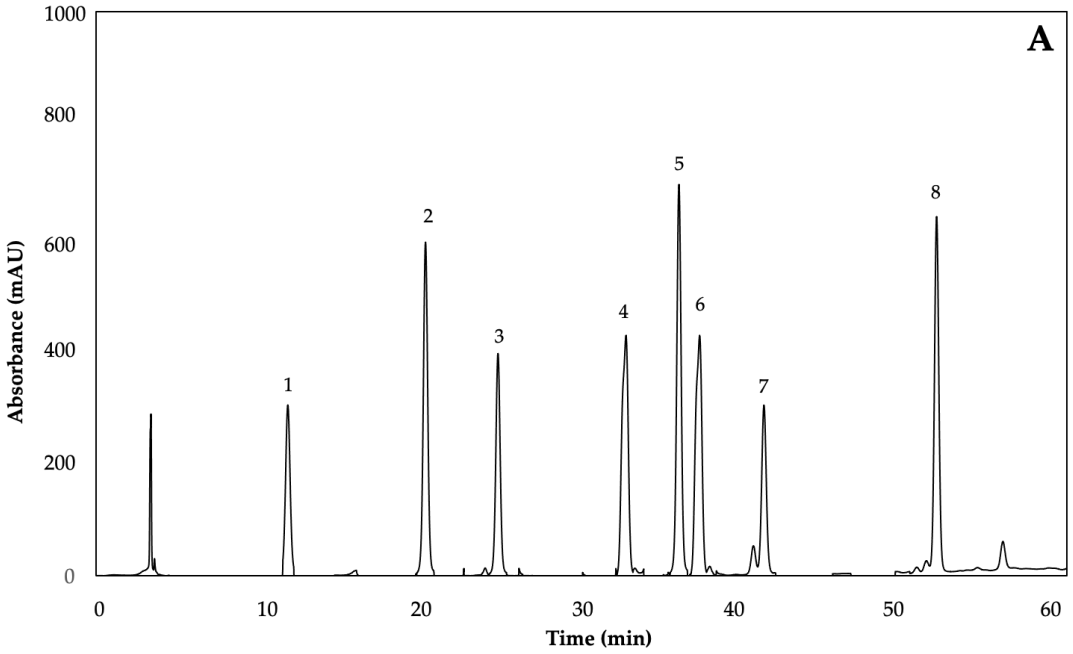


Figure 17. Concentration of individual glucosinolates in untreated Dwarf Green kale sprouts and treated with sulfur during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test (p < 0.050) between the control and treated samples. Abbreviations: sulfur (S), harvest time (HT), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). Results are expressed as desulfogucoraphanin equivalents in dry weight basis.

In general, the maximum accumulation of glucosinolates was observed in Red Russian kale sprouts harvested at 7 d of germination. The four main glucosinolates overproduced by S 120 mg/L, Se 40 mg/L and MeJA 25 µM stresses were the aliphatic glucosinolates glucoiberin (1150%, 978% and 900%), glucoeurocin (510.8%, 407.8% and 282.2%), and glucoraphanin (262.4%, 397.9% and 371.5%), as well as the indolyl glucosinolate methoxy-glucobrassicin (430.7%, 225.6% and 147.1%) in the Red Russian variety, respectively.

3.3.3. Phenolic Compounds

Phenolics are the most abundant secondary metabolites in plants, with a structure that possesses an aromatic ring with at least one hydroxyl substituent (Ortega-Hernández et al., 2021). The phenolic content of kale sprouts treated with MeJA, Se, and S was also investigated. Eight major phenolic compounds were identified in both control and treated kale sprouts from day 5–7 (**Figure 18**) including 4-O-caffeoylquinic acid (4-O-CQA); 3-O-hexoside kaempferol (3-O-H-K); sinapic acid; ferulic acid; kaempferol 3-O-sinapoyl-sophoroside 7-O-glucoside (K-3-O-S-so-7-O-g); 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG); 1,2-disinapoyl-2-ferulolylgentiobiose (1,2- diS-2-FG); and quercetin.



B

Peak number[a] (retention time)	λmax[b] (nm)	Tentative identification	Identification method[c]
1 (12.1)	(218), 326	*4-O*-caffeoylquinic acid	a,b,c
2 (20.6)	222, 265, 330	3-O-hexoside kaempferol	a,b
3 (25.1)	235, 324	Sinapic acid	a,b,c
4 (32.1)	324	Ferulic acid	a,b,c
5 (36.2)	(238), 270, 330	Kaempferol 3-O-sinapoyl-sophoroside7-O-glucoside	a,b
6 (38.9)	(240), 330	1-sinapoyl-2′-ferulolylgentiobiose	a,b
7 (42.9)	(240), 331	1,2-disinapoyl-2-ferulolylgentiobiose	a,b
8 (50.3)	269, 330	Quercetin	a,b,c

Figure 18. (A) HPLC-DAD chromatogram, shown at 320 nm of identified phenolics from ethanol/water (70:30, v/v) extracts of 7-day-old control (water) kale sprouts. (B) Tentative identification of individual phenolics in kale sprouts was obtained by HPLC-DAD. [a] Number of peak assigned according to the order of elution from the C_{18} reverse phase. [b] Wavelengths of maximum absorption in the UV/Vis spectra of each chromatographic peak. [c] Methods considered for identification of each chromatographic peak: (a) Identification by comparison of UV–Visible spectra and wavelengths of maximum absorbance reported in prior literature (M. Lefsrud et al., 2007; Moreira-Rodríguez, Nair, Benavides, Cisneros-Zevallos, & Jacobo-Velázquez, 2017b; Ortega-Hernández et al., 2021), (b) Identification by comparison with the order of chromatographic elution reported by previous authors; (c) Identification by comparison with the retention time and UV–Visible spectra of authentical standard.

Changes in total and individual phenolic content can be observed in **Tables 6-7**. Most of the phenolic compounds were not detected before day five. Regarding the phenolic content, neither S nor Se caused a significant increase compared to control sprouts; however, all three MeJA (25 µM (−41.3% and −36.3%), 50 µM (−32.3% and −39.4%), and 100 µM (−27.4% and −19.4%)) treatments caused a significant decrease in phenolic concentration for Red Russian (**Table 6**) and Dwarf Green (**Table 7**) varieties, respectively, at 7 d of germination. The individual phenolics causing a decrease in overall concentration were sinapic acid; ferulic acid; 4-O-CQA; 1-S-2-FG; and 1,2-diS-2-FG, in both varieties. K-3-O-s-so7-O-g (135.2% and 76.3%) and quercetin (152% and

Table 6. Concentration of individual and total phenolic compounds in Red Russian kale sprouts treated with MeJA, Se, and S after 7 days of germination.

	Phenolic Compound Concentration (mg/100 g DW) [1,2,3]								
Treatment	**4-O-CQA**	**3-O-H-K**	**Sinapic acid**	**Ferulic acid**	**1-S-2-FG**	**1,2-diS-2-FG**	**K-3-O-s-so7-O-g**	**Quercetin**	**Total**
CTRL	47.69 ± 0.65 a	80.12 ± 2.77 a	68.27 ± 1.12 a	21.59 ± 0.96 a	4.00 ± 0.84 a	29.37 ± 1.26 a	27.10 ± 1.04 c	12.71 ± 0.66 bc	290.84 ± 9.31 a
MeJA 25 µM	18.68 ± 0.97 c	17.52 ± 5.61 d	18.48 ± 2.82 e	9.53 ± 0.26 c	1.42 ± 0.58 c	10.29 ± 2.28 b	63.67 ± 1.85 a	32.02 ± 1.48 a	171.59 ± 15.85 e
MeJA 50 µM	29.88 ± 0.85 bc	72.24 ± 4.61 a	26.76 ± 2.25 d	6.20 ± 0.90 c	0.76 ± 0.10 d	15.37 ± 2.29 b	34.81 ± 0.36 b	11.47 ± 0.40 bc	197.77 ± 11.77 d
MeJA 100 µM	33.01 ± 0.96 b	62.99 ± 5.44 b	39.23 ± 3.47 c	6.09 ± 0.74 c	1.51 ± 0.64 c	12.04 ± 0.85 b	31.19 ± 1.51 b	22.95 ± 2.80 b	212.31 ± 16.40 d
Se 10 mg/L	36.43 ± 1.09 b	61.74 ± 0.62 b	24.95 ± 1.38 d	17.15 ± 0.97 ab	3.84 ± 0.13 a	24.18 ± 0.90 a	17.65 ± 1.67 d	14.93 ± 0.77 b	200.87 ± 7.53 d
Se 20 mg/L	39.61 ± 2.13 b	66.13 ± 4.13 b	27.22 ± 2.73 c	18.25 ± 0.93 ab	3.62 ± 0.13 a	24.36 ± 0.50 a	26.03 ± 1.69 c	23.75 ± 1.57 b	228.97 ± 13.81 d
Se 40 mg/L	33.69 ± 2.69 b	49.65 ± 2.23 c	32.60 ± 1.56 c	23.92 ± 1.40 a	3.98 ± 0.29 a	25.61 ± 1.85 a	19.35 ± 0.92 d	26.86 ± 0.57 b	215.67 ± 11.51 d
S 30 mg/L	46.69 ± 1.32 a	71.41 ± 3.68 ab	51.36 ± 6.27 b	16.15 ± 0.87 ab	3.20 ± 0.05 a	26.00 ± 0.44 a	21.78 ± 1.57 d	13.54 ± 0.12 bc	250.12 ± 14.33 c
S 60 mg/L	41.33 ± 3.7 a	86.14 ± 4.13 a	73.22 ± 3.28 a	19.25 ± 0.86 a	2.95 ± 0.85 ab	27.13 ± 0.60 a	26.69 ± 1.28 b	19.76 ± 1.62 b	296.46 ± 16.32 a
S 120 mg/L	32.83 ± 6.68 b	79.36 ± 2.23 a	58.6 ± 5.27 b	17.92 ± 1.69 a	3.23 ± 0.51 a	26.63 ± 1.01 a	39.73 ± 0.06 b	21.08 ± 0.49 b	279.39 ± 17.94 b

[1] Concentrations are reported as each individual standard. Compounds were quantified at 320 nm: sinapic acid; ferulic acid; 4-O-caffeoylquinic acid (4-O-CQA); 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG); and 1,2-disinapoyl-2-ferulolylgentiobiose (1,2- diS-2-FG); 360 nm: kaempferol 3-*O*-sophoroside-7O-glucoside (K-3-O-s-so7-O-g), 3-O-hexoside kaempferol (3-O-H-K), and quercetin. [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Concentrations of K-3-O-s-so7-O-g and 3-O-H-K are expressed as kaempferol equivalents; concentrations of 1-S-2-FG and 1,2-diS-2-FG are expressed as ferulic acid equivalents. Authentic standards were used to determine the concentrations of 4-O-CQA, sinapic acid, ferulic acid, and quercetin.

Table 7. Concentration of individual and total phenolic compounds in Dwarf Green kale sprouts treated with MeJA, Se, and S after 7 days of germination.

	Phenolic compound concentration (mg/100 g DW) [1,2,3]																					
Treatment	**4-O-CQA**		**3-O-H-K**		**Sinapic acid**		**Ferulic acid**		**1-S-2-FG**		**1,2-diS- 2-FG**		**K-3-O-s-so7-O-g**		**Quercetin**		**Total**	
CTRL	43.34 ± 2.90	a	91.18 ± 4.16	a	84.68 ± 4.02	a	28.04 ± 1.11	a	6.32 ± 0.42	a	3.51 ± 2.95	ab	32.86 ± 2.36	cd	23.97 ± 0.99	c	313.91 ± 18.89	a
MeJA 25 µM	17.16 ± 0.97	d	32.52 ± 3.03	d	28.48 ± 2.22	e	13.96 ± 0.26	c	2.42 ± 0.83	c	0.93 ± 2.28	c	57.92 ± 1.85	a	46.10 ± 1.72	a	199.96 ± 13.16	d
MeJA 50 µM	25.88 ± 0.85	c	40.45 ± 7.61	c	36.27 ± 2.11	e	12.20 ± 0.48	c	2.52 ± 0.77	c	0.37 ± 0.97	c	41.98 ± 0.10	b	31.11 ± 0.31	b	190.23 ± 13.2	d
MeJA 100 µM	29.32 ± 0.49	c	67.23 ± 3.35	b	46.97 ± 4.22	d	11.97 ± 1.94	c	2.35 ± 0.83	c	1.30 ± 0.56	c	41.19 ± 1.23	b	26.95 ± 1.61	bc	227.90 ± 14.22	c
Se 10 mg/L	39.98 ± 1.24	a	72.10 ± 6.68	b	62.34 ± 3.27	c	34.28 ± 0.97	a	6.71 ± 0.05	a	4.76 ± 0.39	a	16.88 ± 1.57	d	27.00 ± 0.01	bc	264.03 ± 14.18	b
Se 20 mg/L	31.13 ± 2.70	b	74.35 ± 7.30	b	72.04 ± 3.28	b	28.50 ± 0.93	a	5.58 ± 0.82	b	3.13 ± 0.03	ab	28.87 ± 1.03	cd	30.75 ± 1.62	b	274.35 ± 17.72	b
Se 40 mg/L	38.32 ± 2.07	ab	94.36 ± 5.33	a	69.11 ± 4.33	c	23.89 ± 1.40	ab	4.56 ± 0.61	b	4.86 ± 0.50	a	24.32 ± 0.21	d	22.86 ± 0.90	c	282.28 ± 15.34	ab
S 30 mg/L	40.98 ± 1.37	a	99.99 ± 7.60	a	73.74 ± 5.96	a	27.83 ± 0.87	a	7.76 ± 0.40	a	4.56 ± 0.85	a	19.32 ± 1.92	d	18.93 ± 0.98	c	293.11 ± 19.95	a
S 60 mg/L	33.65 ± 2.21	b	75.70 ± 7.33	b	88.00 ± 4.88	a	32.82 ± 0.86	a	8.13 ± 0.62	a	3.30 ± 0.10	ab	34.97 ± 1.73	c	21.75 ± 1.79	c	298.32 ± 19.53	a
S 120 mg/L	39.89 ± 2.47	a	92.99 ± 9.96	a	74.11 ± 7.44	a	24.95 ± 1.69	ab	9.84 ± 0.78	a	4.60 ± 1.51	b	35.63 ± 0.84	c	27.58 ± 0.98	bc	309.59 ± 25.68	a

[1] Concentrations are reported as each individual standard. Compounds were quantified at 320 nm: sinapic acid and ferulic acid, 4-O-caffeoylquinic acid (4-O-CQA), 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG) and 1,2-disinapoyl-2-ferulolylgentiobiose (1,2-diS-2-FG); 360 nm: kaempferol 3-*O*-sophoroside-7O-glucoside (K-3-O-s-so7-O-g), 3-O-hexoside kaempferol (3-O-H-K) and quercetin. [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$).

92.3%) increased significantly in 7-day old Red Russian and Dwarf Green kale sprouts, compared with controls, respectively, with the application of MeJA 25 µM.

3.3.4. Antioxidant Activity

The antioxidant activity of all the extracts was determined in terms of the proportion (%) of DPPH scavenged, and values are shown in **Figure 19**. The application of Se, S or MeJA treatments did not induce an immediate increment in radical scavenging activity in Red Russian and Dwarf Green kale sprouts. After 4 d of germination, significant increments in the DPPH activity of Red Russian and Dwarf Green kale sprouts treated with Se 40 mg/mL (244.3% and 136.2%), S 120 mg/L (100.1% and 102.4%) and MeJA 25 µM (135.8% and 89.4%) were detected, respectively. Moreover, the highest stress-induced antioxidant activity occurred in the Red Russian kale sprouts treated with S 120 mg/L (296.1%) at 7 d, followed by MeJA 25 µM (214.8%) and Se 40 mg/L (93.9%). The maximum percentage of scavenging capacity achieved was 83.5%, 94.3%, and 66.3% in the Red Russian kale sprouts treated with S 120 mg/L, Se 40 mg/L and MeJA 25 µM, respectively, at 7 d.

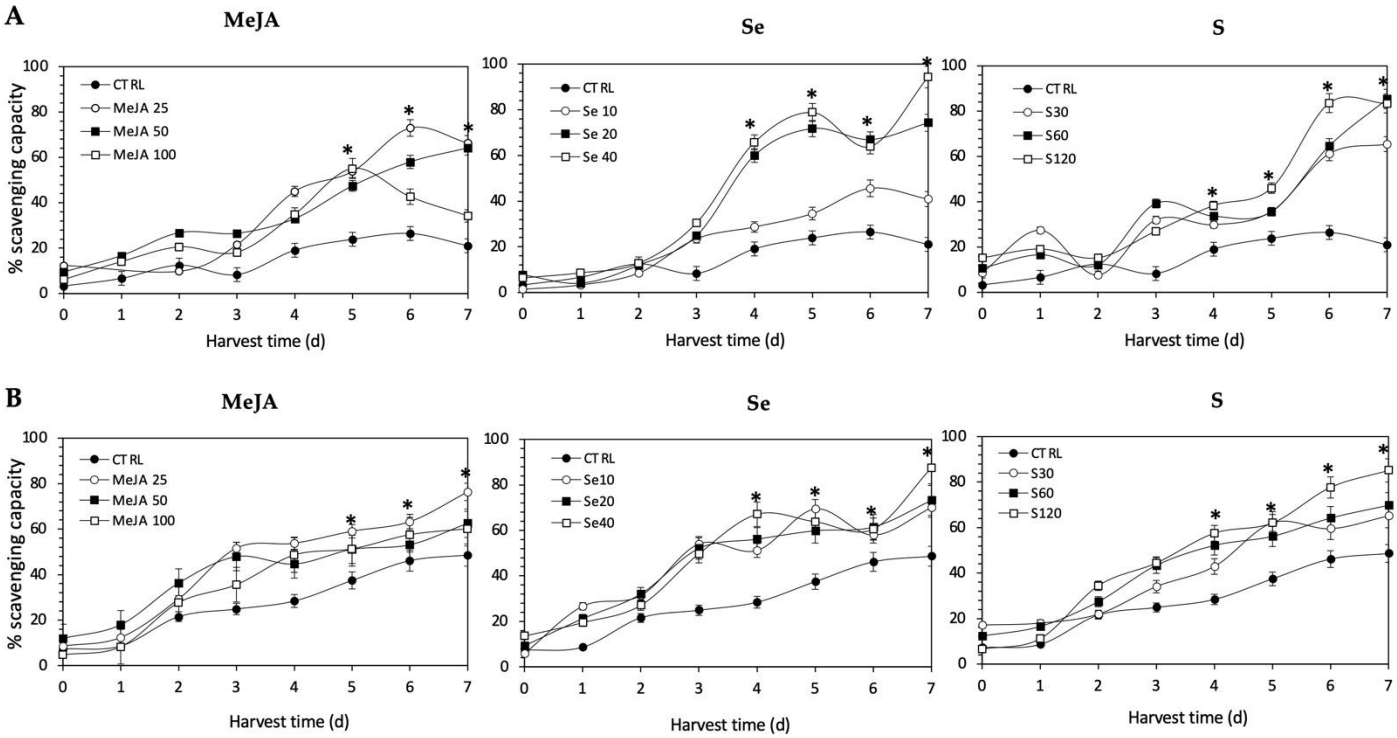


Figure 19. 1, 1-diphenyl-2-picrylhydrazyl scavenging activity of ethanolic extract of (A) Red Russian and (B) Dwarf Green kale sprouts treated with (1) methyl jasmonate, (2) selenium, and (3) sulfur during 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se), harvest time (HT).

Regarding the Fe^{2+}-chelating activity (**Figure 20**), the application of Se, S or MeJA treatments did not induce an immediate increment in kale sprouts. After 5 d of germination, S 120 mg/L (161.9% and 157.8%), Se 40 mg/L (115.7% and 164.7%), and MeJA 25 µM (45.4% and 103.5%) induced a significant increment in Fe^{2+}-chelating activity in Red Russian and Dwarf Green kale sprouts, respectively. Fe^{2+}-chelating activity increased relatively constantly in all treated samples until the end of germination. Furthermore, the highest increment in chelating activity was observed at 7 d in the Red Russian kale sprouts treated with S 120 mg/L (128.5%), followed by Se 40 mg/L (118.3%) and MeJA 25 µM (77.2%), reaching a percentage of inhibition between 70.8% and 91.3%.

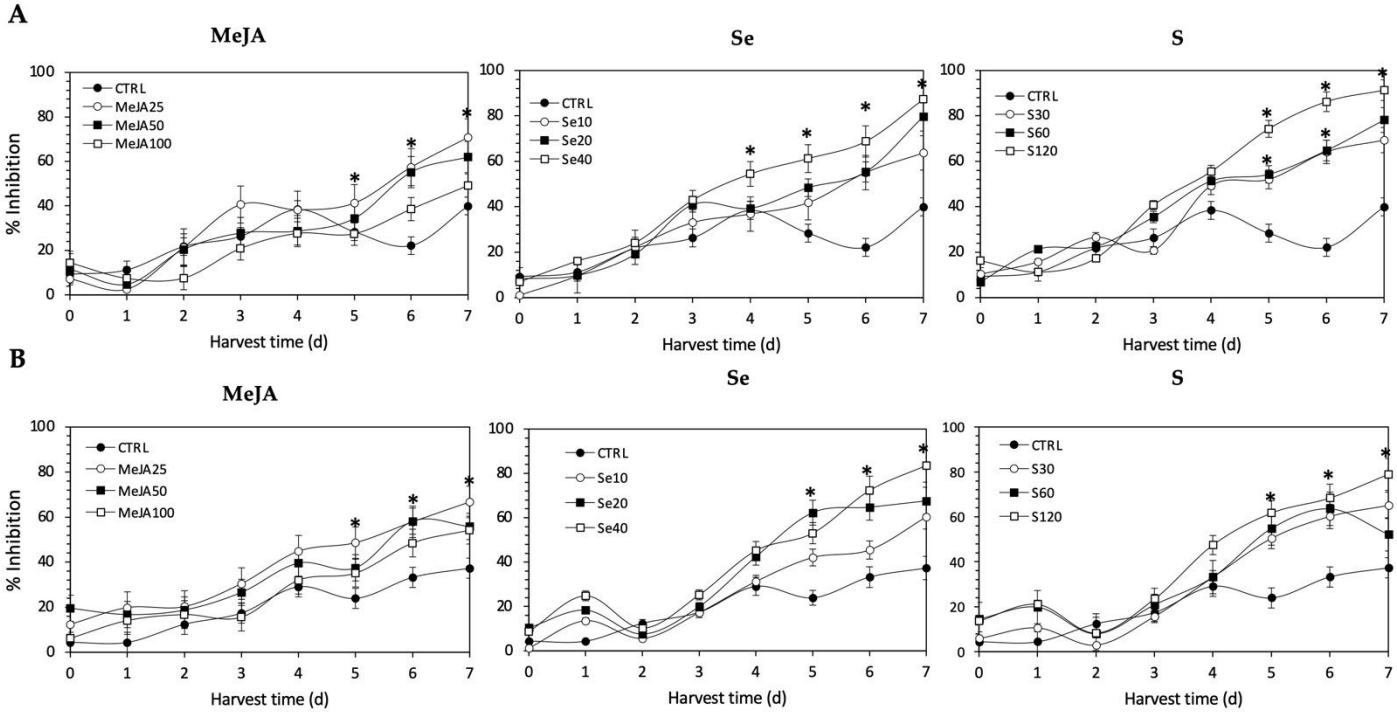


Figure 20. Iron chelating activity of ethanolic extract of (A) Red Russian and (B) Dwarf Green kale sprouts treated with (1) methyl jasmonate, (2) selenium, and (3) sulfur during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se), harvest time (HT).

As observed for radical scavenging and Fe^{2+}-chelating activities, the application of Se, S or MeJA treatments did not induce an immediate increment of nitric oxide scavenging activity in kale sprouts (**Figure 21**). After 4 d of germination, S 120 mg/L (117.1% and 49.7%), Se 40 mg/L (101.6% and 55.28%), and MeJA 25 µM (65.4% and 15.9%) induced significant increments in nitric oxide scavenging activity in Red Russian and Dwarf Green kale sprouts, respectively. Moreover, the highest increment in nitric oxide scavenging activity was observed in the Red Russian kale sprouts treated with S 120 mg/L (109.4%) at 7 d, followed by Se 40 mg/L (106.6%) and MeJA 25 µM (76.3%).

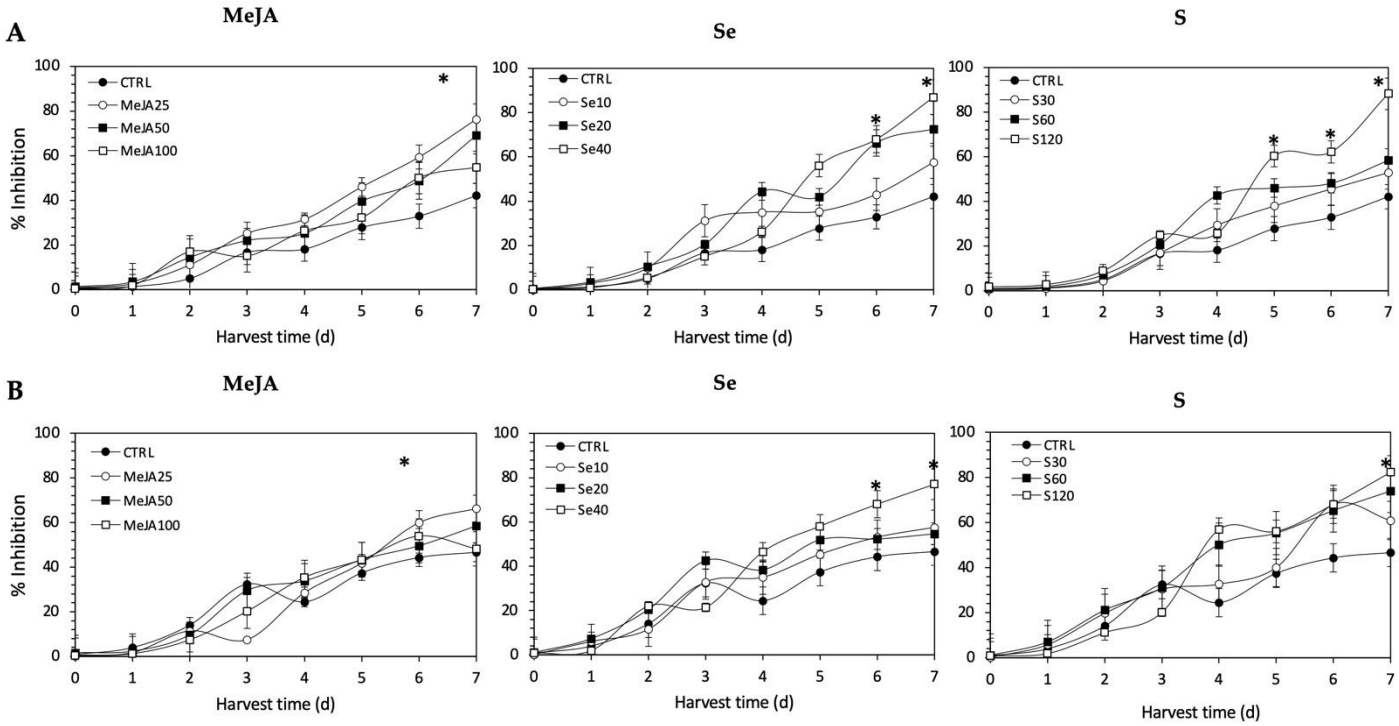


Figure 21. Nitric oxide scavenging activity of ethanolic extract of (A) Red Russian and (**B**) Dwarf Green kale sprouts treated with (1) methyl jasmonate, (2) selenium, and (3) sulfur during the first 7 days of germination. Bars are means of 3 replicates ± standard error. Data points with an asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between the control and treated samples. Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se), harvest time (HT).

3.4. Discussion

3.4.1. Lutein

The performance of S, Se, and MeJA as abiotic stressors for phytochemical accumulation during the germination of kale was tested in the present study. It was demonstrated that lutein was accumulated in an effective manner with all the evaluated stressors. These results are not consistent with previous reports in kale treated with S or Se, where no differences in carotenoids were found (Kim et al., 2018; Kopsell et al., 2003; Lefsrud, Kopsell, Kopsell, & Randle, 2006). In previous research, the carotenoid content in kale leaf (Winterbor, Redbor, and Toscano) showed no response to increasing S levels when supplied at concentrations of 4, 8, 16, 32, and 64 mg of S/L in media (Kopsell et al., 2003). In a similar study, kale leaf lutein accumulation did not respond to treatments with 0–3.5 mg of Se/L (Lefsrud et al., 2006). However, these differences could be due to a difference in cultivars used as well as in the growing conditions and growth stage/age of the plant itself.

Moreover, Sams et al. (2011) demonstrated that Se application in *Arabidopsis* can differentially trigger the expression of phytoene synthase (PSY), a major enzyme involved

in the biosynthesis of carotenoids. Additionally, it is well known that lutein plays a central role in the deactivation of $^1O_2$ through complexes of photosystem I and photosystem II (Jahns & Holzwarth, 2012; Pospíšil, 2016). Therefore, in the present study, the accumulation of lutein in kale sprouts was probably stimulated to keep ROS levels low and to prevent lipid peroxidation in the thylakoid membrane produced under high levels of Se or S. It is worth mentioning that the current study represents the first attempt to measure the influence of Se on the accumulation of lutein in kale sprouts. Data obtained herein demonstrate that neither S nor Se negatively affected the growth of kale plants within the ranges provided in this study (**Figure S1** and **S2** in **Appendix A**).

Regarding MeJA results, a further increase in lutein content was observed in MeJA 100 µM treatment. Previous studies have reported that exogenous treatment with MeJA has a dose effect on the biosynthesis of carotenoids in maize (Luo et al., 2020), different varieties of broccoli (Sirhindi et al., 2020), moringa (Saini, Harish Prashanth, Shetty, & Giridhar, 2014), tomatoes (Saniewski & Czapski, 1983), and apples (Pérez, Sanz, Richardson, & Olías, 1993). MeJA has been related to the photosynthetic efficiency and expression of some genes of photosystem II in different cultivars of *Brassica oleracea* L. (Sirhindi et al., 2020), suggesting that exogenous application of MeJA before germination could influence the assembly, stability, and repair of PS II machinery through the accumulation of carotenoids and sugars. The direct role of MeJA in the enhancement of lutein may be due to the fact that carotenoids are needed to quench several types of pro-oxidants formed continuously during photo-oxidation (Divya, Puthusseri, & Neelwarne, 2014).

On the other hand, there are no previous reports that MeJA application induces lutein accumulation in kale sprouts. MeJA has been used as a stressor in kale close to commercial maturity in higher concentrations (100 µM to 250 µM) with a focus on the induction of other phytochemicals (Ku & Juvik, 2013; Sun et al., 2012; Yi et al., 2016). Since MeJA plays an important role in plant growth and development (Luo et al., 2020; Sirhindi et al., 2020), differences in the results reported herein and previous reports could be attributed to plant variety and maturity stage.

3.4.2. Glucosinolates

The results revealed that the addition of various concentrations of S, Se, and MeJA solution exhibited comparative differences in the glucosinolate contents among the Red Russian and Dwarf Green kale sprouts. The aliphatic glucosinolates were predominant, representing 76.1–87.2% of the total glucosinolate content.

The present investigation showed that S 120 mg/L was an adequate concentration for inducing the synthesis of glucosinolates in kale. Increments induced by S on total glucosinolate content were mainly attributed to accumulation of the aliphatic glucosinolate glucoraphanin, whereas indolyl 4-methoxyglucobrassicin and glucobrassicin contributed a mean less than 20% of the total glucosinolates. These findings are in agreement with previous reports that indicate that S (2 mM) treatment under greenhouse conditions significantly increased aliphatic glucosinolates and total glucosinolates in ten-week-old kale by 67% and 35%, respectively (Park et al., 2018). Similarly, the application of 32 and

64 mg of S/L has been shown to be effective for inducing the accumulation of glucoiberin, glucobrassicin, neoglucobrassicin, and 4-hydroxygluco-brassicin content in kale leaves (Kopsell et al., 2003). It is proposed that the reason why aliphatic glucosinolates have shown a differential increase in S-treated kale sprouts is that aliphatic glucosinolates are derived from sulfur-containing amino acid methionine, instead of indolic glucosinolates which are synthesized from tryptophan (Falk, Tokuhisa, & Gershenzon, 2007).

Moreover, the production of the aliphatic glucosinolates in this study appears to follow patterns based on the R-group and regulation of specific regions of the plant chromosome responsible for the modification of their R-group side chains. The R-groups of glucoiberin and glucoraphanin are both derived from methionine and retain the S atom from methionine. In addition, both are controlled by the same genetic alleles at the Gsl-oxid locus of the plant's chromosome (Kliebenstein et al., 2001). The R-groups of gluconapin and progoitrin are also derived from methionine. However, these glucosinolates do not retain the S from methionine, and their final side-chain modification is controlled by alleles at the Gsl-alk and Gsl-oh loci of the plant's chromosome (Kliebenstein et al., 2001). On the other hand, the R-group of glucobrassicin, 4-hydroxy-glucobrassicin, and neoglucobrassicin is tryptophan, rather than methionine (Kliebenstein et al., 2001). This difference in R-group amino acid precursor may help to explain the differences in production of glucobrassicin and neoglucobrassicin, relative to the other glucosinolates analyzed.

Given that Se and S share the same transporters, and the application of Se can influence the expression of genes coding for these proteins, Se uptake was expected to influence glucosinolate concentrations (Hsu et al., 2011; McKenzie et al., 2017). These results agree with Kim et al. (2018), who reported that Se (2 mg/L) treatment induces the accumulation of glucosinolates in 6-week-old kale.

Although the effect of MeJA on glucosinolate accumulation during kale germination has not been characterized, treatment with MeJA 250 µM has been demonstrated to be successful in inducing the accumulation of total glucosinolates in broccoli sprouts (Pérez-Balibrea, Moreno, & García-Viguera, 2011). In support of this argument, treatment with MeJA may stimulate the expression of genes associated with the biosynthesis of glucosinolates (Mikkelsen et al., 2003; Ana M. Torres-Contreras et al., 2021).

Compared to S and Se supplementation, a smaller increase in glucosinolate content was observed with MeJA treatment. However, a similar trend was observed in the proportion of the indolyl and aliphatic glucosinolates, with glucoiberin, glucoeurocin, glucoraphanin, glucobrassicin, and methoxy-glucobrassicin being overproduced. These results are in agreement with Moreira-Rodríguez et al. (2017) and Mikkelsen et al. (2003), who suggest that MeJA treatment induces the flux of carbon from indolic glucosinolates to aliphatic glucosinolates in the biosynthesis pathway. Moreira-Rodríguez et al. (2017) and Wisner et al. (2013) reported a significant increase in glucoraphanin in sprouts of broccoli and pak choi treated with MeJA 25µM and 200µM, respectively. An induction of transcription factors (i.e., *MYB51*, *MYB34*, and *OBP2*) involved in the biosynthesis of indolic glucosinolates has been reported in the presence of MeJA treatment (Skirycz et al., 2006).

Regarding indolic synthesis, it has been reported that glucobrassicin is synthesized by enzymes sulfotransferases 16 and 18, and then it is converted into 1-hydroxy-3-indoylmethyl or 4-hydroxybrassicin by hydroxylation reactions by the enzymes CYP81F1–3 and CYP81F4, respectively. Then, these glucosinolates are methylated to neoglucobrassicin and 4-methoxyglucobrassicin by 2-O-methyltransferases, respectively (Wiesner et al., 2013). Therefore, the observations presented here suggest that the application of MeJA treatment stimulates the methylation of glucobrassicin and that such an effect involves the formation of highly unstable intermediates, i.e., neoglucobrassicin and 4-methoxyglucobrassicin.

3.4.3. Phenolic Compounds

The evaluation of Se and S on phenolic acids in kale sprouts showed that it may not be profitable or even possible to enhance the content of multiple phytochemicals of a single plant. Since Se and S follow similar metabolic routes, the opposing response between glucosinolates and phenolics may be related to interference in carbon assimilation pathways, or alternatively favoring one pathway over the other. In agreement with our results, Finley et al. (2005) have reported inhibition of phenolic acids in broccoli in the presence of Se.

Furthermore, Moreira-Rodríguez (2017) and Villareal-García (2016) have also reported a decrease in the accumulation of specific phenolic compounds due to MeJA-spraying in broccoli including 1-S-2-FG, 1,2,2-triSG, 1,2-diS-2-FG, and 4-O-CQA. Previous works have shown that MeJA and JA treatments may prevent lignification in a concentration-dependent manner (Denness et al., 2011) and may downregulate genes involved in the biosynthesis of phenolics and lignification (PAL, 4-coumarate-CoA ligase (4CL), and caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) (Jacobo-Velázquez et al., 2015). Additionally, a large increase in kaempferol and quercetin compounds with MeJA 25 µM suggests a redirection of the carbon source towards the biosynthesis of flavonoids.

Although glucosinolates and phenylpropanoids are synthesized through distinct biosynthetic pathways, it has been confirmed that indole glucosinolate biosynthesis limits phenylpropanoid accumulation in *Arabidopsis* due to an overproduction of the substrate indole-3-acetaldoxime (Kim, Dolan, Anderson, & Chapple, 2015; Moreira-Rodríguez et al., 2017a). This is an intermediate in tryptophan-derived glucosinolate biosynthesis which limits phenylpropanoid accumulation mainly through decreased PAL activity and substrate availability (J. I. Kim et al., 2015).

3.4.4. Antioxidant Activity

Due to the complex nature of phytochemicals and their mechanisms of action, no single testing method can provide a full representation of the antioxidant profile. The antioxidant capacities of the treated and control ethanolic kale extracts were determined by measuring free radical scavenging activity, chelating activity, and nitric oxide assays.

All the antioxidant activity determination methods used in this study showed the same trends, with the content of glucosinolates and lutein differentially stimulated by the different treatments. The antioxidant activities showed an increasing trend in all methods

as the concentrations of the extracts increased. The highest scavenging capacity was recorded from Se 40 mg/L-treated kale sprouts after 7 d of germination (94.3%), followed by S 120 mg/L (83.4%) and MeJA 25 μM (66.3%). Likewise, the highest percentage of the inhibition of formation of the Ferrozine-Fe^{2+} complex, around 91.3%, was found with S 120 mg/L-treated Red Russian kale sprouts at 7 d of germination, followed by Se 40 mg/L (87.3%) and MeJA 25 mg/L (70.8%). On the other hand, the extracts from treated kale were checked for their inhibitory effects on nitric oxide release from sodium nitroprusside. The highest percentage of inhibition, 88.2%, was found with S 120 mg/L-treated Red Russian kale sprouts at 7 d of germination, followed by Se 40 mg/L (87.0%) and MeJA 25 μM (76.3%).

The bioactivity of glucosinolates and their hydrolysis products have been reported in the Brassicaceae family (Baek et al., 2021; Barillari et al., 2008; Endo, Usuki, & Kaneda, 1985; Lanfer-Marquez, Barros, & Sinnecker, 2005). Although the direct antioxidant action of glucosinolates is controversial, due to the fact that their antioxidant activity is mainly associated with transcription triggering of phase II metabolic enzymes (Sánchez-Pujante, Borja-Martínez, Pedreño, & Almagro, 2017), the direct antioxidant potential of glucoerucin in arugula extract (*Eruca sativa*) (Barillari et al., 2005), glucobrassicin in cauliflower (*Brassica oleracea* L.) (Cabello-Hurtado, Gicquel, & Esnault, 2012), and glucoraphasatin in radish (*Raphanus sativus* L.) (Papi et al., 2008) has been demonstrated. Some authors have proposed that the antioxidant capacities of glucosinolates is quite specific and has a structural explanation. The sulfur atom in the methyl thiol group present in the side chain of some glucosinolates can act as an electron donor, switching from a reduced form (the sulphide group) to an oxidized form (the sulphinyl group), generating redox pairs (Barillari et al., 2005; Papi et al., 2008). Moreover, the indole glucosinolates, such as glucobrassicin, probably act as proton donors, whereas gluconasturtiin possesses metal-ion-chelating activity (Natella, Maldini, Leoni, & Scaccini, 2014). In addition, it is important to consider that the *in vivo* antioxidative potential of glucosinolates is greatly increased by myrosinase-mediated breakdown to highly active derivatives.

Likewise, the results obtained from antioxidant activity assays of ethanolic kale extracts correlate with the response of lutein content in the samples. It is known that lutein is a pigment which plays a major role in the protection of plants against photooxidative processes (Stahl & Sies, 2003). Like other carotenoids, lutein has the ability to quench singlet oxygen and scavenge toxic free radicals through its double carbon–carbon bonds and cyclic substituents (Merhan, 2017). In agreement with these results, the influence of lutein in the antioxidant activity of kale has also been previously reported by Ligor et al., (Ligor, Trziszka, & Buszewski, 2013) and Sikora & Bodziarczyk (Sikora & Bodziarczyk, 2012).

Although there was no significant increase in the content of most phenolic compounds (except for kaempferol and quercetin) related to the different treatments applied, their intrinsic levels in kale may possibly contribute to antioxidant activity, likely through an interaction (antagonistic, additive or synergistic) with the other bioactive molecules. It has been reported that the antioxidant capacity of phenolic compounds is mainly attributed to their methoxy, hydroxyl, and carboxylic acid groups (Jinxiang Chen et al., 2020), which can play an important role in neutralizing free radicals, quenching singlet and triplet

oxygen, or decomposing peroxides (Pereira, Valentão, Pereira, & Andrade, 2009). Furthermore, the antioxidant properties of polyphenol extracts in several Brassica plants such as broccoli (Naguib et al., 2012), kale (Sikora & Bodziarczyk, 2012), and *B. incana* (Natalizia Miceli et al., 2020) have been confirmed. Thus, the overall antioxidant activity can be the result of the combination of all three induced phytochemicals, where the induced contents of glucosinolates >> phenolics > lutein.

3.5. Conclusions

The recent identification of kale sprouts as a rich source of phytochemicals with anti-inflammatory, anti-cancer, and antioxidant attributes has directed scientific efforts towards improving its content. Results obtained herein demonstrate that MeJA, Se, and S treatments can be used as simple steps during germination to enhance levels of specific secondary plant metabolites including glucosinolates and lutein.

Dwarf Green kale treated with Se 40 and harvested after 7 d resulted in the highest increase of lutein, followed by Red Russian treated with S 120 and harvested after 7 d.

In addition, S 120 could be used as an effective treatment for inducing the accumulation of total and individual glucosinolates during the first 7 d of germination of kale sprouts. Treatments led to the accumulation of aliphatic and indolyl glucosinolates in both varieties; however, S and Se favored mainly the accumulation of GRA, GER, 4-MGBS, and GIB, whereas the latter favored overproduction of 4-MGBS, GIB, and GBS.

Data herein presented suggest that the phytochemicals accumulated as a stress response are supported mainly in a complex carbon flux model acting on the biosynthesis pathway of specific glucosinolates. It is important to study the effects of these stresses on other varieties of kale to study their behavior due to differences in phytochemical profiles and required growing conditions.

The stressed kale sprouts with enhanced levels of glucosinolates and lutein could be used as foods or as raw materials to produce nutraceutical foods, dietary supplements, or cosmeceuticals. Moreover, stressed kale sprouts could be subjected to downstream processing to extract and purify the phytochemicals accumulated, which can be further commercialized as high-value, health-promoting biomolecules. In order to commercialize kale sprouts treated with MeJA, Se, and S as ready-to-eat food with enhanced levels of glucosinolates and lutein, further experiments should be performed to determine shelf-life stability and to validate the bioactivity of the enhanced phytochemicals. Moreover, further experiments should also determine the accumulation of Se and S in the sprouts to determine their levels and ensure their safety before commercialization.

CHAPTER 4. BIOACCESSIBILITY AND POTENTIAL BIOLOGICAL ACTIVITIES OF LUTEIN, GLUCOSINOLATES AND PHENOLIC COMPOUNDS IN KALE

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4.1. Introduction

Increasing the consumption of fruit and vegetables is a key to reducing the risk of cardiovascular disease, stroke, arthritis, inflammatory bowel diseases, and some types of cancers (Aune et al., 2017; Wang et al., 2014). The benefits of a diet based on vegetables have been associated with their numerous secondary metabolites or phytochemicals content.

Brassica vegetables, such as kale, cabbage, broccoli, and cauliflower, constitute a popular food crop of nutritional and economic significance worldwide (Fahey, Zalcmann, & Talalay, 2001). Kale (*Brassica oleracea* var. acephala), known as a superfood, contains a high level of dietary fiber, vitamins, carotenoids, phenolic compounds, and glucosinolates.

Even though kale sprouts contain nutraceutical compounds in significantly greater quantities in comparison with mature kale leaves (Fahey et al., 2001), most of the population in emerging countries do not consume the recommended target of vegetables per day (Perez-Moral et al., 2018). In this context, the application of abiotic stresses on kale sprouts may be an attractive strategy to increase their nutraceutical content; in this way, the consumer could ingest adequate amounts of these functional compounds without the need for high food intakes. Recently, Kim et al., 2018; Kopsell et al., 2003; Ku & Juvik, 2013; Ortega-Hernández, Antunes-Ricardo, Cisneros-Zevallos, & Jacobo-Velázquez, 2022; Park et al., 2018; Sun, Yan, Zhang, & Wang, 2012; Yi et al., 2016 reported the application of Se, S and exogenous phytohormones to increase the levels of glucosinolates, carotenoids and phenolic compounds in kale sprouts.

The premise that plants growing under field conditions are exposed to a wide range of different abiotic stresses that occur sequentially or simultaneously has broadened the field of research towards the combination of common stresses to achieve a better elicitation of phytochemicals (Mittler and Blumwald 2010, Suzuki et al. 2014). However, the combination of different abiotic stresses may act synergistically or antagonistically in terms of impact on plant growth, biosynthesis and accumulation of secondary metabolites (Prasad, et al., 2005). For instance, Kim et al., (2018) demonstrated that when NaCl is combined with Na_2SeO_3 in 6-week-old kale, concentration of isothiocyanates increase 30% after 14 days. Yet, the combined effect of selenium, sulfur and methyl jasmonate on accumulation of phytochemicals in kale has not been studied.

Since phenolic compounds and glucosinolates are believed to be the main responsible of the biological effects attributed to the Brassica vegetables, abiotic stress seems to be a good strategy to increase the antioxidant and anti-inflammatory properties of kale (López-Chillón et al., 2018). However, it would be important to consider the effects of the digestion process on the bioactivity of phytochemicals in kale sprouts. For instance, conditions such as pH, light, temperature, and presence of oxygen can produce many changes in the chemical structure of bioactive compounds during gastrointestinal digestion, decreasing their absorption by epithelial cells and producing the loss of their potential health benefits (Gómez-Maqueo, Antunes-Ricardo, Welti-Chanes, & Cano, 2020).

The aim of this study was to characterize the digestive stability of the individual bioactive compounds of 7-day-old kale sprouts treated alone or in combination with Se, S and MeJA during germination through an *in vitro* simulated gastrointestinal digestion.

4.2. Materials and Methods

4.2.1. Chemicals

Methyl jasmonate (MeJA), sulfatase (from Helix pomatia), diethylaminoethyl (DEAE)-sephadex A-25, sinigrin hydrate, sodium acetate, acetonitrile (HPLC grade), methanol (HPLC grade), methyl tert-butyl ether (MTBE; HPLC grade), phosphoric acid, porcine pancreatin (Cat. No. P1750), porcine pepsin (Cat. No. P7000), porcine pancreatic lipase (Cat. No. L3126), α-amylase from porcine pancreas (Cat. No. A3176), bile salts (Cat. No. B8631), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), dichloro-dihydro-fluorescein diacetate (DCFH-DA), lipopolysaccharide (LPS) (L4391), 4-O-caffeoylquinic acid, ferulic acid, sinapic acid, quercetin, kaempferol and lutein standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco Modified Eagle Medium, fetal bovine serum and Penicillin-Streptomycin antibiotic (Pen-Strep) were acquired from GIBCO (Carlsbad, CA, USA). Griess Reagent System and CellTiter 96 AQueous One Solution Cell Proliferation Assay kits were acquired from Promega (G2930, Madison, WI, USA). Ethanol (HPLC grade), sodium selenite (Se; Na_2SeO_3), potassium sulfate (S; K_2SO_4), sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), urea, calcium chloride dihydrate ($CaCl2*2H_2O_2$), ammonium chloride (NH_4Cl), dibasic sodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), and magnesium chloride ($MgCl_2$) were obtained from Desarrollo de Especialidades Quimicas, S.A. de C.V. (Monterrey, NL, Mexico). Desulfoglucoraphanin was acquired from Santa Cruz Biotechnology (Dallas, TX, USA).

4.2.2. Plant Material

Red Russian kale (*Brassica oleracea* var. acephala) seeds were provided by La Semilleria (Queretaro, Qro, Mexico). Kale seeds were sanitized for 15 min in sodium hypochlorite (1.5%, v/v) and rinsed with Milli-Q water.

4.2.3. MeJA, Se, and S Treatments

Kale seeds were soaked in Milli-Q water (control) or the treatment solutions for 5 h. The treatment solutions used were Se (40 mg/L), S (120 mg/L), MeJA (25 μM), and their

corresponding interactions (Se×S; Se×MeJA; S×MeJA; Se×Se×MeJA). Afterwards, the soaking solutions were discarded, and the seeds were placed on germination trays in a dark chamber set at 25 °C and 85% of relative humidity for 7 days. Three replicates of each treatment were run concurrently. Each replica consisted of a tray containing 30 g of kale seeds sprayed with 5 mL of different treatment concentrations or control every 12 h throughout the experiment. Samples were collected after 7 days of germination, freeze-dried (Labconco, Kansas City, MO, USA), ground into a powder, and stored at −80 °C until further analysis of phytochemicals.

4.2.4. Phytochemical Analyses

4.2.4.1. Extraction of Secondary Metabolites

Extraction of glucosinolates, phenolic compounds and lutein from kale sprouts powder was carried out according to the protocol described by Moreira-Rodríguez et al. (2017). Briefly, 10 mL of ethanol/water (70:30,v/v), preheated to 70 °C for 10 min, were added to 0.2 g of kale samples and 50µL of sinigrin (3 mM) as internal standard (I.S.) were added.

Samples were heated at 70 °C in a bath water (VWR, Radnor, PA, USA) and manually stirred every 5 min for 30 min to inactivate myrosinase. Then, the extracts were left to cool at 25 °C and centrifuged (18,000× g, 10 min, 4 °C) (SL16R, Thermo Scientific, GER) to recover the supernatant. The supernatant obtained was used for the identification and quantification of individual carotenoids and phenolic compounds by direct injection on HPLC-DAD (1260 Infinity, Agilent Technology, Santa Clara, CA, USA). A purification step was carried out before quantification of glucosinolates. .

4.2.4.2. Identification and Quantification of Lutein

The identification and quantification of lutein were performed as described by Moreira-Rodríguez et al. (2017). A HPLC-DAD system (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) and YMC C30 column (4.6 mm × 150 mm, 3 µm particle size) (YMC, Wilmington, NC, USA) were used for lutein detection. The mobile phase program was (A) 50% methanol, (B) 45% MTBE, and (C) 5% water with a flow rate of 0.5 mL/min. Lutein was detected using a 450 nm wavelength and identified by comparing its retention time and absorption spectra with a reference standard. Lutein was quantified using a calibration curve of lutein standard (0–12 ppm) and expressed as mg of lutein per 100 g of kale sprouts in dry weight (DW) basis.

4.2.4.3. Identification and Quantification of Phenolic Compounds

Phenolic compounds in kale extract were quantified using an HPLC system (Agilent Technologies 1260 series, Santa Clara, CA, USA) coupled with a diode array detector (DAD) working at 280, 320, and 360 nm, and a C18 reverse phase column, 4.6 mm × 250 mm, 5 µm (Luna, Phenomenex, Torrance, CA, USA). The mobile phases were: (A) water adjusted to pH 2.4 with phosphoric acid and (B) methanol-water (60:40, v/v). The gradient used was 0/0, 3/30, 8/50, 35/70, 40/80, 45/100, 50/100 and 60/0 (min/% phase B). The solvent flow rate was 0.8 mL/min. The injection volume was 10 µL. The analysis was

performed at and integrated by the OpenLAB CDS ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

Peak identification was based on retention time, UV-visible spectra and wavelengths of maximum absorption, as compared with the reported literature (Aguilar-Camacho et al., 2019; Moreira-Rodríguez et al., 2017a) and commercial standards. For the quantification of phenolic compounds, standard curves of 4-O-caffeoylquinic acid (0.1–50), ferulic acid (0.4–40 ppm), sinapic acid (0.1–50 ppm), quercetin (0.1–12 ppm), and kaempferol (0.1–12 ppm) were prepared. Results were expressed as mg of each individual phenolic compound per 100 g of kale sprouts (mg/ 100 g) in dry weight (DW) basis.

4.2.4.4. Desulfation of Glucosinolates

Columns were prepared with 0.5 mL of hydrated DEAE-sephadex A-25 and washed with 0.5 mL of water. Then, columns were loaded immediately with 3 mL of kale supernatant. After removing excess supernatant by elution, the columns were washed twice with 0.5 mL of water, followed by 1 mL of sodium acetate (0.02 M) and 75 µL of purified sulfatase Columns were incubated at 25 °C for 12 h before elution of desulfoglucosinolates with two 0.5 mL + one 0.25 mL volumes of water.

4.2.4.5. Identification and Quantification of Glucosinolates

The desulfoglucosinolate content of each sample (20 µL) was analyzed according to the method of Moreira-Rodríguez et al. (2017). Evaluation was performed on a HPLC system with a photodiode array detector (DAD) set at 227 nm and a C18 reverse phase column, 4.6 mm × 250 mm, 5 µm (Luna, Phenomenex, Torrance, CA). Desulfoglucosinolates were eluted with at a flow rate of 1.5 mL/min. Separation was achieved with an initial *A* (water) concentration of 100%, adjusting the *B* (acetonitrile) concentration to 20% at 28 min and to 0% at 35 min.

Glucosinolate were identified by their retention time, UV-visible spectra and wavelengths of maximum absorption as compared with the reported literature (Aguilar-Camacho et al., 2019; Moreira-Rodríguez et al., 2017) and commercial standards. For the quantification of glucosinolates, a standard curve of desulfoglucoraphanin (0–1250 ppm) was prepared. Results were expressed as mmol of desulfoglucoraphanin equivalents per 100 g of kale sprouts (mM/ 100 g) (DW).

4.2.5. *In vitro* digestibility of kale sprouts

In order to evaluate the bioavailability of the phytochemicals present in kale, an *in vitro* study was carried out. The *in vitro* digestion process was performed as reported by Flores et al., (2014) with treated and untreated kale sprouts. The process consisted of a 3-step procedure that simulated digestion in mouth, stomach and small intestine. Composition of artificial digestive juices are organized in **Table 8**.

First, dry kale sprouts (0.5 g) were mixed with a stock solution of simulated salivary fluid (FSS) (3 mL). Then the salivary solution was completely homogenized for 2 min at 37 ° C. The resulting oral bolus was mixed with a stock solution of simulated gastrointestinal

fluid (FGS) (6 mL) and the required amount of HCl (1M) until reaching a pH of 2.0. The mixture was incubated for 2 h at 37 °C, adjusting the pH every 30 min.

For the intestinal stage, the resulting gastric chyme was mixed with a stock solution of simulated intestinal fluid (FIS) (6 mL), bile juices (3 mL) and the required amount of NaOH (1 M) to reach a pH of 7.4. This mixture was incubated for 2 h at 37 ° C. At the end of the *in vitro* digestion process, the digestion tubes were centrifuged at 10,000 rpm for 10 min at 4°C, and the resulting supernatants were kept at –80°C until freeze-dried (Labconco, Kansas City, MO, USA). Samples were stored at −80 °C until further glucosinolate, lutein and phenolic profile analysis by HPLC-DAD using the protocol proposed in section (**4.2.4**).

Table 8. Composition of artificial digestive juices

Compound	Artificial saliva	Gastric juice	Intestinal juice	Bile juice
Distilled water	500 mL	500 mL	500 mL	500 mL
NaCl	58.5 mg	2.752 g	7.012 g	5.259 g
KCl	74.5 mg	0.824 g	0.564 g	0.375 g
$NaHCO_3$	1.05 g		3.388 g	5.785 g
Urea	0.2 g	0.085 g	0.1 g	0.25 g
Na_2HPO_4		0.266 g		
$CaCl_2*2H_2O_2$		0.399 g		
NH_4Cl		0.306 g		
KH_2PO_4			80 mg	
$MgCl_2$			50 mg	
α-amylase	1.0 g			
Pepsin		2.5 g		
Pancreatin			9 g	
Lipase			1.5 g	
Bile salts				30 g
pH	7.0 ± 0.2	2.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2

4.2.6. Biological activity *in vitro* of kale sprouts

The effect of undigested extracts and intestinal-digested fractions of 7-day-old Red Russian kale sprouts on cellular antioxidant activity (CAA) and nitric oxide (NOx) production was evaluated.

4.2.7. Cellular antioxidant activity (CAA)

Cellular antioxidant activity of undigested extracts and digested kale was evaluated in human colorectal adenocarcinoma cells (Caco-2) according to the method described by Gutiérrez-Grijalva et al., (2019). Caco-2 cells were obtained from the American Collection Type Culture (ATCC® TIB-71™, VA, USA). The cells were grown in a solution

of DMEM (Modified Eagle Medium) supplemented with 5% of bovine serum and 1% of Pen-Strep antibiotic in 5% in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded in 96-well plate (5×10^4 cells/well) and allowed to adhere for 16 h. Afterwards, cells were treated with 100 µL of sample at different concentrations containing DCFH-DA (60 µM). Then, cells were incubated for 20 min at 37º C. Afterwards, treatment solutions were removed, and the cells were washed twice with PBS. Finally, 100 µL of 500 µM AAPH solution was added to each well, except for blank and negative control wells. Fluorescence emitted at 538 nm upon excitation at 485nm was measured every 2 min for 90 min at 37 ºC using a microplate reader. CAA values were calculated using the following **equation 4.1**:

$$\text{CAA Unit} = 1 - (\int SA / \int CA) \quad (\textbf{Eq. 4.1})$$

where ∫SA is the integrated area under the sample fluorescence versus time curve, and ∫CA is the integrated area from the control curve

4.2.8. Nitric oxide production

The anti-inflammatory potential of kale sprouts was evaluated through the determination of nitric oxide production following the method described by Antunes-Ricardo, Gutiérrez-Uribe, Martínez-Vitela & Serna-Saldívar (2015).

Mouse macrophage (RAW 264.7) cells were obtained from the American Type Culture Collection (ATCC® TIB-71™, VA, USA). Cells were cultured in a solution of Dulbecco Modified Eagle Medium (DMEM) supplemented with 5% of bovine serum and 1% of Pen-Strep antibiotic in 5% of CO_2 at 37 °C. To evaluate the effects of undigested extracts and intestinal-digested fraction of kale on nitric oxide production, cells were detached with a scraper and resuspended in DMEM medium. Cells were seeded at 5×10^4 cells/well in 96-well plate and incubated for 16 h. After that, different concentrations of the extract were added and incubated for another 4 h. Following, half of wells were activated with 1 *µ*g/mL LPS, while the other half were used as control for each sample.

The production of NOx in the culture supernatants was determined by measuring nitrite increase in the medium using the Griess Reagent System (Promega, Madison, WI, USA) according to manufacturer's instructions. Briefly, 100 µL of supernatant were mixed with 10 µL of sulfanilamide, followed by incubation for 10 min at room temperature. After that, 10µl of N-1-naphthylethylenediamine dihydrochloride (NED) reagent was added. After incubation for 10 min, the absorbance values were read at 550 nm on a Synergy HT plate reader (Bio-Tek Instruments, Inc., VT, USA). The NOx concentration was calculated by comparison with a sodium nitrite standard (1.5-50 µM) curve.

4.2.9. Measurement of RAW 264.7 cell viability

Cell viability was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Absorbance was measured at 490 nm with a 96-well microplate reader (Synergy HT, Bio-Tek, Winooski, VM). Cell viability was

calculated by dividing the absorbance of cells treated by the absorbance of control cells (non-treated cells), and this ratio was expressed as percentage (%).

4.3. Results and Discussion

Se, S and MeJA stresses have been previously reported as elicitor techniques with possible impact on phytochemicals stability (Kim et al., 2018; Kopsell et al., 2003; Ku & Juvik, 2013; Ortega-Hernández, Antunes-Ricardo, Cisneros-Zevallos, & Jacobo-Velázquez, 2022; Park et al., 2018; Sun, Yan, Zhang, & Wang, 2012; Yi et al., 2016). Corresponding results for kale samples are presented in **Figure 22-24**.

4.3.1. Lutein Stability of kale after *in Vitro* simulated Digestion

Concentrations of lutein in untreated kale were compared to treated samples with Se 40 mg/L, S 120 mg/L and MeJA 25 µM (**Figure 22**). The highest increase of lutein was detected in kale sprouts treated with Se (53.2%), followed by S (48.1%) and MeJA (32.8%), compared to control. The combination of these conditions had no significant effect on lutein content.

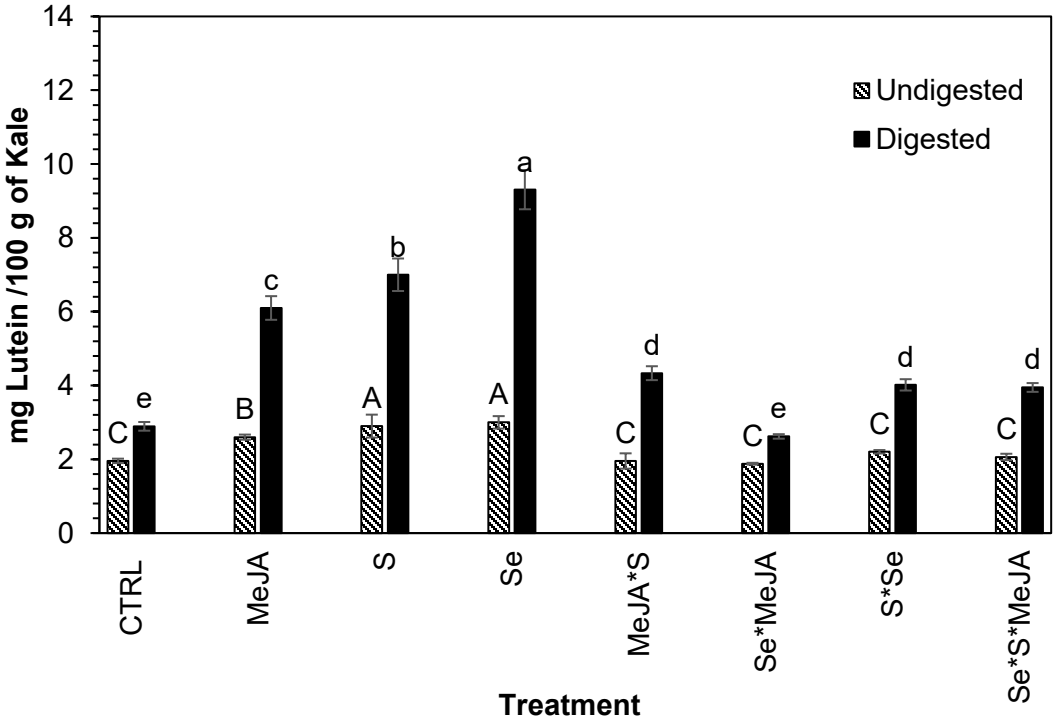


Figure 22. Concentration of lutein in intestinal digested fraction and undigested Red Russian kale sprouts treated with methyl jasmonate, sulfur, selenium or a combination of stresses after 7 days of germination. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Abbreviations: methyl jasmonate (MeJA), sulfur (S) and selenium (Se). Results are expressed in dry weight basis.

As mentioned earlier in chapter 3, to our knowledge, the current study represents the first attempt to measure the influence of Se on the accumulation of lutein in kale sprouts. The accumulation of lutein in kale sprouts was probably stimulated to keep ROS levels low and to prevent lipid peroxidation in the thylakoid membrane produced under high levels of Se and S (Baroli et al., 2004; Havaux, Dall'Osto, & Bassi, 2007).

When the bioaccessibility of carotenoid extract was analyzed, a significant liberation of lutein was observed in all samples of kale after intestinal digestion, in relation to initial concentration. These results are in agreement with previous findings which proved that after *in vitro* intestinal digestion, there were significant increase in lutein concentration (Eriksen, Luu, Dragsted, & Arrigoni, 2017). Kale samples treated with Se (210.3%) and S (141.5%) during germination showed higher levels of lutein compared to control (47.2%) after simulated digestion. Since dietary carotenoids are not substrates for digestive enzymes (Furr & Clark, 1997), mechanical disruption of the food matrix may be the dominating influencing factor for carotenoid liberation resulting in significantly higher values of lutein from kale sprouts.

4.3.2. Phenolic Stability of kale after *in Vitro* simulated Digestion

Eight individual phenolic compounds were detected, mainly 4-O-caffeoylquinic acid, 3-O-hexoside kaempferol (3-O-H-K) and sinapic acid, which is in agreement with previous studies on kale (Ortega-Hernández et al., 2022). Phenolic contents of the different samples before and after digestion are shown in Table 9 and 10, respectively.

Results showed that, Se and S did not cause a significant change in phenolic content compared to control sprouts; however, all five MeJA (35%), MeJA x Se (46%), MeJA x S (47%), MeJA x Se x S (44%) and Se x S (21%) treatments caused a significant decrease in phenolic concentration.

After *in vitro* gastric digestion, phenolic levels showed high losses in comparison to the initial fraction after the gastrointestinal digestion in all kale samples. Significant decreases in total phenolic of untreated (73%) and treated kale sprouts with Se (71%), S (76%) and MeJA (62%), regarding the initial concentration were found (Table 10). This general trend after gastrointestinal digestion has been previously reported for other food products (Gil-Izquierdo, Zafrilla, & Tomás Barberán, 2002; Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002).

Concerning to individual compounds, similar loss rates were found for total flavonoids and hydroxycinnamic acids. This high loss could be associated with the instability of the phenolic acids in aqueous solution (Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera, 2004). In addition, the pH value of 7.5 and the bile salts could contribute to the increase in this loss.

4.3.3. Glucosinolates Stability of kale after *in Vitro* simulated Digestion

In agreement with previous reports (Hahn et al., 2016; Kim et al., 2017), 9 glucosinolates were found in kale sprouts, namely, glucoiberin, progoitrin, glucoraphanin, 4-hydroxy-glucobrassicin, glucobrassicin, 4-methoxy-glucobrassicin, glucoeurocin, gluconapin,

Table 9. Concentration of individual and total phenolic compounds in Red Russian kale sprouts treated with methyl jasmonate, sulfur, selenium or a combination of stresses after 7 days of germination.

	Phenolic Compound Concentration (mg/100 g DW) [1,2,3]								
Treatment	**4-O-CQA**	**3-O-H-K**	**Sinapic acid**	**Ferulic acid**	**1-S-2-FG**	**1,2-diS-2-FG**	**K-3-O-s-so7-O-g**	**Quercetin**	**Total**
CTRL	48.27 ±0.95 a	87.69 ±2.93 a	65.27 ± 3.43 a	29.37 ±2.44 a	6.00 ±0.34 b	26.13 ± 2.43 b	20.07 ± 1.34 c	18.16 ±0.98 c	300.95 ± 10.34 a
MJ	21.95 ±0.23 e	16.43 ±1.43 e	12.20 ± 1.43 d	14.18 ±1.44 b	1.60 ±0.18 c	25.35 ± 2.54 b	67.65 ± 2.67 a	34.23 ±1.99 b	193.60 ± 12.34 c
S	39.22 ±0.78 b	48.68 ±4.12 c	33.95 ± 3.43 c	27.36 ±2.44 a	3.28 ±0.40 c	30.83 ± 2.43 ab	36.03 ± 1.56 b	31.95 ±0.43 bc	251.30 ± 19.43 b
SE	38.60 ±0.98 b	72.88 ±5.09 b	55.30 ± 3.45 b	21.61 ±2.00 ab	3.61 ±0.34 c	31.01 ± 2.43 ab	39.35 ± 1.89 b	29.33 ±2.43 bc	291.69 ± 15.34 a
MJ*S	28.48 ±1.93 d	13.01 ±1.13 e	4.74 ± 3.54 e	14.29 ±0.97 b	14.48 ±1.22 a	36.50 ± 2.43 a	19.35 ± 1.23 c	26.28 ±1.43 bc	157.13 ± 11.48 d
SE*MJ	25.76 ±2.41 d	23.01 ±1.33 d	4.06 ± 2.45 e	10.37 ±0.93 b	11.57 ±0.42 a	26.77 ± 1.54 b	14.81 ± 1.98 c	44.44 ±1.54 a	160.79 ± 13.98 d
S*SE	34.23 ±3.21 c	37.75 ±1.43 c	25.80 ± 2.65 cd	27.04 ±1.00 a	18.34 ±0.34 a	25.71 ± 1.09 b	31.19 ± 0.34 b	36.10 ±1.43 b	236.16 ± 18.53 b
SE*S*MJ	41.86 ±2.09 b	48.34 ±2.43 c	2.69 ± 2.34 e	24.06 ±2.00 a	2.50 ±0.32 c	4.50 ± 2.09 c	0.00 ± 0.93 d	43.21 ±2.53 a	167.16 ± 14.43 d

[1] Concentrations are reported as each individual standard. Compounds were quantified at 320 nm: sinapic acid; ferulic acid; 4-O-caffeoylquinic acid (4-O-CQA); 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG); and 1,2-disinapoyl-2-ferulolylgentiobiose (1,2- diS-2-FG); 360 nm: kaempferol 3-*O*-sophoroside-7O-glucoside (K-3-O-s-so7-O-g), 3-O-hexoside kaempferol (3-O-H-K), and quercetin. [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Concentrations of K-3-O-s-so7-O-g and 3-O-H-K are expressed as kaempferol equivalents; concentrations of 1-S-2-FG and 1,2-diS-2-FG are expressed as ferulic acid equivalents. Authentic standards were used to determine the concentrations of 4-O-CQA, sinapic acid, ferulic acid, and quercetin.

Table 10. Concentration of individual and total phenolic compounds in intestinal digested fraction of Red Russian kale sprouts treated with methyl jasmonate, sulfur, selenium or a combination of stresses after 7 days of germination.

	Phenolic compound concentration (mg/100 g DW) [1,2,3]																							
Treatment	**4-O-CQA**		**3-O-H-K**		**Sinapic acid**		**Ferulic acid**		**1-S-2-FG**		**1,2-diS- 2-FG**		**K-3-O-s-so7-O-g**		**Quercetin**		**Total**	
CTRL	46.36 ± 2.90	a	15.35 ± 4.16	b	15.45 ± 4.02	a	0.01 ± 1.11	c	0.01 ± 0.42	c	2.01 ± 2.95	d	0.01 ± 2.36	e	0.01 ± 0.99	e	79.21 ± 18.89	a
MJ	45.70 ± 0.97	a	4.96 ± 3.03	c	11.70 ± 2.22	b	0.01 ± 0.26	c	0.01 ± 0.83	c	2.01 ± 2.28	d	4.56 ± 1.85	c	4.01 ± 1.72	c	72.97 ± 13.16	ab
S	8.91 ± 0.85	c	13.99 ± 7.61	b	14.21 ± 2.11	a	3.01 ± 0.48	a	0.01 ± 0.77	c	6.01 ± 0.97	b	8.09 ± 0.10	a	6.01 ± 0.31	b	60.24 ± 13.2	b
SE	17.17 ± 0.49	b	27.31 ± 3.35	a	5.94 ± 4.22	c	4.01 ± 1.94	a	1.01 ± 0.83	b	11.01 ± 0.56	a	6.58 ± 1.23	b	9.01 ± 1.61	a	82.04 ± 14.22	a
MJ*S	12.17 ± 1.24	b	3.40 ± 6.68	d	0.01 ± 3.27	d	0.99 ± 0.97	c	3.56 ± 0.05	a	3.59 ± 0.39	c	1.67 ± 1.57	d	3.98 ± 0.01	d	29.37 ± 14.18	d
SE*MJ	44.60 ± 2.70	a	3.11 ± 7.30	d	0.01 ± 3.28	d	0.78 ± 0.93	c	0.00 ± 0.82	b	4.42 ± 0.03	b	1.64 ± 1.03	d	3.91 ± 1.62	d	58.47 ± 17.72	c
S*SE	44.40 ± 2.07	a	6.66 ± 5.33	c	3.76 ± 4.33	c	3.26 ± 1.40	a	1.07 ± 0.61	b	5.40 ± 0.50	b	8.68 ± 0.21	a	3.35 ± 0.90	d	76.59 ± 15.34	ab
SE*S*MJ	13.00 ± 1.37	b	11.98 ± 7.60	b	0.01 ± 5.96	d	2.91 ± 0.87	b	0.01 ± 0.40	c	0.01 ± 0.85	e	0.00 ± 1.92	e	0.01 ± 0.98	e	27.92 ± 19.95	d

[1] Concentrations are reported as each individual standard. Compounds were quantified at 320 nm: sinapic acid and ferulic acid, 4-O-caffeoylquinic acid (4-O-CQA), 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG) and 1,2-disinapoyl-2-ferulolylgentiobiose (1,2-diS-2-FG); 360 nm: kaempferol 3-*O*-sophoroside-7O-glucoside (K-3-O-s-so7-O-g), 3-O-hexoside kaempferol (3-O-H-K) and quercetin. [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$).

Table 11. Concentration of individual and total glucosinolates in Red Russian kale sprouts treated with methyl jasmonate, sulfur, selenium or a combination of stresses after 7 days of germination.

	Glucosinolate Concentration (mM/100 g DW) [1,2,3]								
Treatment	**GIB**	**PRO**	**GRA**	**4-HGB**	**GBS**	**4-MGBS**	**GER**	**GNP**	**Total**
CTRL	0.79 ±0.05 c	0.61 ±0.01 b	25.95 ± 0.34 d	0.22 ±0.32 c	4.64 ±0.34 c	29.31 ± 0.13 b	0.29 ± 0.34 c	10.53 ±0.98 b	72.34 ± 1.34 d
MJ	2.55 ±0.03 a	0.40 ±0.02 b	98.63 ± 0.43 b	2.23 ±0.32 a	23.69±0.11 a	14.98 ± 0.14 c	0.69 ± 0.67 c	1.07 ±0.99 c	144.23 ± 10.34 c
S	1.85 ±0.01 b	1.90 ±0.05 a	96.00 ± 0.36 b	1.17 ±0.32 c	30.93±0.20 a	42.34 ± 1.33 a	5.35 ± 0.56 a	0.59 ±0.43 c	180.13 ± 10.43 a
SE	1.25 ±0.01 b	1.24 ±0.12 a	116.63± 0.48 a	0.27 ±0.30 c	11.97±0.14 b	27.95 ± 0.13 b	0.12 ± 0.89 c	0.88 ±0.43 c	160.31 ± 11.34 b
MJ*S	1.48 ±0.12 b	1.04 ±0.12 a	75.39 ± 0.57 c	0.78 ±0.17 b	16.56±0.12 b	15.66 ± 0.33 c	0.55 ± 0.23 c	10.97 ±0.43 b	122.43 ± 10.48 c
SE*MJ	1.68 ±0.14 b	1.49 ±0.13 a	99.18 ± 0.74 b	0.22 ±0.03 c	12.45±0.02 b	18.65 ± 1.14 c	1.74 ± 0.98 c	12.85 ±0.54 b	148.27 ± 11.98 c
S*SE	1.01 ±0.15 b	0.31 ±0.03 b	70.56 ± 0.32 c	0.22 ±0.01 c	12.04±0.14 b	30.83 ± 1.29 b	0.43 ± 0.04 c	10.21 ±0.43 b	125.60 ± 11.53 c
SE*S*MJ	0.80 ±0.01 c	0.55 ±0.02 b	39.52 ± 0.34 d	1.80 ±0.01 a	6.12 ±0.02 c	3.65 ± 1.49 d	3.40 ± 0.13 b	35.53 ±0.53 a	91.36 ± 6.43 d

[1] Results are expressed as desulfogucoraphanin equivalents in dry weight basis. Compounds were quantified at 270 nm: glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$).

Table 12. Concentration of individual and total glucosinolates in intestinal digested fraction of Red Russian kale sprouts treated with methyl jasmonate, sulfur, selenium or a combination of stresses after 7 days of germination.

	Glucosinolate concentration (mM/100 g DW) [1,2,3]								
Treatment	**GIB**	**PRO**	**GRA**	**4-HGB**	**GBS**	**4-MGBS**	**GER**	**GNP**	**Total**
CTRL	ND	ND	15.95 ± 1.02 c	ND	1.64 ± 0.43 c	7.42 ± 0.03 d	ND	1.03 ± 0.03 b	26.03 ± 2.89 e
MJ	ND	ND	38.63 ± 1.32 b	ND	11.69 ± 1.54 a	4.22 ± 0.22 e	ND	1.97 ± 0.34 b	56.50 ± 3.16 c
S	ND	ND	42.00 ± 2.93 b	ND	15.93 ± 1.34 a	9.73 ± 0.11 C	ND	0.15 ± 0.02 c	67.82 ± 3.24 b
SE	ND	ND	82.63 ± 3.43 a	ND	6.97 ± 0.75 b	11.64 ± 1.54 b	ND	0.13 ± 0.02 c	101.36 ± 11.22 a
MJ*S	ND	ND	18.39 ± 3.27 c	ND	2.56 ± 0.24 c	6.20 ± 0.65 d	ND	0.18 ± 0.01 c	27.33 ± 1.18 e
SE*MJ	ND	ND	22.18 ± 1.24 c	ND	8.45 ± 0.31 b	11.92 ± 0.43 b	ND	0.23 ± 0.02 c	42.78 ± 2.72 d
S*SE	ND	ND	39.56 ± 2.34 b	ND	6.04 ± 0.44 b	16.76 ± 0.34 a	ND	2.95 ± 0.02 a	65.31 ± 4.34 b
SE*S*MJ	ND	ND	16.52 ± 1.43 c	ND	2.31 ± 0.32 c	1.23 ± 0.13 f	ND	2.99 ± 0.01 a	23.05 ± 1.95 e

[1] Results are expressed as desulfogucoraphanin equivalents in dry weight basis. Compounds were quantified at 270 nm: glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS). [2] Values represent the mean of three replicates ± standard error of the mean. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$).

and neoglucobrassicin, representing a total amount of 72.34 mM/100 g of dry weight. Regarding the abiotic stress, the application of S 120 mg/L induced a significant increase in the content of progoitrin (211.1%), glucoraphanin (269.9), glucobrassicin (566.5%), 4-methoxy-glucobrassicin (44.4%), glucoeurocin (1744%) and total glucosinolate content (149%) in kale sprouts (**Table 11**). Additionally, Se treatment significantly increased the concentration of glucoraphanin (349.4%), progoitrin (103.2%) and total glucosinolate content (121.6%) in kale compared with control sprouts.

After intestinal digestion, total glucosinolate values showed a significant decrease in all kale samples (**Table 12**). Aliphatic glucosinolates were much more stable than indolic derivatives in all treated kale samples; only 38% was lost on the gastric digestion in contrast to indolic, which suffered losses of 74.8% in kale treated with Se during germination. The preserved amount of glucosinolates in the evaluated kale sprouts, even after hydrolysis may be associated to the selenium capacity to suppress glucosinolates breakdown (Finley, Sigrid-Keck, Robbins, & Hintze, 2005).

S and MeJA treatments did not show significant effects in glucosinolate degradation compared to control samples in intestinal digested fractions.

Additionally, no glucoiberin, progoitrin, 4-hydroxy-glucobrassicin and glucoeurocin were found in any kale sample after intestinal digestion. A possible explanation for the high percentage of loss during digestion could be the low stability of glucosinolates at intestinal pH (=7) led to a decomposition into their secondary reaction products (isothiocyanates) (Barba et al., 2016).

4.3.4. Cellular antioxidant activity

Prior to running the cellular experiments, the cytotoxicity of kale extract from different varieties was tested on Caco-2 cells at the maximum concentration used in the experiments (240 µg/mL) to ensure that the viability of the cells would not be affected. All the extracts showed a cellular viability over 90%. The CAA from the undigested extract and intestinal digesta fraction is shown in **Figure 23**.

The maximum percentage of CAA was achieved in the undigested extract (89.3%) and intestinal digested fraction (80.5%) from kale sprouts treated with Se. The increased total antioxidant activity has been reported in several Se-enriched brassica plants such as kale and broccoli (Ortega-Hernández et al., 2022; Ramos, Yuan, Faquin, Guilherme, & Li, 2011; Schiavon & Pilon-Smits, 2017). However, there is no significant difference in antioxidant activity before and after digestion.

Our results indicate that antioxidant activity of the tested sprouts, was positively correlated (95%, $p<0.05$) with the release of lutein as well as the presence of glucosinolates and their breakdown products, ITCs. Isothiocyanates have been well documented for their indirect antioxidant activity by activating the Keap1/Nrf2/ARE pathway and inducing production of protective phase II enzymes (Dinkova-Kostova and Talalay, 2008;Tsuji et al., 2013).

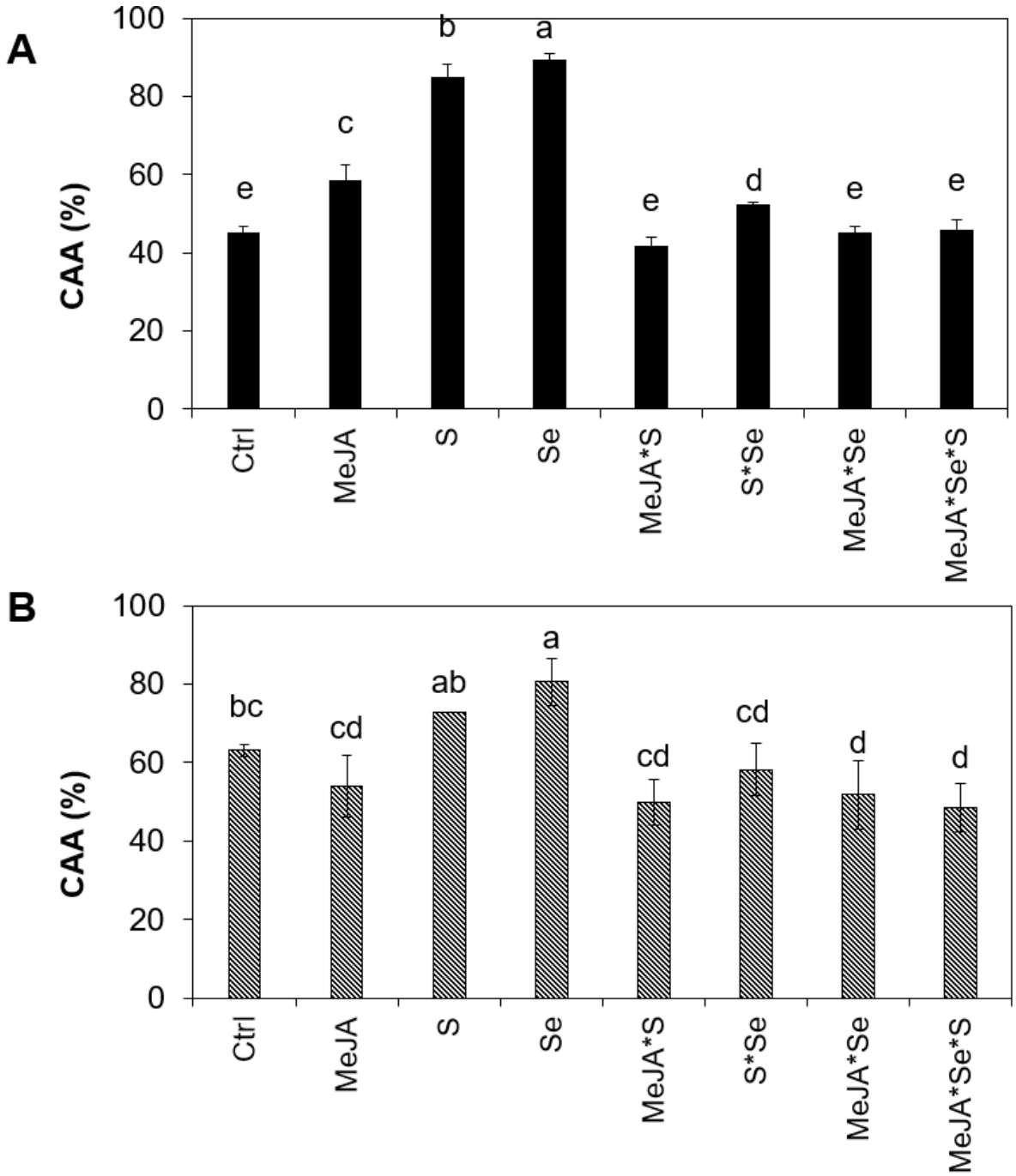


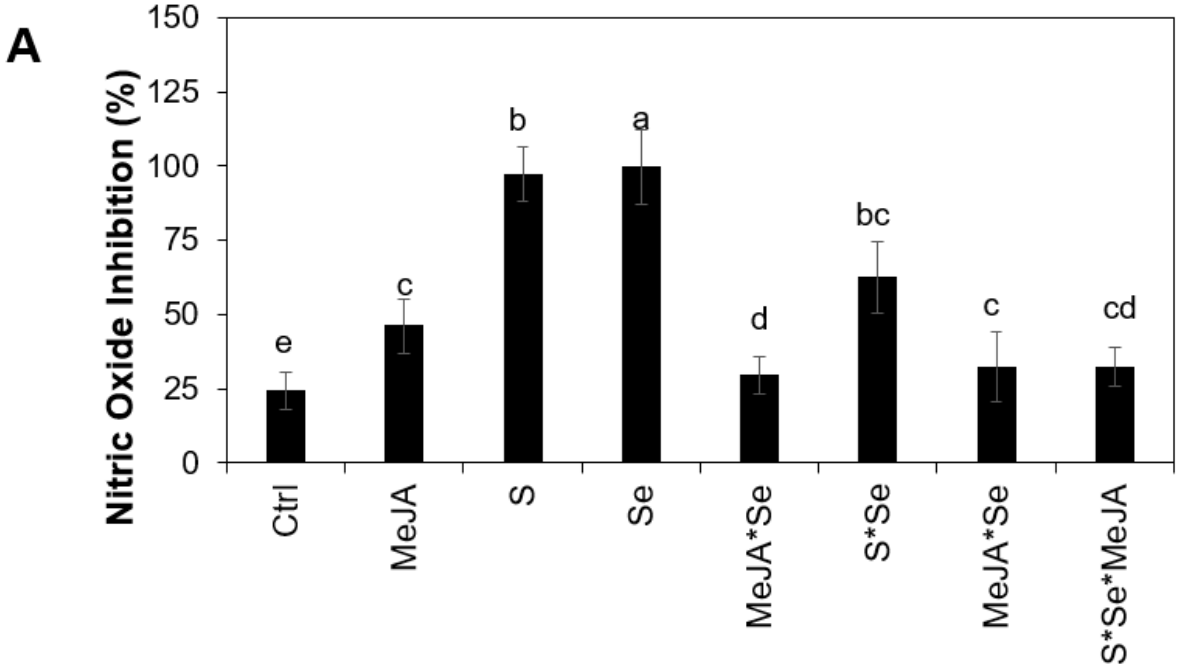
Figure 23. Cellular antioxidant activity of (A) ethanolic undigested extract and (B) intestinal digested fraction of Red Russian kale sprouts treated with methyl jasmonate, selenium, and sulfur after 7 days of germination in Caco-2 cells. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se).

4.3.5. Nitric oxide production

Figure 24 shows the results about the ability of kale sprouts to inhibit NO production by LPS-stimulated RAW 264.7 cells. In cells treated with 1 μg/mL LPS, NOx production decreased in a significant manner in undigested extract (98.3%) and intestinal digested fraction (91.5%) from kale sprouts treated with selenium. The nitric oxide production was directly correlated (92%) to the lutein and glucosinolate changes.

Lutein has been shown as an anti-inflammatory compound in several *in vitro* and *in vivo* studies. The anti-inflammatory properties of lutein were reported in a study conducted by Lee et al. (2004) where a lutein enriched diet reduced UV radiation-induced inflammation in the ears of mice. In another study, Gonzalez et al. (2003) have shown that UVB-induced skin inflammation in the ears of mice was suppressed with a diet supplemented with lutein and zeaxanthin. The capacity of carotenoids for scavenging singlet oxygen species and per oxyradicals has been associated with the presence of conjugated double bonds in their molecules (Stahl, 2005).

On the other hand, the bioactive properties of glucosinolates lies in their structure and in their hydrolysis products, which are significant regulators in oxidative stress.



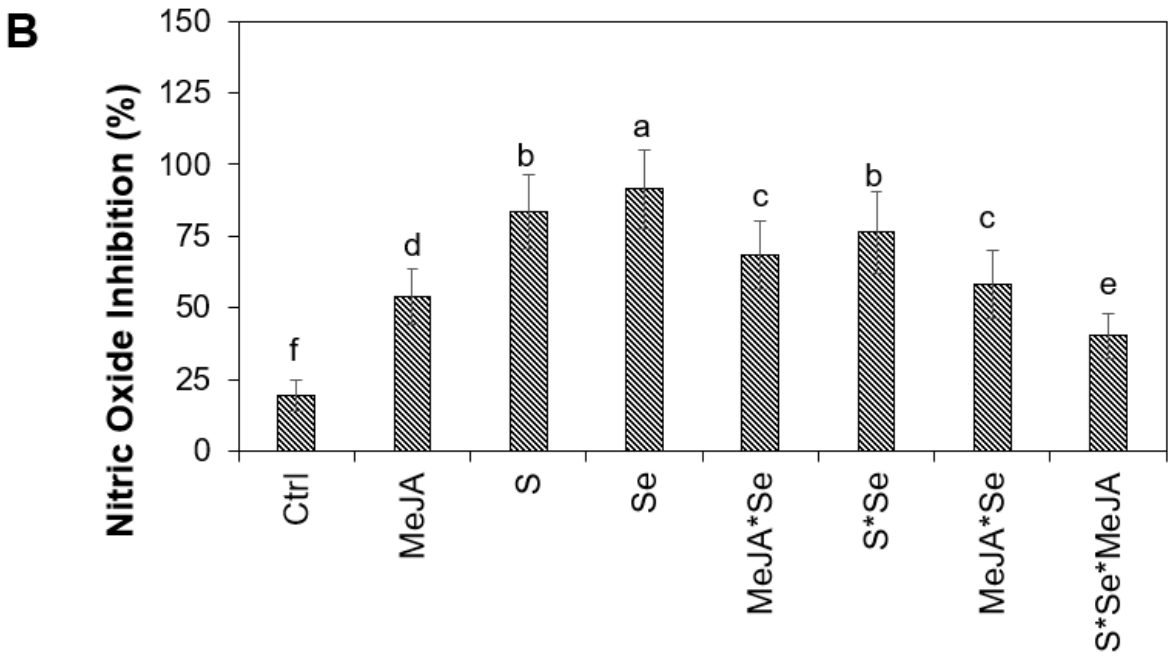


Figure 24. Nitric oxide production of (A) ethanolic undigested extract and (B) intestinal digested fraction of Red Russian kale sprouts treated with methyl jasmonate, selenium, and sulfur after 7 days of germination in Raw 264.7 cells. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Abbreviations: methyl jasmonate (MeJA), sulfur (S), selenium (Se).

4.4. Conclusions

A total of 9 phenolic compounds, 9 glucosinolates and 1 carotenoid were identified and quantified in *Brassica oleracea var.* acephala samples. The greatest accumulation of phytochemicals was observed in 7-day-old kale sprouts treated with Se and S. In contrast, the MeJA application and the combination of treatments was not interesting to use as strategy to increase the nutraceutical content of kale due to it antagonist effect or lower accumulation of glucosinolates, lutein and phenolics compounds. Furthermore, I found that the phytochemical accumulation was correlated (90%) with cellular antioxidant activity and nitric oxide production by Caco-2 and Raw 264.7 cells, respectively. On the other hand, the results of *in vitro* digestion process indicated that an additional process must be proposed to avoid the phytochemical degradation and to conserve the bioactivity of kale. It is necessary the incorporation of experiments directed to the encapsulation of kale extract for phytochemical stability.

CHAPTER 5. ANTIOXIDANT AND IMMUNOMODULATOR EFFECT OF SECONDARY METABOLITES OF KALE SPROUTS UNDER STRESS CONDITIONS

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5.1. Introduction

Inflammatory bowel disease (IBD) is a discontinuous and transmural inflammatory process that can affect any segment of the digestive tract and can even evolve with luminal stenosis or with the formation of abscesses or fistulas (Baumgart & Sandborn, 2012). Despite advances in research in recent years, the exact etiology of the disease is still unknown, available therapies are expensive and a definitive cure has not been found (Figueroa, 2019). IBD has been increasing worldwide and this is especially remarkable in developing countries. Although the incidence is highest in North America and Europe, epidemiological data show that the incidence has increased in Asia and Latin America (Ananthakrishnan, 2015).

It is known that fruits and vegetables are good sources of dietary fiber, vitamins, minerals, and secondary metabolites that are beneficial for our health, such as carotenoids, glucosinolates, and phenolic compounds. This is due to the potential properties of their phytochemical content such as antioxidation and inflammatory biomarkers modulation (Lapuente, Estruch, Shahbaz, & Casas, 2019).

Among crucifers, members of the Brassica genus (e.g. broccoli, cabbage, kale, cauliflower, and Brussels sprouts), are regarded as some of the most health-promoting, commonly grown and consumed vegetables worldwide (Podsędek, 2007). Particularly, kale (*Brassica oleracea* L. var acephala) is a very important crop in economic terms. Particularly, young kale sprouts constitute an exceptionally rich source of phenolic compounds, carotenoids and glucosinolates, with concentrations several times greater than those found in kale leaves (Fahey, Zhang, & Talalay, 1997; Maldini, Baima, Morelli, Scaccini, & Natella, 2012; Pająk, Socha, Gałkowska, Rożnowski, & Fortuna, 2014; W. T. Park et al., 2012; Vale et al., 2015).

However, environmental and physiological factors such as oxygen, temperatures and pH changes directly affect the stability of these compounds and their bioavailability (Dias, Ferreira, & Barreiro, 2015). Therefore, it is necessary to develop delivery systems for these components that are inert but allow these active ingredients to be protected during storage and during gastrointestinal digestion to favor their bioactivity.

Encapsulation is a technique that aims to stabilize specific compounds for their application as nutraceutical or in food-grade matrices, in addition to controlling their release in the gastrointestinal tract, thereby increasing their bioavailability (Wang et al., 2015). Among the encapsulation methods available, a notable procedure is spray-drying, a scalable and low-cost method that can result in the formation of a stable, free-flowing powder (Dias et al., 2015). Commonly, these phytochemicals are trapped in a

matrix of polysaccharides or proteins formed when the atomized liquid comes into contact with hot air. Compared with conventional drying methods, the drying time is generally shorter (1–20 s) (Guiné, 2018; Díaz-Torres, Alonso-Castro, Carrillo-Inungaray, & Carranza-Alvarez, 2021). Published reports on spray-drying of Brassicaceae vegetables involve the effect of drying conditions and wall materials on the stability and encapsulation efficiency of glucosinolates and phenolic compounds (Radünz et al., 2020; Saavedra-Leos, Leyva-Porras, Toxqui-Terán, & Espinosa-Solis, 2021; Tian et al., 2015; Wu, Zou, Mao, Huang, & Liu, 2014). Radünz et al., (2020) have reported the influence of encapsulation on broccoli extracts during digestive processes. The capsule mainly protected the 5-caffeoylquinic acid, 4-hydro-xybenzoic acid glucoside, p-coumaroylquinic acid, 4-methoxy-gluco-brassicin, 1-O-sinapoyl-β-D-glucose, and coumaric acid; at the same time, inhibitory activity for the nitric oxide radical showed an increase (74.8%) in the intestine fractions. Later, Radünz et al., (2020) informed that encapsulation of glucosinolates and phenolic compounds of broccoli extract showed a greater protection of the phytochemicals (86.9%) without affecting the active sites against glial tumor cells. Yet, the effect encapsulation on phytochemicals of kale during gastrointestinal digestion and their bioactive properties has not been studied.

To address this, the human digestion of fresh kale was simulated using an *in vitro* digestion model. This system was sampled at mouth, stomach and intestinal stages. The range of secondary metabolites in each digesta were identified and quantified by HPLC−DAD. The transport of the identified carotenoids, glucosinolates and phenolic compounds in intestinal digesta was evaluated in Caco-2 cell monolayers differentiated into intestinal epithelial cells. Measuring the glucosinolates and glucosinolate hydrolysis products in intestinal digesta provides key information on the bioaccessibility of these compounds, while measuring the transport across Caco-2 cell monolayers models provides key information on the bioavailability of these compounds.

The objective of this chapter was to evaluate the protector effect of encapsulation on the phytochemical content of kale treated with S and S during simulated gastrointestinal digestion, as well as its impact on the cellular antioxidant activity (CAA), nitric oxide (NOx) production and immunomodulatory activity.

5.2. Materials and Methods

5.2.1. Chemicals

Methyl jasmonate (MeJA), sulfatase (from *Helix pomatia*), diethylaminoethyl (DEAE)-sephadex A-25, sinigrin hydrate, sodium acetate, acetonitrile (HPLC grade), methanol (HPLC grade), methyl tert-butyl ether (MTBE; HPLC grade), phosphoric acid, porcine pancreatin (Cat. No. P1750), porcine pepsin (Cat. No. P7000), porcine pancreatic lipase (Cat. No. L3126), α-amylase from porcine pancreas (Cat. No. A3176), bile salts (Cat. No. B8631), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), dichloro-dihydro-fluorescein diacetate (DCFH-DA), lipopolysaccharide (LPS) (L4391), lucifer yellow fluorescent stain (LY), maltodextrin, DL-sulforaphane, 4-O-caffeoylquinic acid, ferulic acid, sinapic acid, quercetin, kaempferol and lutein standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco Modified Eagle Medium, fetal bovine

serum, phosphate-buffered saline (PBS, pH 7.4), Penicillin-Streptomycin antibiotic (Pen-Strep), antibiotic-antimycotic and Hank's buffered saline solution (HBSS) were acquired from GIBCO (Carlsbad, CA, USA). Griess Reagent System and CellTiter 96 AQueous One Solution Cell Proliferation Assay kits were acquired from Promega (G2930, Madison, WI, USA). Ethanol (HPLC grade), sodium selenite (Se; Na_2SeO_3), potassium sulfate (S; K_2SO_4), sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), urea, calcium chloride dihydrate ($CaCl_2*2H_2O_2$), ammonium chloride (NH_4Cl), dibasic sodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), and magnesium chloride ($MgCl_2$) were obtained from Desarrollo de Especialidades Quimicas, S.A. de C.V. (Monterrey, NL, Mexico). Desulfoglucoraphanin was acquired from Santa Cruz Biotechnology (Dallas, TX, USA). MILLIPLEX MAP Mouse Cytokine / Chemokine panel, Human/Mouse kit was obtained from Millipore (Billerica, MA, USA). Total COX-2 DuoSet IC ELISA and Mouse IL-10 ELISA were purchased from R&D Systems (Minneapolis, MN, USA).

5.2.2. Plant Material

Red Russian kale (*Brassica oleracea* var. acephala) seeds were provided by La Semilleria (Queretaro, Qro, Mexico). Kale seeds were sanitized for 15 min in sodium hypochlorite (1.5%, v/v) and rinsed with Milli-Q water.

5.2.3. MeJA, Se, and S Treatments

Kale seeds were soaked in Milli-Q water (control) or the treatment solutions for 5 h. The treatment solutions used were Se (40 mg/L) and S (120 mg/L). Afterwards, the soaking solutions were discarded, and the seeds were placed on germination trays in a dark chamber set at 25 °C and 85% of relative humidity for 7 days. Three replicates of each treatment were run concurrently. Each replica consisted of a tray containing 30 g of kale seeds sprayed with 5 mL of different treatment concentrations or control every 12 h throughout the experiment. Samples were collected after 7 days of germination, freeze-dried (Labconco, Kansas City, MO, USA), ground into a powder, and stored at −80 °C until further analysis of phytochemicals.

5.2.4. Phytochemical Analyses

5.2.4.1. Extraction of Secondary Metabolites

Extraction of glucosinolates, phenolic compounds and lutein from kale sprouts powder was carried out in a single process based on the protocol published by Moreira-Rodríguez et al. **(**2017**)**. Kale samples (0.2 g) were spiked with 50µL of internal standard consisting in sinigrin 3 mM and mixed with 10 mL of pre-heated (70°C) ethanol 70%, to inactivation of myrosinase.

Samples were immersed in a water bath (VWR, Radnor, PA, USA) at 70 °C and vortexed every 5 min for 30 min to inactivate myrosinase. Then, the extracts were left to cool at 25 °C and centrifuged (18,000× g, 10 min, 4 °C) (SL16R, Thermo Scientific, GER) to recover the supernatant. The supernatant obtained was used for the identification and

quantification of individual glucosinolates, lutein, and phenolic compounds by HPLC-DAD (1260 Infinity, Agilent Technology, Santa Clara, CA, USA).

5.2.4.2. Identification and Quantification of Lutein

The identification and quantification of lutein were performed according to a previous work (Moreira-Rodríguez et al. 2017) using a HPLC-DAD system (1260 Infinity, Agilent Technologies, Santa Clara, CA). Samples (25 μL) analysis were achieved on a YMC C30 column (4.6 mm × 150 mm, 3 μm particle size) (YMC, Wilmington, NC, USA). The mobile phase was (A) 50% methanol, (B) 45% MTBE, and (C) 5% water set at a flow rate of 0.5 mL/min. The detector wavelength was set at 450 nm. Lutein was identified by comparing its retention time and absorption spectra with a reference standard. Lutein was quantified using a calibration curve of lutein standard (0–12 ppm) and expressed as mg of lutein per 100 g of kale sprouts in dry weight (DW) basis.

5.2.4.3. Identification and Quantification of Phenolic Compounds

Phenolic compounds in kale extract were quantified using an HPLC system (Agilent Technologies 1260 series, Santa Clara, CA, USA) coupled with a diode array detector (DAD). The chromatographic separations were carried out on a a C18 reverse phase column, 4.6 mm × 250 mm, 5 μm (Luna, Phenomenex, Torrance, CA, USA). The mobile phases were used for chromatographic separation: (A) water containing 1% (v/v) phosphoric acid and (B) methanol-water (60:40, v/v). Gradient elution consisted of 0/0, 3/30, 8/50, 35/70, 40/80, 45/100, 50/100 and 60/0 (min/% phase B) at a flow rate of 0.8 mL/min. The injection volume was 10 μL. The peaks were determined at 280, 320, and 360 nm and integrated by the OpenLAB CDS ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

Compounds identification was based on retention time, UV-visible spectra and wavelengths of maximum absorption, as compared with the reported literature (Aguilar-Camacho et al., 2019; Moreira-Rodríguez et al., 2017) and commercial standards. Quantification was accomplished using standard curves of 4-O-caffeoylquinic acid (0.1–50), ferulic acid (0.4–40 ppm), sinapic acid (0.1–50 ppm), quercetin (0.1–12 ppm), and kaempferol (0.1–12 ppm) were prepared. Results were expressed as mg of each individual phenolic compound per 100 g of kale sprouts (mg/ 100 g) in dry weight (DW) basis.

5.2.4.4. Desulfation of Glucosinolates

The ethanolic extracts (3 mL) were added into Bio-Rad polypropylene columns, filled with 0.5 mL of DEAE-Sephadex A-25 resin pre-activated for at least 12 h in sodium acetate (0.02 M, pH 5). After removing excess supernatant by elution, the columns were washed twice with 0.5 mL of water followed by 1 mL of sodium acetate (0.02 M). Purified sulfatase (75 μL) was added to each column and was left at 25 °C for 12 h. Desulfoglucosinolates were diluted with 2x0.5 mL and 0.25 mL of water and analyzed directly using HPLC.

5.2.4.5. Identification and Quantification of Glucosinolates

Glucosinolates were identified and quantified using a HPLC system equipped with a diode array detector (DAD) set at 227 nm. Separation was performed on a C18 reverse phase column, 4.6 mm × 250 mm, 5 µm (Luna, Phenomenex, Torrance, CA). The mobile phases were (A) water and (B) acetonitrile at a flow rate of 1.5 mL/min with a gradient program as: a linear step from 100% to 80% of solvent A within 28 min, followed by linear up to 100% A in 7 min. The injection volume was 20 µL.

Glucosinolate identification was based on retention time, UV-visible spectra and wavelengths of maximum absorption as compared with the reported literature (Aguilar-Camacho et al., 2019; Moreira-Rodríguez et al., 2017) and commercial standards. For the quantification of glucosinolates, a standard curve of desulfoglucoraphanin (0–1250 ppm) was prepared. Results were expressed as mmol of desulfoglucoraphanin equivalents per Kg of kale sprouts (mM/ 100 g) (DW).

5.2.4.6. Sulforaphane analysis

The extraction and analysis of sulforaphane was carried out according to the method reported by González et al. (2021) with slight modifications. Kale powder (1 g) was suspended in 10 mL of methylene chloride, which was combined with 0.5 g anhydrous sodium sulfate and 5 µL of butyl isothiocyanate (0.5 mg/mL) as an internal standard. After agitation for 60 min in shaker in the absence of light, sample was filtered, and the solution was vaporized at 30°C under vacuum (EZ-2.3, Genevac Ltd., Ipswich, EN) until dryness. For quantification, the residue was dissolved in 1 mL of ethanol and processed as described below.

Determination of sulforaphane was performed as reported by Torres-Contreras et al., (2017).A HPLC-DAD (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA), and a C18 reverse phase column (5µm particle size, 250×4.6 mm; Luna, Phenomenex, Torrace, CA, USA) were used. Mobile phase consisted in water (phase A) and methanol (phase B). The gradient solvent system was 0/100, 10/90, 35/0, 40/0, and 50/100 (min/% phase A) with an injection volume of 20 µL. Flow rate of the mobile phase was kept at 0.8 mL/min. The detector wavelength was set at 227 and 254 nm. A standard curve of sulforaphane was prepared in the range of 0–100 ppm for quantification, and results were expressed as mg per kg of kale (DW).

5.2.5. Encapsulation process

For the preparation of the phytochemical extract, 18 g of kale untreated and treated with Se (40 mg/mL) and S (120 mg/mL) were mixed with 200 mL of ethanol/water (70:30,v/v), previously heated for 10 min at 70 °C to inactivation of myrosinase. Then, samples were additionally heated in a water bath at 70 °C for 5 min and filtered to remove the fiber from the liquid. The supernatant was recovered.

Spray drying was employed in the preparation of the kale powders according to the method reported by Radünz et al., (2020). Feeding solutions were prepared by mixing a maltodextrin solution (MD, 9% w/v) with kale extracts assaying three different ratios

30:70, 50:50 and 70:30 w/w (Saavedra-Leos et al., 2021; Wu et al., 2014). Microencapsulation was carried out in a Yamato Spray Dryer ADL311S (Yamato Scientific Co., Ltd., Tokyo, Japan) under the following operating conditions: inlet temperature of 120 °C, pump flow rate of 8.5 mL/min, outlet temperature of 60 °C and pressure of 0.15 MPa. The operating conditions were selected based on preliminary tests. Powders were individually placed in plastic containers and stored in darkness at 4 °C.

5.2.5.1. Efficiency of Encapsulation

The efficiency of encapsulation (EE) was determined by the glucosinolate content evaluated by HPLC-DAD (Radünz et al., 2020). For this, quantification of glucosinolates on kale extract microcapsules (0.25 g) was carried out according to the method described in section 5.2.4. EE was expressed as a percentage and was calculated according to Eq.(5.1).

$$\mathbf{EE}(\%) = \frac{\mathbf{Glucosinolates\ of\ kale - Glucosinolates\ of\ the\ capsule}}{\mathbf{Glucosinolates\ of\ kale}}$$

5.2.5.2. Storage stability

The samples of each treatment and wall proportion were stored at 35 °C for 28 days. Afterwards, sampling for quantification of glucosinolates, lutein and phenolic compounds by HPLC-DAD (section 5.2.4) as carried out . The retention percentage was used to evaluate the storage stability of phytochemical microcapsules.

5.2.5.3. Scanning electron microscopy

The microcapsule morphology was evaluated using a scanning electron microscope (SEM) (JSM6360LV, Jeol). Analyzes of SEM were carried out by the Academic Service of Scanning Electron Microscopy from Instituto de Ciencias del Mar y Limnología (UNAM, MX.)

5.2.6. *In vitro* bioaccessibility of kale sprouts

In order to evaluate the bioaccessibility of the encapsulated and unencapsulated phytochemicals present in kale, an *in vitro* study was carried out according to the method reported by Flores et al., (2014). Briefly, 3 mL of simulated salivary fluid (FSS) was added to 0.5 g of sample. After homogenizing for 2 min at 37 ° C, 6 mL of simulated gastrointestinal fluid (FGS) was added, pH adjusted to 2.0 ± 0.2, and the sample re-incubated for 2 h at 37°C. The final step in the digestion procedure included the addition of 6 mL of simulated intestinal fluid (FIS) and 3 mL of bile juices. The pH was adjusted to 7.0 ± 0.2 and samples incubated for another 2 h. Composition of artificial digestive juices are organized in **Table 8**.

Aliquots were taken after each phase (oral, gastric, and intestinal) of the simulated digestion. Samples were centrifuged at 10,000 rpm for 10 min at 4°C, and the resulting supernatants were kept at –80°C until freeze-dried (Labconco, Kansas City, MO, USA).

Samples were stored at −80 °C until further glucosinolate, lutein and phenolic profile analysis by HPLC-DAD using the protocol proposed in section (5.2.4).

Table 8. Composition of artificial digestive juices

Compound	Artificial saliva	Gastric juice	Intestinal juice	Bile juice
Distilled water	500 mL	500 mL	500 mL	500 mL
NaCl	58.5 mg	2.752 g	7.012 g	5.259 g
KCl	74.5 mg	0.824 g	0.564 g	0.375 g
$NaHCO_3$	1.05 g		3.388 g	5.785 g
Urea	0.2 g	0.085 g	0.1 g	0.25 g
Na_2HPO_4		0.266 g		
$CaCl_2*2H_2O_2$		0.399 g		
NH_4Cl		0.306 g		
KH_2PO_4			80 mg	
$MgCl_2$			50 mg	
α-amylase	1.0 g			
Pepsin		2.5 g		
Pancreatin			9 g	
Lipase			1.5 g	
Bile salts				30 g
pH	7.0 ± 0.2	2.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2

5.2.7. Cell Permeability of phytochemicals of kale

Human colon (Caco-2) cell monolayers were used to evaluate the *in vitro* cell permeability of lutein, glucosinolates and phenolics compounds after the simulated gastrointestinal digestion of encapsulated and unencapsulated kale treated with Se (40 mg/mL) and S (120 mg/mL).

Caco-2 cells were cultured at 37°C and 5% CO_2 atmosphere in DMEM medium supplemented with 5% (v/v) fetal bovine serum and 0.5 % antibiotic-antimycotic. The medium was replaced every 2 days during cell growth and differentiation. For the experiments, cells were seeded in 12-well transwell inserts at a density of 1×10^6 cells/well. Monolayers were used 21 days after reaching confluence to allow for full differentiation into intestinal epithelial cells (Hwang, Bornhorst, Oteiza, & Mitchell, 2019). Cells were washed twice with PBS. An aliquot of 0.5 mL of intestinal-digested fractions of encapsulated and unencapsulated kale (120 µg/mL) containing lucifer yellow stain (LY) 100 µM was added to the upper chamber (apical side) and 1.5 mL of HBSS (pH 7.4) was added to the lower chamber (basolateral side). Cells were incubated at 37 °C for 2 h at 100 rpm.

After that, medium from both sides of the inserts was collected, taken to dryness at 30 °C, and stored at −80 °C until HPLC-DAD analysis (Section 5.4). For chromatographic analysis, powders were reconstituted in 0.5 mL of ethanol/water (70:30,v/v).

The integrity of the cell monolayers was assessed by monitoring lucifer yellow permeation. The fluorescence of apical and basolateral samples was measured at 530 nm (emission) and 485 nm (excitation) using a microplate reader (Synergy HT, BioTek, Winooski, VM). The apical to basal LY permeation (%) was calculated according to Eq.(5.2):

$$\boldsymbol{LY}\ (\%) = \frac{(\boldsymbol{F\ test - F\ blank})}{(\boldsymbol{Fo - F\ blank})}$$

Where, F_{test} is the fluorescence intensity of LY from the basolateral side; F_0, the initial fluorescence intensity of LY on the apical side, F_{blank} is the fluorescence intensity of the blank sample (HBSS alone). Only those monolayers with permeability lower than 5% were considered for the experiment.

5.2.8. Biological activity *in vitro* of phytochemical kale extract

The effect of intestinal-digested fractions of encapsulated and unencapsulated kale treated with Se (40 mg/mL) and S (120 mg/mL) on cellular antioxidant activity (CAA) and nitric oxide (NOx) production was evaluated.

5.2.9. Cellular antioxidant activity (CAA)

To evaluate cellular antioxidant activity of intestinal-digested fractions of encapsulated and unencapsulated kale, the method described by Gutiérrez-Grijalva et al., (2019) was used. Human colorectal adenocarcinoma cells (Caco-2) were obtained from the American Type Culture Collection (ATCC® TIB-71™, VA, USA). Cells were grown in a solution of DMEM (supplemented with 5% of fetal bovine serum and 1% of Pen-Strep antibiotic) and were maintained at 37°C and 5% CO_2 at 37 °C. Cells were seeded at a density of 5 × 10^4 cells/well on a 96-well plate and allowed to adhere for 16 h. Afterwards, cells were treated with 100 µL of sample at different concentrations containing DCFH-DA (60 µM). Then, cells were incubated for 20 min at 37º C. Afterwards, treatment solutions were removed, and the cells were washed twice with PBS. Finally, 100 µL of 500 µM AAPH solution was added to each well, except for blank and negative control wells. Fluorescence emitted at 538 nm upon excitation at 485nm was measured every 2 min for 90 min at 37 ºC using a microplate reader. CAA values were calculated using the equation 5.3:

$$\boldsymbol{CAA\ Unit} = \mathbf{1} - \left(\frac{\int \boldsymbol{SA}}{\int \boldsymbol{CA}}\right)$$

where ∫SA is the integrated area under the sample fluorescence versus time curve, and ∫CA is the integrated area from the control curve

5.2.10. Nitric oxide production

Nitric oxide production was evaluated according to the method described by Antunes-Ricardo, Gutiérrez-Uribe, Martínez-Vitela & Serna-Saldívar (2015).

5.2.10.1. Cell culture

Murine macrophages RAW 264.7 cells will be obtained from the American Collection Type Culture (ATCC® TIB-71™, VA, USA). The cells were seeded in a solution of DMEM (Dulbecco Modified Eagle Medium) supplemented with 5% of bovine serum and

1% of Pen-Strep antibiotic in 5% of CO_2 at 37 °C. To evaluate the effects of extracts and digested kale on cellular antioxidant activity, cells were plated in 96-well plate (5×10^4 cells/well) and allowed to adhere for 16 h. After that, different concentrations of intestinal-digested fractions of encapsulated and unencapsulated kale were added and incubated for another 4 h. Following, half of wells were stimulated with LPS at 1 *μ*g/mL, while the other half were used as control for each sample.

5.2.10.2. Measurement of nitric oxide

The nitrite concentration in the cell culture supernatant was used as a measure of NOx production. The production of NOx in the culture supernatants was determined by measuring nitrite increase in the medium (100 µL) using the Griess Reagent System (Promega, Madison, WI) following the manufacturer´s instructions. After incubation for 10 min, the absorbance values were read at 550 nm on a Synergy HT plate reader (Bio-Tek Instruments, Inc., VT, USA). For the quantification of nitrite concentration, a standard curve of sodium nitrite (1.5-50 µM) was prepared.

5.2.10.3. Measurement of RAW 264.7 cell viability

Cell viability was determined by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Absorbance was measured at 490 nm with a 96-well microplate reader (Synergy HT, Bio-Tek, Winooski, VM). Cell viability was calculated by dividing the absorbance of cells treated by the absorbance of control (non-treated) cells, and this ratio was expressed as percentage (%).

5.2.11. Measurement of COX-2, IL-1β, IL-2, IL-6, IL-10 and TNF-α

The effect of intestinal-digested fractions of encapsulated and unencapsulated kale treated with Se (40 mg/mL) and S (120 mg/mL) on proinflammatory and anti-inflammatory cytokines was evaluated in RAW 264.7 cells. After incubation of cells with the different treatments, the spontaneous release of COX-2 and IL-10 was determined using the Human/Mouse Total COX-2 DuoSet IC and Mouse IL-10 ELISA kits according to the manufacturer's instructions, respectively. The absorbance values of cytokines were read at 450 nm on a Synergy HT plate reader (Bio-Tek Instruments, Inc., VT, USA). MILLIPLEX MAP Mouse Cytokine / Chemokine panel was used to measure IL-1β, IL-2, IL-6, and TNF-α in supernatant on a Luminex^R 200^{TM} System with xPONENT@3.0 software (Luminex, TX, USA). From the immunoassay, Median Fluorescent Intensity (MFI) data using a polynomial curve-fitting method was used to calculate cytokine/chemokines concentrations as per manufacturers' guidelines.

5.3. Results

5.3.1. Encapsulation efficiency and capsule morphology

The effective encapsulation of phytochemical extracts should result in an encapsulated formula with maximum retaining of the core material inside the dry powder (Jafari et. al, 2008)

Encapsulation with maltodextrin 50/50 showed the highest encapsulation efficiency (69.29-88.43%) (**Table 13**). **Figure 25** shows the morphology of capsules with proportions of 50:50 of the hydroalcoholic extract of kale and maltodextrin, which formed spherical structures and smooth surface, confirming a good encapsulation. Particle size was in the range of 2–600 μm.

Table 13. Encapsulation Efficiency based on the glucosinolate content

	Encapsulation Efficiency (%)		
CTRL 30/70	52.48	±	4.22
CTRL 50/50	69.29	±	3.43
CTRL 70/30	57.50	±	5.09
S 30/70	66.28	±	5.32
S 50/50	85.97	±	7.63
S 70/30	61.23	±	4.56
SE 30/70	58.27	±	4.76
SE 50/50	88.43	±	6.43
SE 70/30	75.01	±	6.23

5.3.2. Influence of encapsulation and storage on lutein, phenolic and glucosinolate compounds

The effect of core-to-wall ratio between kale extract and wall maltodextrin in the encapsulation process is an important factor (**Figure 26**), that may affect the stability of microcapsules during their digestion and storage.

An intermediate core (50:50) ratio resulted in higher stability of phytochemicals in all tested samples, followed by 30:70 and 70:30 core-to-wall ratios. Lutein (54.2%, 25.4%, 9.6%), phenolic compounds (13.5%, 10.1%, 11.4%) and glucosinolates (16.9%, 6.4%, 13.1%) presents in untreated and treated kale sprouts with sulphur and selenium, respectively, suffered a small but significantly loss during encapsulation process with maltodextrin 50:50 compared to non-encapsulated samples.

After storage, there was an additional loss in phytochemicals; however, the 50:50 maltodextrin encapsulation treatment remained as the optimal treatment for their protection. Results are shown in **Figure 27**. Lutein (78.2%, 35.6%, 28.2%), phenolic compounds (34.1%, 20.3%, 25.7%) and glucosinolates (30.2%, 15.4%, 18.9%) presents in encapsulated (50:50) untreated and treated kale sprouts with sulphur and selenium, respectively, suffered a significantly loss after storage of capsules compared to non-encapsulated samples.

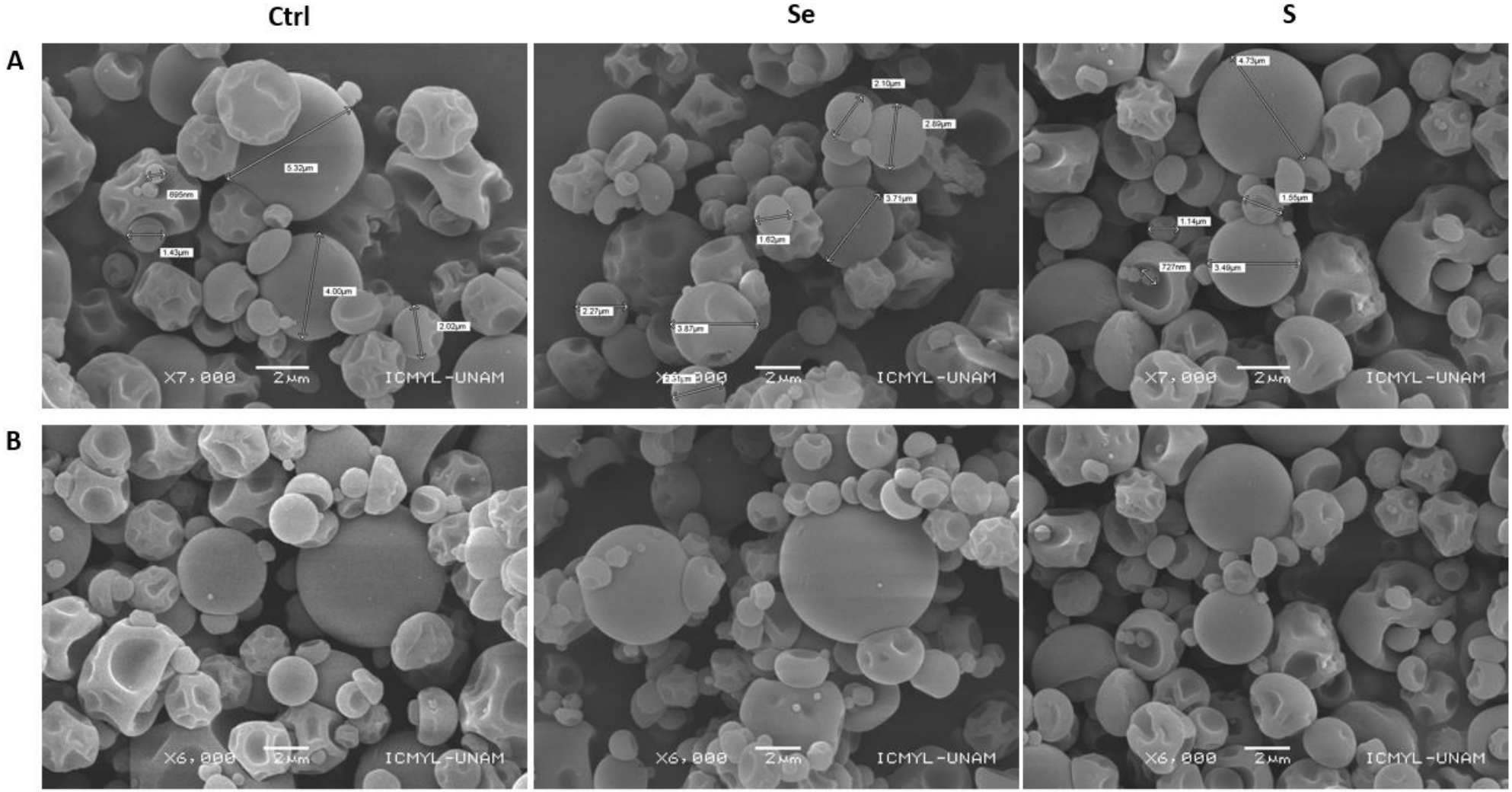

Figure 25. Electron microscopic images showing the morphology of encapsulated ethanolic extracts of 7-day-old Red Russian kale untreated and treated with sulfur (S) and selenium (Se) generated by spray-drying at 7,000× (A) and 6,000× (B) magnifications. Extracts were encapsulated with maltodextrin in ratio 50/50.

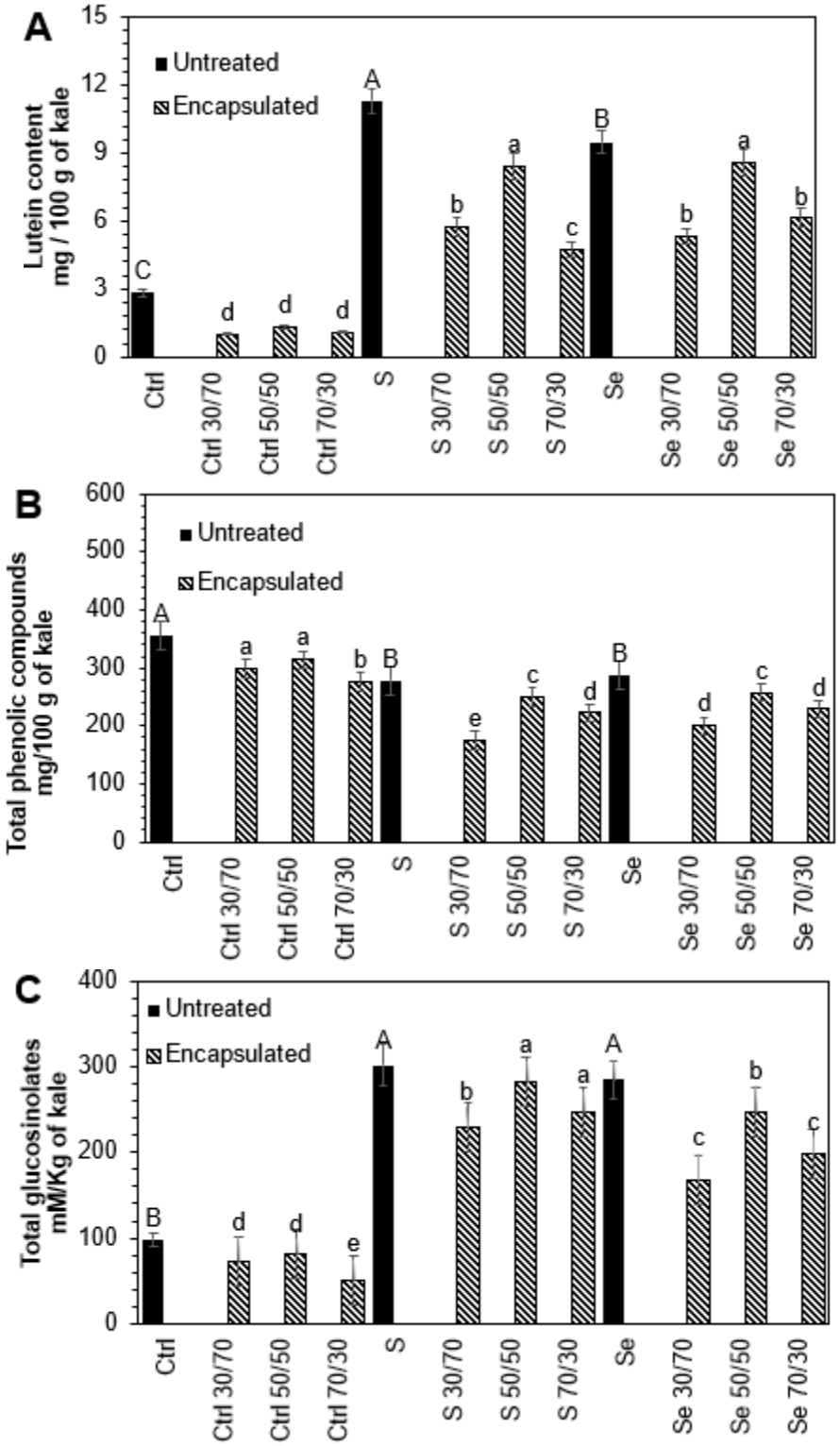

Figure 26. Concentration of A) lutein B) total phenolic compounds and C) total glucosinolates in encapsulated and non-encapsulated extracts of 7-day-old Red Russian kale sprouts untreated and treated with sulfur and selenium. Extracts were encapsulated with maltodextrin in ratio 30/70, 50/50 and 70/30. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical

difference between treatments using the Tukey's HSD test ($p < 0.05$). Abbreviations: control (Ctrl), sulfur (S), selenium (Se).

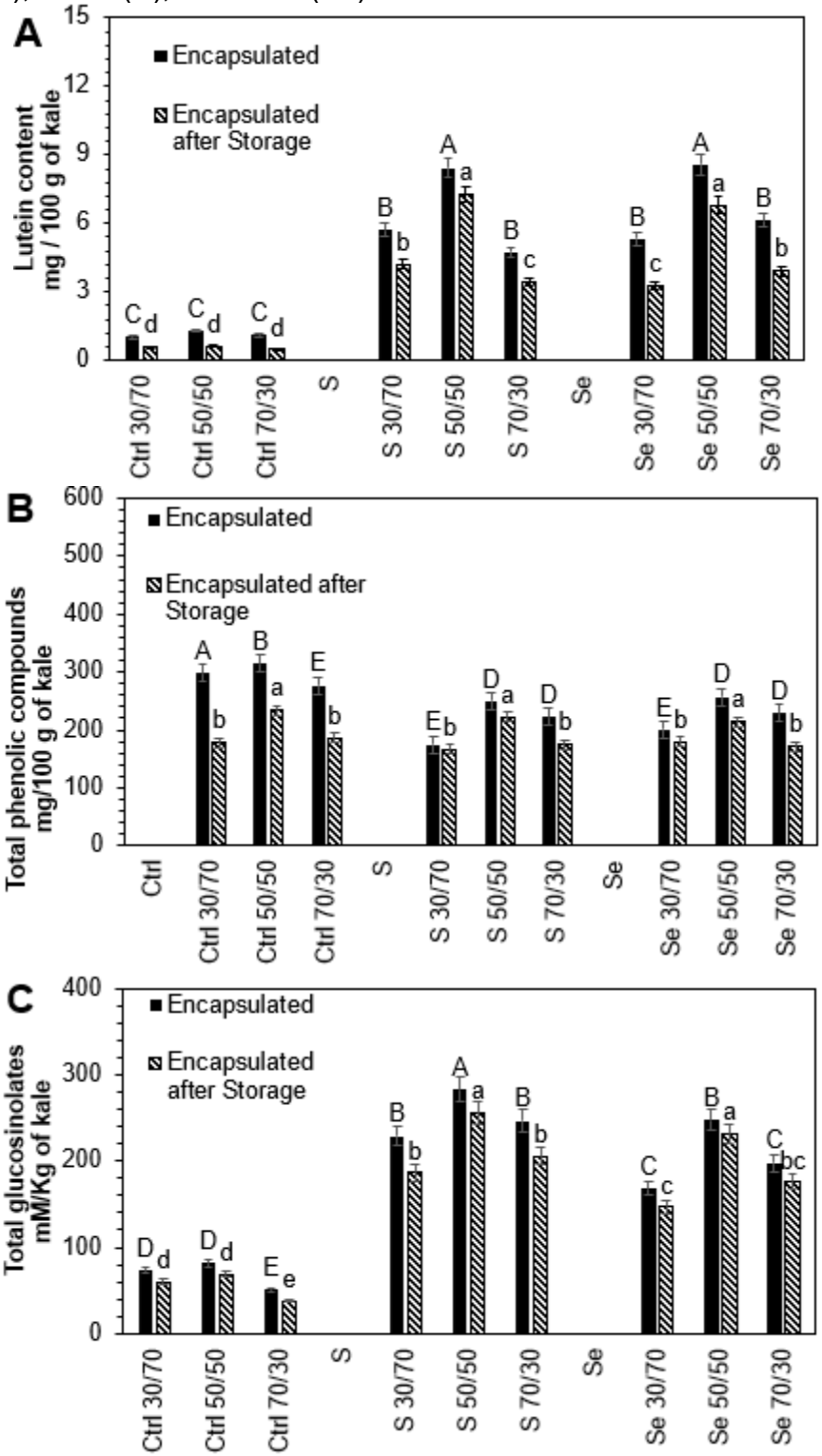

Figure 27. Concentration of A) lutein B) total phenolic compounds and C) total glucosinolates in encapsulated extracts of 7-day-old Red Russian kale sprouts untreated and treated with sulfur and selenium before and after 28 days of storage.

Extracts were encapsulated with maltodextrin in ratio 30/70, 50/50 and 70/30. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Abbreviations: control (Ctrl), sulfur (S), selenium (Se).

5.3.3. Bioaccessibility on lutein, phenolic and glucosinolate compounds

In vitro simulation of gastrointestinal digestion provides important information of how chosen compounds can behave in a physiological environment (Wang et al.,2015).

Liberation of phytochemicals from the food matrix is the first step in the absorption process. Liberation of lutein after simulated saliva solubilization and *in vitro* digestion of non-encapsulated and encapsulated kale sprouts is presented in **Table 14**.

Liberation of lutein from the S and Se-rich kale sprouts and capsule matrix significantly increase its content, through the mouth (24.2%, 35.2%), stomach (31.7%, 40.6%), and intestine (40.1%, 45.5%) fractions, compared with the concentrations of undigested fractions, respectively. Besides, only capsule of Se-rich kale sprout presents a significantly liberation of lutein in contrast with non-encapsulated sample, during *in vitro* digestion: mouth 23.9%, stomach 19.7% and intestine 22.6%.

Comparing the concentrations of phenolic compounds in the undigested fractions of the encapsulated and unencapsulated kale extract with the mouth, stomach, and intestine fractions of digestion in vitro, all 8 identified compounds were found to be significantly lost at all stages. The observed reductions in phenolic compounds are available in **Table 15**. The greatest reduction at the intestinal level was observed in the non-encapsulated control samples: 4-O-CQA (87%), 3-O-H-K (89%), sinapic acid (89%), ferulic acid (88%), 1-S-2-FG (94%), 1,2-diS-2-FG (90%), K-3-O-s-so7-O-g (90%), quercetin (90%)

On the other hand, the Se-rich encapsulated samples had a better stability of phenolic compounds compared to non-encapsulated samples during *in vitro* digestion. At the intestinal level, the additional reductions of phenolic compounds content in non-encapsulated samples compared to Se-rich encapsulated samples were: 4-O-CQA (30%), 3-O-H-K (18%), sinapic acid (26%), ferulic acid (15%), 1-S-2-FG (20%), 1,2-diS-2-FG (22%), K-3-O-s-so7-O-g (43%), quercetin (33%).

Also, individual glucosinolates were identified and quantified in in undigested kale. The observed reductions in glucosinolates are available in **Table 16**. The greatest reduction at the intestinal level was observed in the non-encapsulated control samples: GIB (100%), PRO (100%), GRA (50%), GNP (100%), 4-HGB (100%), GER (100%), GBS (60%), 4-MGBS (75%)

On the other hand, the S and Se-rich encapsulated samples showed a higher stability than non-encapsulated samples at intestinal level for PRO (29%, 35%), GNP (32%, 27%), and 4-MGBS (10%, 13%), respectively.

Table 14. Bioaccessibility of lutein in encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur.

	Non-Encapsulated						Encapsulated					
	Ctrl		S		Se		Ctrl		S		Se	
Undigested	2.8 ± 0.1 b		11.3 ± 1.2 c		9.5 ± 0.4 c		1.3 ± 0.1 b		8.4 ± 0.5 b		8.57 ± 0.2 b	
Mouth	2.9	3.4% ± 0.2 b	12.85	12.1% ± 2.4 b	10.8	11.7% ± 1.5 b	1.4	5.9% ± 0.1 b	11.1	24.2% ± 0.7 b	13.3	35.2% ± 0.4 a
Stomach	3.7	23.4% ± 0.1 a	14.74	23.2% ± 1.7 a	12.0	20.9% ± 1.5 a	1.6	20.9% ± 0.1 a	12.3	31.7% ± 1.6 a	14.4	40.6% ± 1.3 a
Intestine	4.1	31.4% ± 0.1 a	16.67	32.4% ± 2.2 a	12.4	23.1% ± 1.5 a	1.8	28.3% ± 0.1 a	14.2	40.1% ± 1.4 a	15.9	45.4% ± 1.5 a

[1] Concentrations are reported mg per 100 g of kale (DW). [2] Percentage values represents the proportion of liberation of lutein with respect to the control. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Abbreviations: non-encapsulated (NE), encapsulated (E), selenium (S) and sulfur (S).

Table 15. Bioaccessibility of individual phenolic compounds in encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur.

	Non-Encapsulated[1,2,3]						Encapsulated					
	Ctrl	S	Se	Ctrl	S	Se
4-O-CQA						
Undigested	89.2 ± 8.0 a	41.7 ± 3.8 a	68.9 ± 6.2 a	79.2 ± 7.1 a	40.1 ± 3.6 a	59.9 ± 5.4 a
Mouth	68.6 23% ± 6.2 b	39.2 6% ± 3.5 a	53.7 22% ± 4.8 ab	63.3 20% ± 5.7 b	34.8 13% ± 3.1 a	50.5 16% ± 4.5 b
Stomach	46.3 48% ± 4.2 c	24.6 41% ± 2.2 b	39.9 42% ± 3.6 b	44.4 44% ± 4.0 c	27.0 32% ± 2.4 b	41.9 30% ± 3.8 c
Intestine	11.3 87% ± 1.0 d	16.3 61% ± 1.5 c	19.4 72% ± 1.7 c	29.4 63% ± 2.6 d	19.0 53% ± 1.7 c	34.9 42% ± 3.1 d
3-O-H-K						
Undigested	58.2 ± 5.2 a	41.2 ± 3.7 a	36.6 ± 3.3 a	45.2 ± 4.1 a	37.2 ± 3.3 a	33.6 ± 3.0 a
Mouth	43.6 25% ± 3.9 b	33.5 19% ± 3.0 ab	33.2 9% ± 3.0 a	37.1 18% ± 3.3 b	31.6 15% ± 2.8 b	27.4 18% ± 2.5 ab
Stomach	24.5 58% ± 2.2 c	21.8 47% ± 2.0 b	24.6 33% ± 2.2 b	23.3 48% ± 2.1 c	24.1 35% ± 2.2 c	22.5 33% ± 2.0 b
Intestine	6.2 89% ± 0.6 d	15.1 63% ± 1.4 c	17.0 54% ± 1.5 c	15.5 66% ± 1.4 d	19.7 47% ± 1.8 d	21.5 36% ± 1.9 b
Sinapic acid						
Undigested	78.3 ± 7.0 a	37.0 ± 3.3 a	50.3 ± 4.5 a	69.3 ± 6.2 a	24.0 ± 2.2 a	43.3 ± 3.9 a
Mouth	63.0 19% ± 5.7 ab	30.4 18% ± 2.7 b	43.2 14% ± 3.9 a	54.4 21% ± 4.9 b	19.4 19% ± 1.7 b	35.8 17% ± 3.2 a
Stomach	39.6 49% ± 3.6 b	20.2 45% ± 1.8 c	26.6 47% ± 2.4 b	33.8 51% ± 3.0 c	17.8 26% ± 1.6 b	29.3 32% ± 2.6 b
Intestine	8.4 89% ± 0.8 c	14.4 61% ± 1.3 d	17.1 66% ± 1.5 c	27.3 61% ± 2.5 d	8.8 63% ± 0.8 c	25.8 40% ± 2.3 c
Ferulic acid						
Undigested	32.4 ± 2.9 a	29.4 ± 2.6 a	24.6 ± 2.2 a	28.4 ± 2.6 a	27.1 ± 2.4 a	21.6 ± 1.9 a
Mouth	25.2 22% ± 2.3 ab	26.4 10% ± 2.4 a	21.0 15% ± 1.9 a	24.7 13% ± 2.2 a	24.1 11% ± 2.2 a	17.4 20% ± 1.6 b
Stomach	18.8 42% ± 1.7 b	18.2 38% ± 1.6 b	6.5 74% ± 0.6 b	14.9 48% ± 1.3 b	20.0 26% ± 1.8 b	14.1 35% ± 1.3 c
Intestine	3.8 88% ± 0.3 c	13.5 54% ± 1.2 c	8.8 64% ± 0.8 b	9.6 66% ± 0.9 c	11.2 59% ± 1.0 c	10.9 50% ± 1.0 d

Table 15. (Continuation) Bioaccessibility of individual phenolic compounds in encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur.

	Non-Encapsulated[1,2,3]									Encapsulated								
	Ctrl			S			Se			Ctrl			S			Se		
1-S-2-FG																		
Undigested	27.1		± 2.4 a	38.0		± 3.4 a	43.4		± 3.9 a	27.1		± 2.4 a	36.7		± 3.3 a	42.4		± 3.8 a
Mouth	20.1	26%	± 1.8 b	31.6	17%	± 2.8 b	37.6	13%	± 3.4 b	20.7	24%	± 1.9 b	32.2	12%	± 2.9 b	33.0	22%	± 3.0 b
Stomach	6.0	78%	± 0.5 c	10.8	72%	± 1.0 c	17.8	59%	± 1.6 c	11.2	59%	± 1.0 c	26.7	27%	± 2.4 c	28.6	32%	± 2.6 c
Intestine	2.6	90%	± 0.2 cd	6.6	83%	± 0.6 d	15.1	65%	± 1.4 d	7.1	74%	± 0.6 d	15.5	58%	± 1.4 d	23.3	45%	± 2.1 d
1,2-diS-2-FG																		
Undigested	12.0		± 1.1 a	7.3		± 0.7 a	11.6		± 1.0 a	9.0		± 0.8 a	6.0		± 0.5 a	8.6		± 0.8 a
Mouth	9.5	21%	± 0.9 b	5.0	32%	± 0.4 ab	10.2	12%	± 0.9 a	8.2	9%	± 0.7 a	5.5	8%	± 0.5 a	7.8	10%	± 0.7 a
Stomach	4.8	60%	± 0.4 c	3.4	54%	± 0.3 b	6.1	48%	± 0.5 b	4.0	55%	± 0.4 b	5.2	13%	± 0.5 a	5.0	42%	± 0.5 b
Intestine	0.8	94%	± 0.1 d	1.4	80%	± 0.1 c	2.5	79%	± 0.2 c	2.7	70%	± 0.2 c	2.5	59%	± 0.2 b	3.7	57%	± 0.3 c
K-3-O-s-so7-O-g																		
Undigested	34.1		± 3.1 a	43.8		± 3.9 a	27.0		± 2.4 a	37.1		± 3.3 a	41.1		± 3.7 a	24.0		± 2.2 a
Mouth	25.7	25%	± 2.3 b	38.5	12%	± 3.5 b	21.6	20%	± 1.9 b	28.7	23%	± 2.6 b	34.5	16%	± 3.1 b	20.9	13%	± 1.9 a
Stomach	7.8	77%	± 0.7 c	18.2	58%	± 1.6 c	9.3	66%	± 0.8 c	24.8	33%	± 2.2 b	29.8	28%	± 2.7 c	15.8	34%	± 1.4 b
Intestine	3.4	90%	± 0.3 d	8.0	82%	± 0.7 d	4.0	85%	± 0.4 d	5.2	86%	± 0.5 c	19.5	53%	± 1.8 d	14.0	42%	± 1.3 b
Quercetin																		
Undigested	24.2		± 2.2 a	39.0		± 3.5 a	24.3		± 2.2 a	19.2		± 1.7 a	38.0		± 3.4 a	23.3		± 2.1 a
Mouth	17.9	26%	± 1.6 b	29.2	25%	± 2.6 b	19.6	19%	± 1.8 ab	14.3	25%	± 1.3 b	32.9	13%	± 3.0 b	19.7	15%	± 1.8 b
Stomach	5.4	77%	± 0.5 c	17.6	55%	± 1.6 c	8.8	64%	± 0.8 c	8.7	54%	± 0.8 c	25.6	33%	± 2.3 c	14.3	39%	± 1.3 c
Intestine	2.3	90%	± 0.2 d	6.1	84%	± 0.6 d	3.8	84%	± 0.3 d	4.8	75%	± 0.4 d	21.1	44%	± 1.9 d	11.3	52%	± 1.0 d

[1] Concentrations are reported mg per 100 g of kale (DW). [2] Values in parentheses represent the percentage of degradation with respect to the control. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Abbreviations: non-encapsulated (NE), encapsulated (E), selenium (S), sulfur (S), 4-O-caffeoylquinic acid (4-O-CQA), 1-sinapoyl-2′-ferulolylgentiobiose (1-S-2-FG), 1,2-disinapoyl-2-ferulolylgentiobiose (1,2-diS-2-FG), kaempferol 3-*O*-sophoroside-7O-glucoside (K-3-O-s-so7) and 3-O-hexoside kaempferol (3-O-H-K).

Table 16. Bioaccessibility of individual glucosinolates in encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur.

	Non-Encapsulated[1,2,3]												Encapsulated											
	Ctrl				S				Se				Ctrl				S				Se			
GIB																								
Undigested	0.1		± 0.0	a	4.0		± 0.4	a	1.2		± 0.1	a	ND		± 0.0	a	2.4		± 0.2	a	0.5		± 0.0	a
Mouth	ND	100%	± 0.0	b	3.1	23%	± 0.3	b	1.0	16%	± 0.1	b	ND		± 0.0	a	2.1	15%	± 0.2	a	0.4	12%	± 0.0	a
Stomach	ND	100%	± 0.0	b	2.1	46%	± 0.2	c	0.7	41%	± 0.1	c	ND		± 0.0	a	1.4	41%	± 0.1	b	0.3	39%	± 0.0	b
Intestine	ND	100%	± 0.0	b	ND	100%	± 0.0	d	ND	100%	± 0.0	d	ND		± 0.0	a	ND	100%	± 0.0	c	ND	100%	± 0.0	c
PRO																								
Undigested	1.9		± 0.2	a	3.6		± 0.3	a	2.0		± 0.2	a	1.6		± 0.1	a	2.2		± 0.2	a	1.4		± 0.1	a
Mouth	1.5	21%	± 0.1	a	3.1	12%	± 0.3	ab	1.8	10%	± 0.2	a	1.4	11%	± 0.1	a	1.9	10%	± 0.2	a	1.3	9%	± 0.1	a
Stomach	1.0	45%	± 0.1	b	2.2	39%	± 0.2	c	1.3	37%	± 0.1	b	1.0	38%	± 0.1	b	1.5	33%	± 0.1	b	0.9	36%	± 0.1	b
Intestine	ND	100%	± 0.0	c	ND	100%	± 0.0	d	ND	100%	± 0.0	c	0.3	81%	± 0.0	c	0.6	71%	± 0.1	c	0.5	65%	± 0.0	c
GRA																								
Undigested	24.7		± 2.2	a	157.1		± 14.1	a	111.5		± 10.0	a	23.5		± 2.1	a	149.6		± 13.5	a	103.5		± 9.3	a
Mouth	18.8	24%	± 1.7	b	138.5	12%	± 12.5	b	93.8	16%	± 8.4	a	18.8	20%	± 1.7	a	132.2	12%	± 11.9	a	88.9	14%	± 8.0	a
Stomach	13.1	47%	± 1.2	c	97.0	38%	± 8.7	c	84.4	24%	± 7.6	b	15.2	35%	± 1.4	b	99.3	34%	± 8.9	b	80.1	23%	± 7.2	b
Intestine	12.3	50%	± 1.1	c	82.9	47%	± 7.5	d	75.1	33%	± 6.8	b	6.5	72%	± 0.6	c	85.0	43%	± 7.7	c	73.3	29%	± 6.6	b
GNP																								
Undigested	8.9		± 0.8	a	9.5		± 0.9	a	11.6		± 1.0	a	6.9		± 0.6	a	8.6		± 0.8	a	10.6		± 1.0	a
Mouth	6.3	29%	± 0.6	b	8.6	9%	± 0.8	b	9.2	21%	± 0.8	a	5.1	26%	± 0.5	a	7.9	8%	± 0.7	a	9.5	11%	± 0.9	b
Stomach	4.4	50%	± 0.4	c	6.0	37%	± 0.5	c	6.5	44%	± 0.6	b	4.6	34%	± 0.4	b	6.4	25%	± 0.6	b	6.6	37%	± 0.6	c
Intestine	ND	100%	± 0.0	d	ND	100%	± 0.0	d	ND	100%	± 0.0	b	2.0	72%	± 0.2	c	2.8	68%	± 0.2	c	2.9	73%	± 0.3	d

Table 16. (Continuation) Bioaccessibility of individual glucosinolates in encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur.

	Non-Encapsulated[1,2,3]												Encapsulated											
	Ctrl				S				Se				Ctrl				S				Se			
4-HGB																								
Undigested	0.1		± 0.0	a	0.7		± 0.1	a	0.4		± 0.0	a	ND		± 0.0	a	0.4		± 0.0	a	1.2		± 0.1	a
Mouth	0.1	20%	± 0.0	a	0.4	49%	± 0.0	b	0.0	100%	± 0.0	b	ND		± 0.0	a	0.3	67%	± 0.0	b	1.1	10%	± 0.1	b
Stomach	0.0	56%	± 0.0	b	0.3	65%	± 0.0	b	0.0	100%	± 0.0	b	ND		± 0.0	a	ND	100%	± 0.0	c	0.8	37%	± 0.1	c
Intestine	ND	100%	± 0.0	c	ND	100%	± 0.0	c	0.0	100%	± 0.0	b	ND		± 0.0	a	ND	100%	± 0.0	c	ND	100%	± 0.0	d
GER																								
Undigested	0.1		± 0.0	a	0.2		± 0.0	a	0.4		± 0.0	a	ND		± 0.0	a	0.5		± 0.0	a	0.4		± 0.0	a
Mouth	0.1	27%	± 0.0	a	0.2	14%	± 0.0	a	0.3	21%	± 0.0	b	ND		± 0.0	a	0.5	9%	± 0.0	a	0.2	34%	± 0.0	a
Stomach	0.1	76%	± 0.0	a	0.1	45%	± 0.0	b	0.1	52%	± 0.0	c	ND		± 0.0	a	0.3	37%	± 0.0	b	ND	100%	± 0.0	b
Intestine	ND	100%	± 0.0	a	ND	100%	± 0.0	c	ND	100%	± 0.0	d	ND		± 0.0	a	ND	100%	± 0.0	c	ND	100%	± 0.0	c
GBS																								
Undigested	5.0		± 0.5	a	20.8		± 1.9	a	10.8		± 1.0	a	2.7		± 0.2	a	16.1		± 1.5	a	9.5		± 0.9	a
Mouth	3.7	26%	± 0.3	b	16.5	21%	± 1.5	b	8.2	24%	± 0.7	b	2.0	24%	± 0.2	a	14.0	13%	± 1.3	a	8.6	9%	± 0.8	a
Stomach	3.4	33%	± 0.3	b	13.2	37%	± 1.2	c	7.8	28%	± 0.7	b	1.8	31%	± 0.2	b	11.2	30%	± 1.0	b	6.0	36%	± 0.5	b
Intestine	2.0	60%	± 0.2	c	12.0	42%	± 1.1	c	4.7	56%	± 0.4	c	1.6	38%	± 0.1	b	9.3	42%	± 0.8	c	5.9	38%	± 0.5	b
4-MGBS																								
Undigested	16.4		± 1.5	a	33.6		± 3.0	a	59.5		± 5.4	a	14.5		± 1.3	a	28.5		± 2.6	a	49.2		± 4.4	a
Mouth	12.5	24%	± 1.1	b	27.6	18%	± 2.5	b	48.5	18%	± 4.4	b	11.9	18%	± 1.1	a	23.3	19%	± 2.1	a	41.9	15%	± 3.8	b
Stomach	8.8	47%	± 0.8	c	19.3	43%	± 1.7	c	34.0	43%	± 3.1	c	9.3	36%	± 0.8	b	18.4	35%	± 1.7	b	32.4	34%	± 2.9	c
Intestine	4.1	75%	± 0.4	d	14.4	57%	± 1.3	d	9.3	84%	± 0.8	d	4.0	72%	± 0.4	c	14.4	49%	± 1.3	c	13.9	72%	± 1.3	d

[1] Concentrations are reported mM per 100 g of kale (DW). [2] Values in parentheses represent the percentage of degradation with respect to the control. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Abbreviations: ND (No detected), non-encapsulated (NE), encapsulated (E), selenium (S), sulfur (S), glucoiberin (GIB), progoitrin (PRO), glucoraphanin (GRA), 4-hydroxy-glucobrassicin (4-HGB), glucobrassicin (GBS), 4-methoxy-glucobrassicin (4-MGBS), glucoeurocin (GER), gluconapin (GNP), and neoglucobrassicin (NGBS).

5.3.4. Cellular permeability

The integrity of the cell monolayers was assessed by monitoring LY permeation. LY cannot freely permeate lipophilic barriers and, therefore, it serves as a marker of paracellular transport. Only monolayers with LY permeability lower than 5% were considered for the experiment. The data obtained are presented in **Table 17**.

After 2 h, the phytochemical compounds present both in the apical and basolateral compartment were analyzed. HPLC-DAD analysis of the polyphenols permeating to the basolateral side after 120 min revealed the presence of 5 identified compounds: lutein, sulforaphane, ferulic acid, 3-O-hexoside kaempferol and quercetin.

The highest basolateral recovery of phytochemicals was found in Se encapsulated kale samples. Lutein, sulforaphane, ferulic acid, 3-O-hexoside kaempferol and quercetin recovery ranged between 8.4%–67.4%.

Besides, results showed the emergence two unidentified peaks, tentatively derived from acid ferulic.

5.3.5. Cellular antioxidant activity

As we known, many biologically active molecules in plants may contribute to antioxidant capacities, and kale is not an exception. Cellular antioxidant activity of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with Se and S in Caco-2 cells is shown in **Figure 28**.

There was a significant increase in cellular antioxidant activity in non-encapsulated and encapsulated samples treated with S 200 (19.7%, 43.7%), S 240 (15.5%, 43.7%), Se 200 (28.9%, 46.5%) and Se 240 (13.8%, 42.3%), compared to control, respectively.

Although Se and S treatments were not significantly different, the process of encapsulation had a positive effect on cellular antioxidant activity. Encapsulated samples S 200 (23.9%), S 240 (28.2%), Se 200 (17.6%) and Se 240 (28.4%) show a significant increase compared with their respective non-encapsulated pair.

Table 17. Cellular permeability of digested encapsulated and non-encapsulated 7-day-old Red Russian kale sprouts treated with selenium and sulfur in Caco-2 cells.

Non-Encapsulated	Lutein		Sulforaphane		Ferulic Acid		3-O-H-K		Quercetin	
Ctrl	1.2	22.4% ± 0.1 c	3.7	17.9% ± 0.4 d	1.9	10.4% ± 0.2 d	1.1	9.4% ± 0.1 d	0.7	8.4% ± 0.1 d
S	1.3	25.2% ± 1.2 c	24.9	34.2% ± 2.4 c	4.5	16.4% ± 0.4 d	4.1	14.8% ± 0.4 c	1.8	13.3% ± 0.2 d
Se	1.6	27.4% ± 0.4 c	52.6	36.3% ± 5.1 b	11.9	25.5% ± 1.2 b	6.2	23.0% ± 0.6 b	2.6	20.7% ± 0.3 c
Encapsulated										
Ctrl	1.1	20.2% ± 0.1 c	3.9	19.2% ± 0.4 d	9.3	38.4% ± 0.9 c	5.8	34.6% ± 0.6 c	2.9	31.1% ± 0.3 c
S	8.7	45.0% ± 0.5 b	59.5	46.3% ± 5.8 a	13.8	43.3% ± 1.3 b	7.8	39.0% ± 0.8 b	14.8	35.1% ± 1.4 a
Se	10.0	57.4% ± 0.2 a	66.0	67.4% ± 6.4 a	19.3	54.5% ± 1.9 a	9.8	49.1% ± 1.0 a	10.2	44.1% ± 1.0 b

[1] Concentrations are reported mg per 100 g of kale (DW). [2] Percentage values represents the proportion of recovery of each compound in basal surface with respect to the initial concentration. [3] Different letters in the same column indicate statistical differences in the concentration of each compound between treatments using the least significant difference (LSD) test ($p < 0.05$). Abbreviations: 3-O-hexoside kaempferol (3-O-H-K), non-encapsulated (NE), encapsulated (E), selenium (S) and sulfur (S).

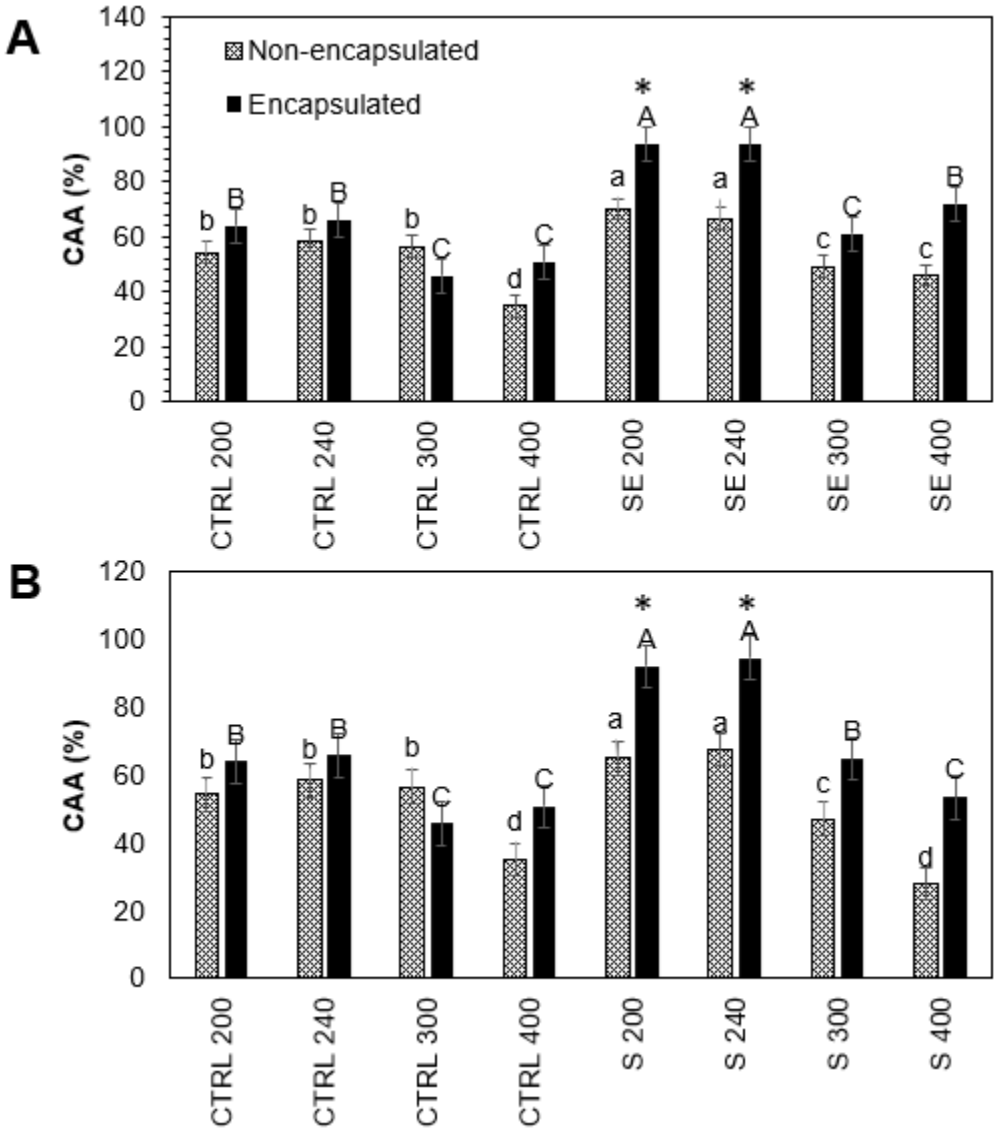

Figure 28. Cellular antioxidant activity of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with A) selenium and B) sulfur after 7 days of germination in Caco-2 cells. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between Se and S treated samples. Abbreviations: control (Ctrl), sulfur (S), selenium (Se).

5.3.6. Nitric oxide inhibition

Nitric oxide inhibition of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with Se and S in Caco-2 cells and Raw 264.7 cells is shown in **Figure 29-30**.

Raw 264.7 cells showed a significant increase in nitric oxide inhibition in non-encapsulated and encapsulated samples treated with all S and Se extracts concentrations tested. The maximum nitric oxide inhibition was observed with non-encapsulated and encapsulated S 50 (51.1%, 115.2%) and Se 50 (58.6%, 143.1%) samples compared to control, respectively. On the other hand, the highest increase of nitric oxide inhibition in Caco-2 cells was detected with non-encapsulated and encapsulated samples S 240 (42.1%, 80.9%) and Se 240 (34.3%, 58.8%), followed S 200 (41.6%, 64.9%) and Se 200 (13.3%, 41.5%), compared to control, respectively.

Although Se and S treatments were not significantly different in Raw 264.7 cells, the process of encapsulation had a positive effect on cellular antioxidant activity. Encapsulated samples S 50 (64.1%) and Se 50 (84.3%) showed a significant increase compared with their respective non-encapsulated pair. In the case of Caco-2 cells, encapsulation showed a significant increase in S 200 (23.2%), S 240 (38.9%), Se 200 (28.2%) and Se 240 (24.5%) samples.

5.4.7. Measurement of COX-2, IL-1β, IL-2, IL-6, IL-10 and TNF-α

Thus, as the final step of our investigation, we decided to verify the influence of the sulphur and selenium-enriched kale sprouts extracts on the release of the selected pro-inflammatory mediators (COX-2, IL-1β, IL-2, IL-6, IL-10 and TNF-α) in LPS-stimulated RAW 264.7 macrophages. Results are shown **Figure 31.**

Raw 264.7 cells showed a significant inhibition in pro-inflammatory cytokines non-encapsulated and encapsulated kale samples, including the control, whereas IL-10 was stimulated. There was a significant increase of 24.1% and 13.9% of IL-10 in S and Se encapsulated kale samples respect to the non-encapsulated samples. COX-2 and TNF-α showed a significant inhibition of 50.6% and 51.4% in S encapsulated kale samples compared to S non-encapsulated kale samples, respectively, whereas a significant inhibition of 21.2% and 32.5% was observed in IL-6 in S and Se-encapsulated kale samples, respectively.

Furthermore, the maximum inhibition was observed with S and Se encapsulated kale samples in the corresponding COX-2 (84.1%, 85.7%), IL-1β (28.5%, 29.6%), IL-2 (31.5%, 23.4%), IL-6 (36.6%, 54.3%) and TNF-α (75.1%, 90.1%) cytokines, respectively, compared to control stimulated with LPS. Whereas IL-10 concentration increased significantly 88.9% and 74.6% with S and Se encapsulated kale samples, respectively.

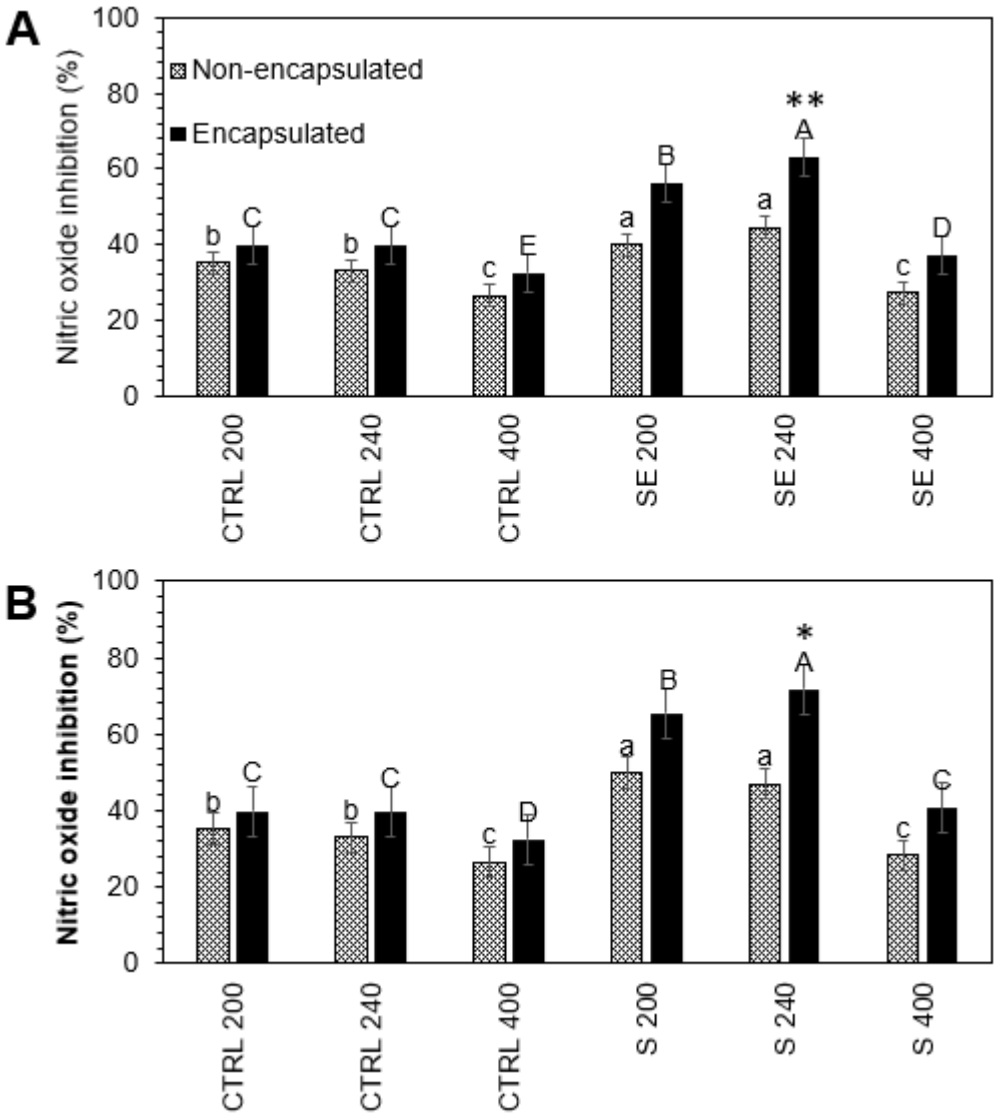

Figure 29. Effect of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with A) selenium and B) sulfur after 7 days of germination on nitric oxide production by Caco-2 cells stimulated with 1 µg/mL lipopolysaccharide. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between Se and S treated samples. Abbreviations: control (Ctrl), sulfur (S), selenium (Se).

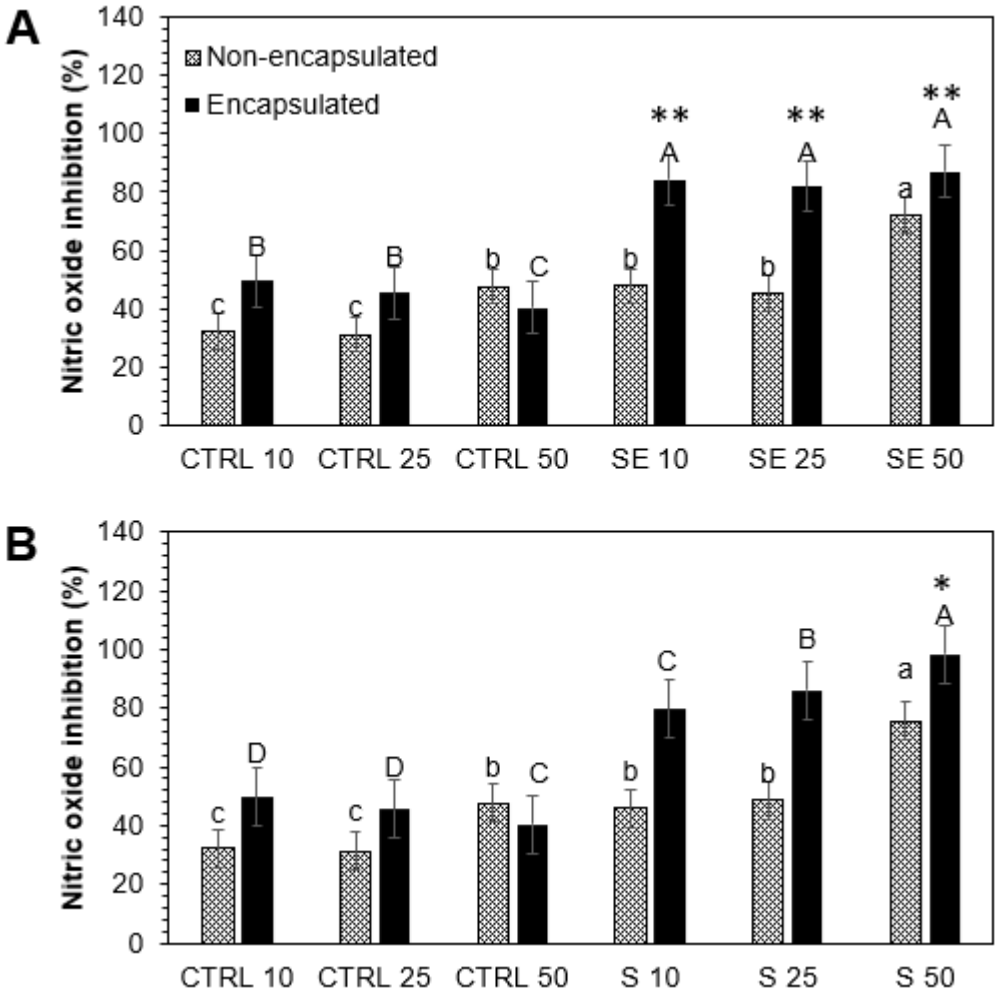

Figure 30. Effect of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with A) selenium and B) sulfur after 7 days of germination on nitric oxide production by macrophage Raw 264.7 cells stimulated with 1 µg/mL lipopolysaccharide. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between treatments using the Tukey's HSD test ($p < 0.05$). Asterisk (*) indicate statistical difference determined by a t-test ($p < 0.050$) between Se and S treated samples. Abbreviations: control (Ctrl), sulfur (S), selenium (Se).

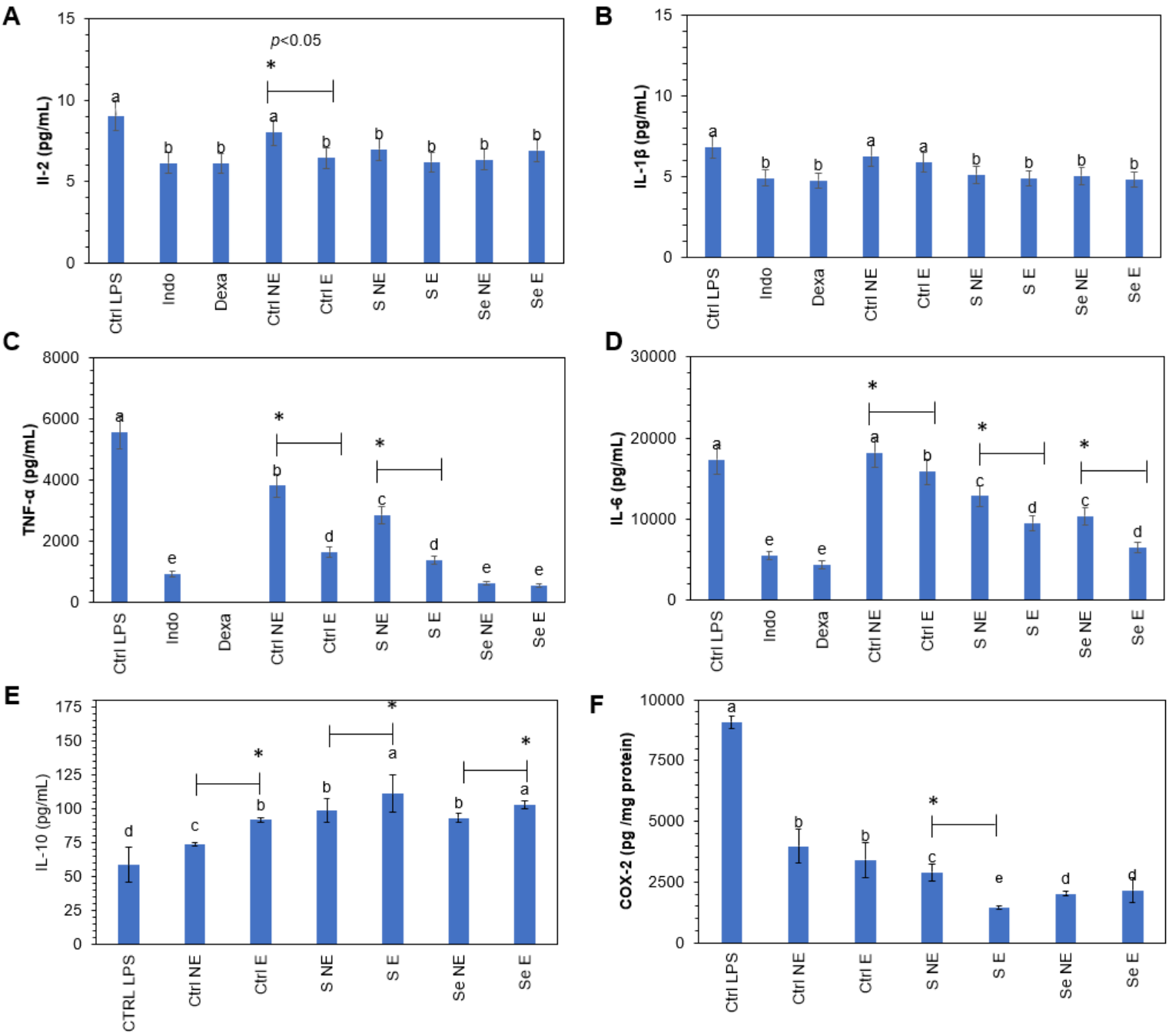

Figure 31. Effect of encapsulated and non-encapsulated intestinal digested fractions of Red Russian kale sprouts treated with selenium and sulfur after 7 days of germination on (A) IL-2, (B) IL-1β, (C) TNF-α, (D) IL-6, (E) IL-10 and (F) COX-2 production by macrophage Raw 264.7 cells stimulated with 1 μg/mL lipopolysaccharide. Bars are means of 3 replicates ± standard error. Different letters among bars indicate statistical difference between all treatments using the Tukey's HSD test ($p < 0.05$). Asterisk (*) indicate high statistical difference between encapsulated and non-encapsulated kale pairs samples. determined by a t-test ($p < 0.050$) Abbreviations: control (Ctrl), sulfur (S), selenium (Se), lipopolysaccharide (LPS), encapsulated (E), non-encapsulated (NE).

5.4. Discussion

5.4.1. Encapsulation characterization

Possibly, it is the optimal concentration for non-saturation of the active maltodextrin sites, which permitted a total encapsulation of the combined extract, as well as an additional protection of the compounds (Radünz et al., 2020). This result was superior to the reported by Tian et al. (2015) and Wu, Zou, Mao, Huang and Liu (2014), who reported encapsulation efficiencies between 12% and 77% of glucosinolate and isothiocyanate extract derived from broccoli by spray drying, using maltodextrin. Possibly, this difference in efficiency is due to coating materials as maltodextrin exhibit greater efficacy for water- soluble compounds (Tomsone et al., 2020).

Previous studies using maltodextrin as wall material of broccoli extract by spray drying have reported irregular and rough surface due to the high temperature employed which causes a rapid evaporation of the droplets (Tian et al., 2015; Wu, Zou, Mao, Huang, & Liu, 2014).

5.4.2. Influence of encapsulation and storage on lutein, phenolic and glucosinolate compounds

The stability of phytochemicals encapsulated is dependent on the interaction between core-to-wall ratio and type of wall material. It has been demonstrated that maltodextrin can provide good oxidative stability to encapsulated olive oil and was also found to be effective in protecting the carotenoids of paprika oleoresin (Calvo, 2012; Barbosa-Santos et al., 2006). Likewise, it has been informed that maltodextrin is an effective protector of phenolic extracts and is able to preserve their antioxidant activity (Sansone et al., 2011).

The degradation of phytochemicals during encapsulation process is mainly due to direct exposition to heat (Wu et al., 2014).

Our results of storage stability may be explained based on maltodextrin is capable of create a dense and oxygen-impermeable wall system. This has been reported previously by Cai et al., (2000), who observed a better storage stability for betacyanins using maltodextrin as encapsulant after 16 weeks. In this way, treatment with 30:70 ratio, where phytochemicals are near to the outer surface of the capsule led to a greater loss. Since, the wall is thinner compared to core, it is possible for phytochemical to react with the outside oxygen.

5.4.3. Bioaccessibility on lutein, phenolic and glucosinolate compounds

Lutein liberation and *in vitro* accessibility were half-fold higher from encapsulated compared to non-encapsulated samples. These results are in accordance with Eriksen, et al., (2017) and Moelants et al., (2012), who reported a carotenoid liberation from spinach and carrots *in vitro* accessibility.

In contrast to lutein, digestion caused a drop in the amount of phenolics. These results are in agreement with what was reported by Oidtmannet al., (2012), Betzet al., (2012), Wanget al., (2013), Flores et al., (2014), who report a decrease in total phenolics in indigestion conditions. However, there is currently a lack of data from the literature on the estimation of phenolics in encapsulated kale extracts after in vitro digestion.

Losses found for almost all phenolic compounds analyzed possibly resulted from a structural breakdown by the action of enzymes and pH changes throughout the digestive processes. Also, the high affinity of phenolics to maltodextrin through non-covalently bind, including interactions between hydrophobic groups (Jakobek et al., 2015), may possibly stabilize the polyphenol structure and properties, such as flexibility and weight of molecules (Nikoli et al., 2019).

Similarly, reductions in glucosinolate compounds in all samples were found during *in vitro* digestion. Changes in concentration may be due to a restructuring with other compounds, such as sulforaphane. Once digestion begins, glucosinolates go through enzymatic hydrolysis by action of myrosinase, and are hydrolyzed to isothiocyanate (Corona et al., 2014)

Thus, an *in vitro* digestion proved that encapsulation is a method for improving phytochemical stability of kale, leading to less reduction, as well to their better bioaccessibility.

5.4.4. Cellular permeability

The basolateral side of the intestinal permeability experiment represents the basal surface of the membrane that mediates the transport of nutrients from cell to surrounding fluids that lead to the circulatory system (X).

In the cases of glucosinolates quantification, both glucosinolates and the hydrolysis products were present in the intestinal digesta at 2 h. However, only the hydrolysis products were analyzed in the Caco-2 cells because intact glucosinolates are not absorbed through the intestinal epithelial cells.

Also, the presence of two unidentified peaks, tentatively derived from acid ferulic, in the basolateral compartment and not in the initial sample suggests it resulted from cell metabolism. Kern et al., (2003) have reported that Caco-2 cells can absorb ferulic acid, p-coumaric acid and sinapic acid, and conjugate them by sulfation or glucuronidation before their excretion.

Although, there is not much information of effect of maltodextrin in cellular permeability, Pham et al., (2018) reported an improved gut barrier integrity due to the use of maltodextrin.

Finally, it is worth to mention that research had shown that organics selenium in natural foods has a high bioavailability and is safe to consume (Mahan et al., 2014). Li et al., (2018) have demonstrated that selenium is easily absorbed in an *in vivo* of

selenium deficiency. However, it is necessary to perform future test to quantify the association of Se to phytochemicals.

5.4.5. Antioxidant and anti-inflammatory activities

Our results indicate also that antioxidant and anti-inflammatory activities of the Se and S tested sprouts, evaluated by cellular method were positively correlated with the release of lutein and sulforaphane in the extracts, as well as the remanent content of phenolic and glucosinolate compounds, which is higher than in control samples. The influence of lutein, glucosinolates and phenolic compounds in neutralizing free radicals, quenching singlet oxygen, or decomposing peroxides through their chemical structure has been reported previously (Merhan et al., 2017; Chen et al., 2020; Natella et al; 2014); whereas the antioxidant activity of sulforaphane is mainly associated with transcription triggering of phase II metabolic enzymes (Sánchez-Pujante et al., 2017). Additionally, the antioxidant properties of phytochemical extracts in S- and Se-enriched Brassica plants such as broccoli (Ramos et al., 2011), kale (Ortega-Hernández, 2022; Paśko et al., 2022), and garden cres (Frias et al., 2010) have been confirmed.

On the other hand, I tested the inhibitory effects of kale on the production of NOx and the cytokines COX-2, IL-1β, IL-2, IL-6, IL-10 and TNF-α by RAW 264.7 macrophage cells to confirm the anti-inflammatory activity *in vitro*.

The extract considerably inhibited NOx production by LPS-induced macrophages. Also, I compared the effect of kale extract with two anti-inflammatory agents (dexamethasone and indomethacin) as reference drugs on the inhibition of cytokines.

Macrophages treated with kale extract showed an inhibition in the production of COX-2, IL-1β, IL-2, IL-6, and TNF-α by LPS-stimulated RAW 264.7cells with the same potency that dexamethasone and indomethacin.

First, the observed tendency to decrease the anti-inflammatory cytokines might be explained by the potential of present phenolics and lutein to suppress iNOS protein by inhibiting nuclear factor-κB (NF-κB), which is a main transcription factor for the activation of genes associated with the inflammatory mediators, such as interleukins, inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs) (Ahn & Kim, 2021; Hwang & Lim, 2014; Shahidi & Yeo, 2018). Additionally, the chemical structure and lutein and phenolics compounds allows them scavenging NOx radicals (Ambriz-Pérez, Leyva-López, Gutierrez-Grijalva, & Heredia, 2016; Stringham & Stringham, 2015; Tsai et al., 2018). The potential of lutein, kaempferol, acid ferulic and quercetin as anti-inflammatory agents has been reported previously (Ahn & Kim, 2021; Shahidi & Yeo, 2018).

On the other hand, lutein and sulforaphane, are capable to activate the nuclear factor erythroid 2–related factor 2 (Nrf2) and Nrf2 signaling-related antioxidant enzymes

(glutathione-s-transferase, glutathione peroxidase, superoxide dismutase and catalase) (Ahn & Kim, 2021; Chang et al., 2011).

Also, the increment in IL-10, an anti-inflammatory cytokine, may possibly inhibit the NF-kB translocation (Arranz, Mes, et al.,2015) and further production of the pro-inflammatory cytokines.

Finally, it is possible that maltodextrin had protected the compounds acting on the inhibition of free radicals during digestion, demonstrating that encapsulation, is an effective method to preserve the antioxidant and anti-inflammatory activities of kale.

5.5. Conclusions

In this study, we characterized the carotenoid, glucosinolate and phenolic profile of kale sprouts (*Brassica oleracea* var. acephala) by HPLC-DAD. Additionally, using an *in vitro* gastrointestinal methodology, we determined the digestive stability and bioaccessibility of their most relevant antioxidants. We reported a total of 19 antioxidants, which were composed of 9 phenolic acids, 9 glucosinolates, and 1 carotenoid. We found that these phytochemicals were more abundant in kale treated with Se and S where they presented a high bioaccessibility and bioactivity.

Furthermore, encapsulation protected carotenoids, phenolics and glucosinolates from its degradation during simulated digestion, without negatively affecting the permeability through the Caco-2 monolayer. Likewise, the encapsulated kale extract demonstrated better anti-inflammatory effects *in vitro* by analyzing mediators of inflammation (IL-2, IL-1β, TNF-α, IL-6, IL-10 and COX-2). Therefore, this kale-based powder could be a better drug candidate or adjunct for the relief of various types of inflammation corticosteroids. Further research evaluating the role of these bioactive compounds in the gut microbiota and *in vivo* studies should be considered, in order to have a better understanding on the potential effect of kale extracts in human health.

CHAPTER 6. GENERAL CONCLUSIONS

In this work, the health benefits of kale related to its main phytochemicals (phenolics, carotenoids and glucosinolates) were discussed. Literature supports that kale can be considered a super-food due to its high content of phytochemicals and several studies supporting their pharmacological activity. Herein, different controlled abiotic stress conditions that affect the content of secondary metabolites of nutraceutical importance in kale were also discussed. For instance, results presented in Chapter 3 showed that the application of selenium, sulfur and methyl jasmonate can influence the biosynthesis and accumulation of secondary metabolites in kale sprouts. Additionally, the study shown in Chapter 4 revealed that Se, S and exogenous MeJA application have a great impact in the bioactivity *in vitro*. Furthermore, in Chapter 5 it was proposed the encapsulation as an additional process to ensure the shelf life of the phytochemical kale extract and stabilize its bioactive properties.

Thus, results presented in Chapter 3 indicate that pre-harvest abiotic stresses could be used as an effective and simple strategy to use kale sprouts as biofactories of high-value compounds. Saline stress (S and Na_2SeO_3) and exogenous phytohormone (MeJA) can be applied to improve glucosinolate and carotenoid concentration (mainly xanthophylls. Results from this study allowed the possibility to test higher doses of salts and phytohormones without negatively affecting plant growth.

The most effective treatments to induce the biosynthesis of lutein were Se 40 and S 120 after 7 d of germination for Dwarf green kale and Red Russian, respectively. The highest glucosinolate accumulation occurred in Red Russian kale variety after 7 days of germination with S 120 mg/L, increasing glucoraphanin, glucoerucin, 4-methoxy-glucobrassicin, and glucoiberin. These results allowed the elucidation of strategies to induce the accumulation of specific phytochemicals.

It would be interesting to evaluate the molecular mechanisms governing the effects of saline stress on the biosynthesis of phytochemicals in kale sprouts. It is important characterize the production of signaling molecules in kale sprouts treated with different doses of Se or S to elucidate their role in the metabolic pathways involved in the biosynthesis of secondary metabolites. Additionally, a study of gene expression and enzymes involved in specific biosynthetic pathways is necessary to better understand the physiological response of kale sprouts towards stress treatments. These recommendations could help to design more efficient strategies to exploit kale as a biofactory of nutraceutical compounds.

On the other hand, despite all the evidence validating secondary metabolism elicitation through abiotic stresses, it is not yet applied at an industrial scale to produce kale. Thus, it is highly relevant to transfer this knowledge to kale producers to generate a product with a higher potential to prevent chronic and degenerative diseases.

The information reviewed in this thesis also is a starting point to validate the effects of abiotically stressed kale on the prevention and treatment of chronic inflammation through *in vitro* bioassays.

In Chapter 4 and 5, the results presented demonstrate that the kale extract presents antioxidant and anti-inflammatory effects *in vitro*. The presence of lutein, glucosinolates and phenolic compounds would explain this activity. Kale have therefore a therapeutic potential that supports its use in traditional medicine and could thus be developed as a commercial anti-inflammatory. The signaling mechanism by which the extract exerts its anti-inflammatory effects on immune cell functions *in vitro* now needs to be investigated.

The results showed that kale sprouts treated with Se and S showed highest encapsulation efficiency and best morphology. Digestion affected the content of compounds in both encapsulated and non-encapsulated extracts. However, the encapsulation promoted the control of oxidative processes during storage, and kale extracts germinated with Se and S showed less degradation of compounds compared to the control. Besides, the encapsulate extract treated with S exerted the highest antioxidant capacity and anti-inflammatory activity by stimulating IL-10 and inhibiting COX-2 and NO production. Therefore, kale sprouts with enhanced levels of phytochemicals could be used as feedstock to produce processed foods or undergo further processing for extraction and purification of high-value health-promoting biomolecules.

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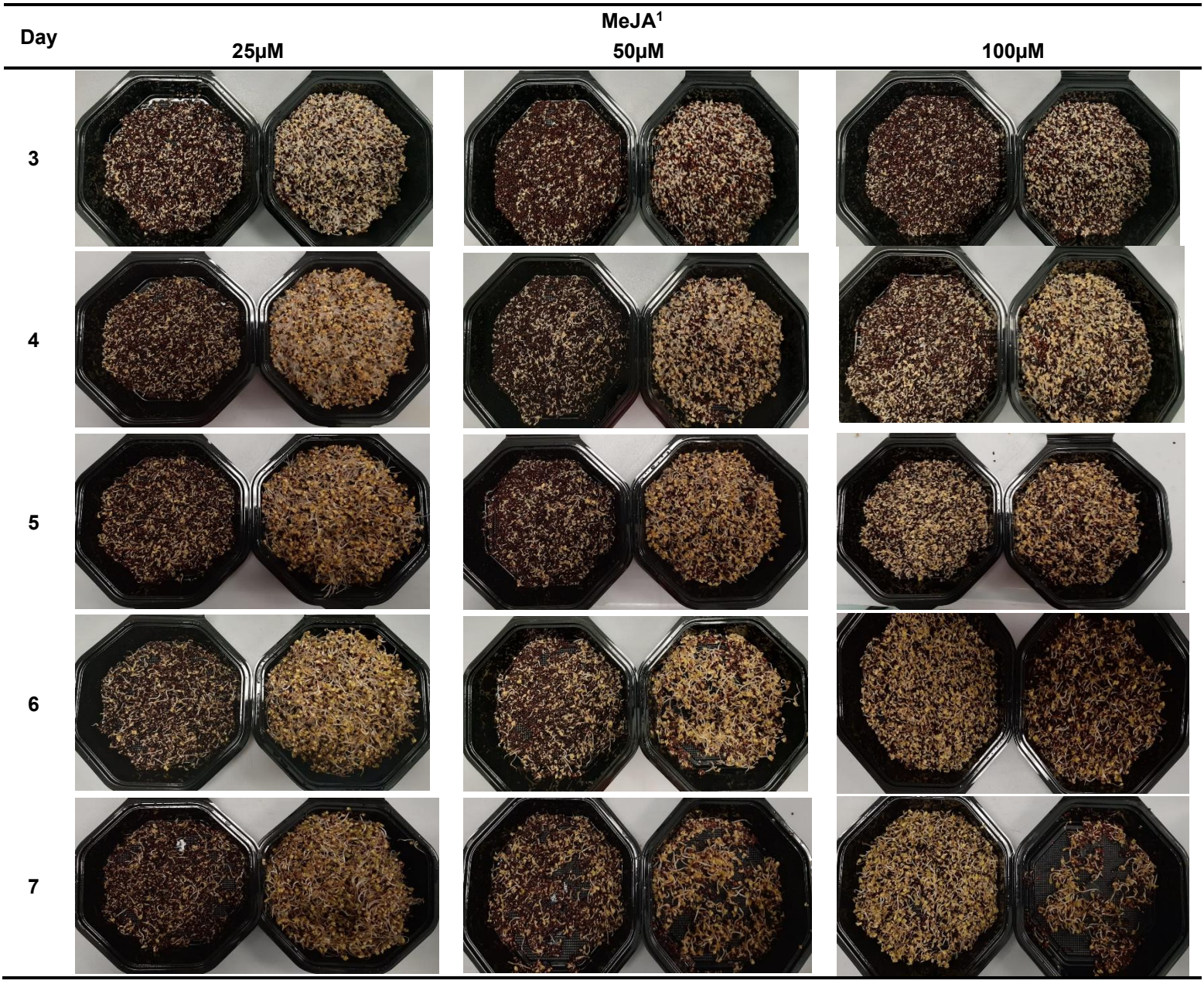
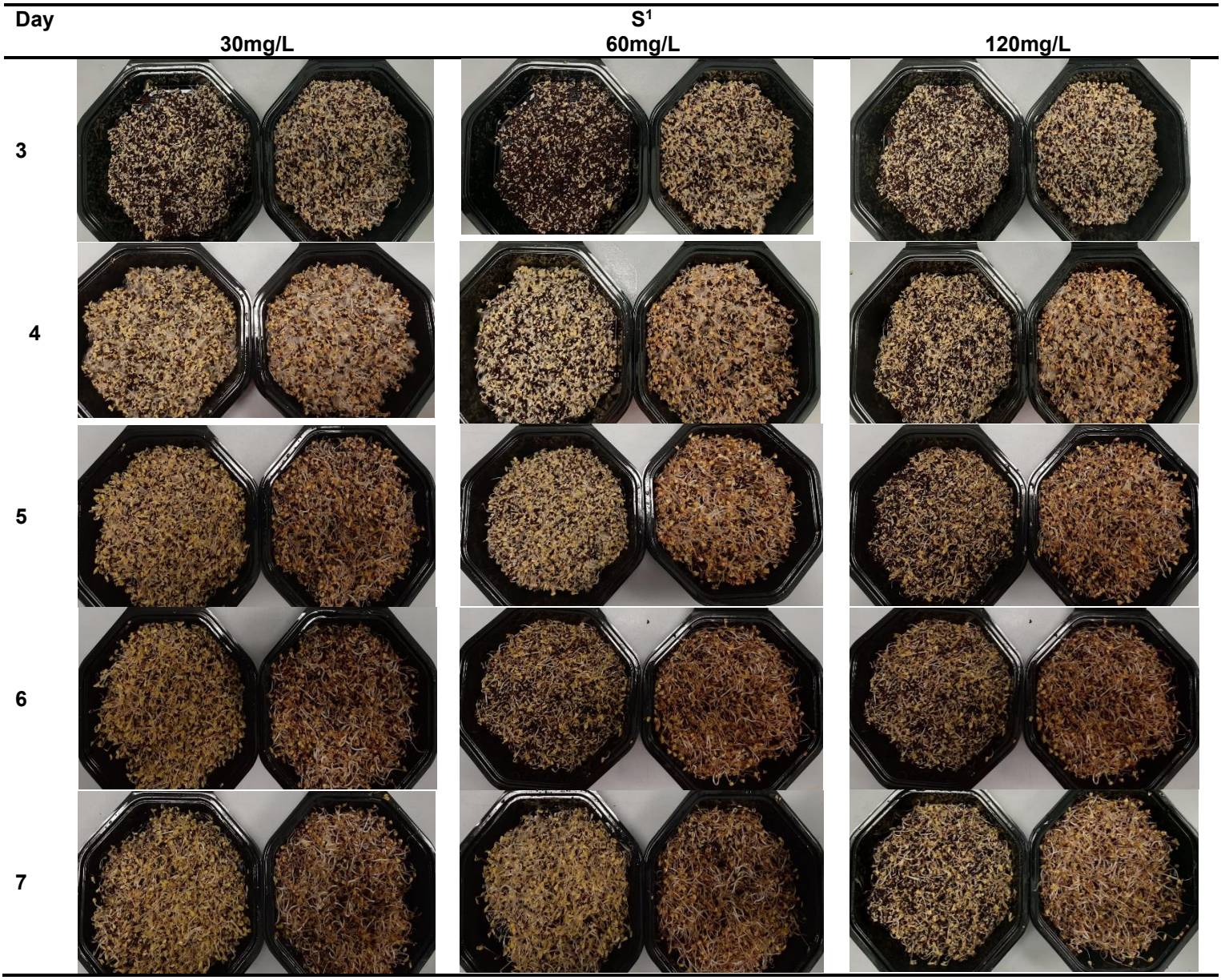
Appendix A

Figure S1. Seed germination of Red Russian and Dwarf Green kale (*Brassica oleracea* var. acephala) treated with methyl jasmonate, selenium, sulfur or water as control for 7 days



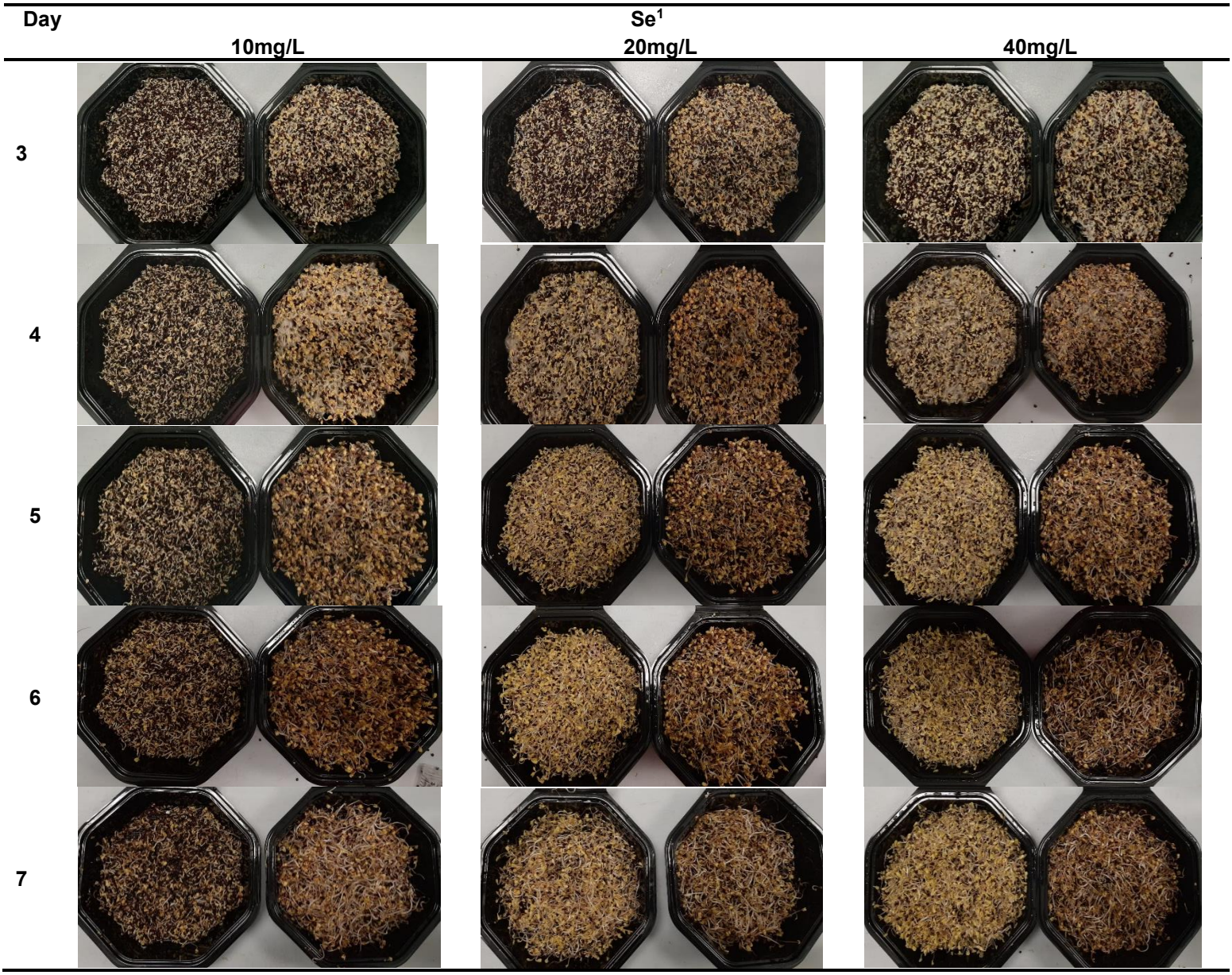
[1] The tray on the left side belongs to the Dwarf Green kale, while the tray on the right side is the Red Russian kale.

Figure S1. (Continuation) Seed germination of Red Russian and Dwarf Green kale (*Brassica oleracea* var. acephala) treated with methyl jasmonate, selenium, sulfur or water as control for 7 days



[1] The tray on the left side belongs to the Dwarf Green kale, while the tray on the right side is the Red Russian kale.

Figure S1. (Continuation) Seed germination of Red Russian and Dwarf Green kale (*Brassica oleracea* var. acephala) treated with methyl jasmonate, selenium, sulfur or water as control for 7 days



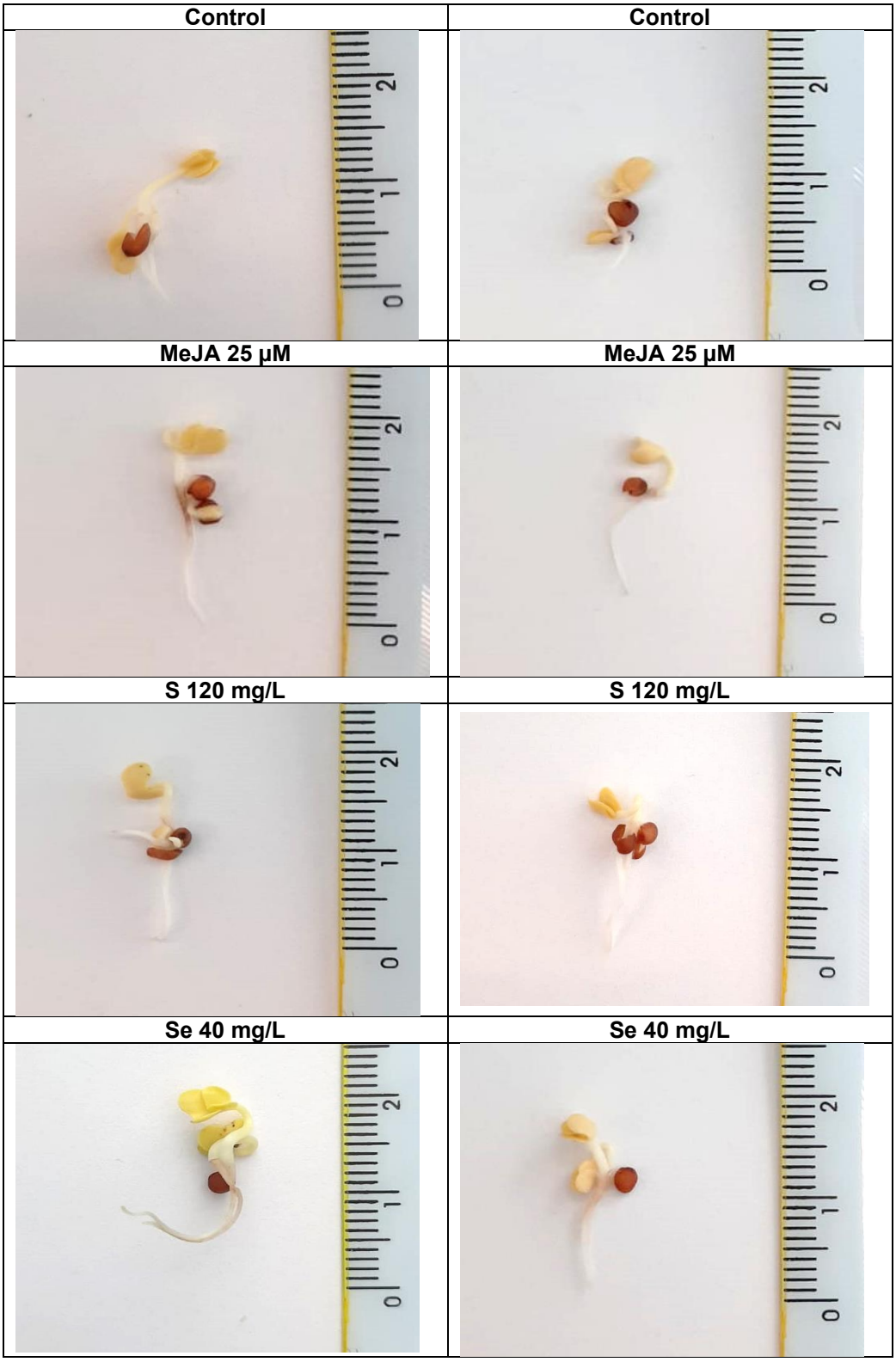
[1] The tray on the left side belongs to the Dwarf Green kale, while the tray on the right side is the Red Russian kale.

Figure S1. (Continuation) Seed germination of Red Russian and Dwarf Green kale (*Brassica oleracea* var. acephala) treated with methyl jasmonate, selenium, sulfur or water as control for 7 days

Day	Control[1]
3	
4	
5	
6	
7	

[1] The tray on the left side belongs to the Dwarf Green kale, while the tray on the right side is the Red Russian kale.

Figure S2. Comparison of Red Russian (left) and Dwarf Green kale (right) sprouts (*Brassica oleracea* var. acephala) germinated with most significant treatments: methyl jasmonate 25 µM, selenium 40 mg/mL and sulfur 120 mg/mL after 7 days.



Published papers

1. Ortega-Hernández, E.; Antunes-Ricardo, M.; Jacobo-Velázquez, D.A. Improving the Health-Benefits of Kales (Brassica oleracea L. var. acephala DC) through the Application of Controlled Abiotic Stresses: A Review. Plants 2021, 10, 2629. https://doi.org/10.3390/plants10122629
2. Ortega-Hernández, E.; Antunes-Ricardo, M.; Cisneros-Zevallos, L.; Jacobo-Velázquez, D.A. Selenium, Sulfur, and Methyl Jasmonate Treatments Improve the Accumulation of Lutein and Glucosinolates in Kale Sprouts. Plants 2022, 11, 1271. https://doi.org/10.3390/plants11091271

Curriculum Vitae

Erika Ortega Hernández was born in Morelia, México, on July 18, 1990. She is the first daughter of Margarita Hernández Fernández and Martin Ortega Saavedra.

She earned the Biotechnology Engineering degree from the *Instituto Tecnológico y de Estudios Superiores de Monterrey*, Campus Monterrey in December 2013. In 2014, Erika joined to Bioprocess and Synthetic Biology Strategic Focus Group in the FEMSA Biotechnology Center of Tecnológico de Monterrey working as support specialist in the areas of Food science and Postharvest biotechnology. She earned her MSc in Biotechnology degree from the Tecnologico de Monterrey (NL, Mexico) in May 2018 working on the biosynthesis of secondary metabolites in plants using abiotic stressors. She was accepted in the Biotechnology doctoral program in August 2018, working under the supervision of Dr. Marilena Antunes Ricardo and Dr. Daniel Alberto Jacobo Velázquez. This thesis represents the termination of her Ph.D studies. So far, Erika has published 9 research articles, 1 review article and 1 chapter book in recognized journals and editorials.

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