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Validation of a selenized chickpea flour as a potential ingredient for functional foods

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Dedication

Y todo lo que hagan, háganlo de buen ánimo como para Yahweh y no para los hombres, sabiendo que de Yahweh recibirán la recompensa de la herencia. ¡Pues le sirven al Mashíaj soberano!

Col 3:23-24 VIN.

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Abstract

Germination of chickpea (*Cicer arietinum L.)* in presence of selenium (Se) is an alternative to increase the mineral and healthy-related properties of the sprouts. This study aimed to produce an Se-enriched chickpea sprouts flour as an ingredient for functional foods. Firstly, the Se-stored protein fraction was identified, and the protein hydrolysates cellular antioxidant activity (CAA) was evaluated. Secondly, the germination in presence of Se was scaled up to obtain high Se and isoflavones concentrations in the chickpea sprouts. In third place, the formulation and evaluation of a food containing germinated chickpea flour was carried out. Finally, the adjuvant capacity against colon cancer in xenografted mice model of a diet containing Se-enriched chickpea sprouts flour was evaluated. For the first step, chickpeas were germinated at laboratory conditions for 4 days soaking with sodium selenite (Na_2SeO_3) (0, 1, or 2 mg/100 g seeds). The protein fractions were digested, identified by Tricine-SDS-PAGE gels, passed through a 10kDa membrane, and evaluated for CAA. Se was accumulated in the order: Glutelin (Glu) > Albumin (Alb) > Globulin (Glo). Glu hydrolysate of less than 10kDa from selenized chickpea sprouts (2 mg of Na_2SeO_3/100 g seeds) increased by 51.5% the CAA related to non-selenized chickpea sprouts. Besides, it was found that Glo digestibility increased with the selenization. For the second step, different doses of Na_2SeO_3 were used to soak the seeds (0, 24, 48, or 96 mg/L) at industrial level. Freeze- and convection-drying (FD, CD) were evaluated on the Se retention, isoflavones, and enzymatic activities (β-glucosidase, phenylalanine-ammonia-lyase, PAL). Increases of 65% and 19% were observed in PAL and phenolics, respectively, when the lowest dose of Se was used. CD did not affect the Se content in the sprouts soaked with 0, 24, and 48 mg/L, and converted isoflavones glycosides into aglycones due to β-glucosidase activation. The highest dose of Na_2SeO_3 (96 mg/L) was found toxic. At the third objective, maize extrudates supplemented with germinated chickpea flour were formulated. Protein augmented with the chickpea supplementation and the interaction between fat, starch, and protein increased the RS in comparison to defatted samples. Germinated chickpea flour increased the water absorption index (WAI) but reduced water solubility index (WSI) when it was combined with maize grits to produce extrudates. The *in vitro* protein digestibility (IVPD) was higher in the germinates chickpea

extrudates and with 20% of supplementation. The supplementation with 20 and 30% of germinated chickpea showed the highest acceptability. Finally, Se-enrichment diet was evaluated as a chemotherapy adjuvant in a xenografted colorectal cancer Nu/Nu mice model. Tumor growth was analyzed by the Gompertz model and the lipid, liprotein and GPx and TrxR were evaluated. The inflection time when the tumor growth rate was deaccelerated (*Ti*) was significantly higher for NC group than the observed in the selenized C15Se and C30Se groups by 1.26-fold. There were significant differences in serum HDL-C between the C30Se group and NC and C30 groups by 1.07- and 1.12-fold. C15NSe did not show inhibitory effect on the tumor growth in contrast to the observed for C60, C15Se, and C30Se and could be related to the differences in LDL-C levels. Triglycerides of basal group was significantly higher than those measured in C15, C30, C15NSe, and C30Se group; the reductions were in a range of 22 to 28%. Almost an increase of 2-, 1.6-, and 1.9-fold were found in the GPx groups in selenized treatments in comparison to basal, NC, and C15 groups, respectively. As a result, C30Se showed the best significant outcomes in the *in vivo* study: lower *Ti* compared to NC, higher HDL-C compared to NC and C30; lower triglycerides compared to basal group; and higher GPx activity compared to basal, NC, C15, and C30 groups.

Keywords: Selenium-enrichment, isoflavones, antioxidant peptides, chickpea germination, food drying, extrudates, xenografted mice model, Se adjuvant.

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Chapter 1. Introduction

Selenium (Se) is an essential micronutrient for mammals and has been related to the prevention of chronic diseases such as cancer, cardiovascular disease, neurodegenerative diseases, type 2 diabetes, and hypertension (Newman *et al*., 2019). Germination of legumes and vegetables in the presence of Se has been explored as an alternative to increase its consumption (Guardado-Félix *et al*., 2017; D´Amato *et al*., 2018; Trolove *et al*., 2018). It is reported that during germination in presence of Se, some genes related to the biosynthesis of polyphenolic compounds such as phenolics and isoflavones are triggered (D´Amato *et al*., 2018; Hawrylak-Nowak *et al*., 2018).

Selenocompounds or Se-enriched diet could play role as chemopreventive agents by different mechanisms that can act simultaneously. These mechanisms include antioxidant effects (Hu *et al*., 2019; Brzacki *et al*., 2019; Wang *et al*., 2020) apoptosis (Guardado-Félix *et al*., 2019), reduced angiogenesis (Saeed *et al*., 2019; Rajkumar *et al*., 2020), among others. Also, selenocompounds have demonstrated several anticancer outcomes in combination with chemotherapeutic agents and natural compounds. Docetaxel (Ertilav *et al*., 2019), cisplatin (Wu *et al*., 2017), adriamycin (Wu *et al*., 2019), and paclitaxel (Guler & Ovey 2020) have been combined with Se in different forms to enhance the chemotherapy.

Promising results have been obtained from selenized chickpea (*Cicer arietinum L.*) sprouts flour in our research group (Guardado-Félix *et al*., 2017; Guardado-Félix *et al*., 2019; Serrano-Sandoval *et al*., 2019; Serrano-Sandoval *et al*., 2021) but little is known about the interaction with chemotherapeutic agents.

To produce an ingredient for functional food; firstly, it was needed to know where Se is stored, identify the protein profile, and asses the antioxidant effect of protein hydrolysates from germinated chickpea flour enriched with Se. Then, it was needed up-scale the germination process of Se-enriched germinated chickpeas flour, and to find an economic drying method to retain the Se content and isoflavones. Subsequently, a food containing germinated chickpeas flour was

formulated, in this case extrudates based on maize. And finally, prove the health-related effect of selenized germinated chickpea flour in an *in vivo* model xenografted with colorectal cancer cells.

1.1 Hypothesis

The selenized chickpea sprouts flour obtained at a pilot-plant scale and maize based extrudates containing it have the potential to be used as chemotherapy adjuvants.

1.2 General and specific objectives

To evaluate the beneficial effects of Se-enriched chickpea sprouts flour obtained at pilot-plant scale in *in vitro* and *in vivo* models to validate its use as chemotherapy adjuvant.

Specific objectives by stage

Stage 1

1.1 To evaluate the changes in the protein profile of the chickpea sprouts in the presence of Se and through the germination time.

1.2 To identify the protein fraction with the highest quantity of Se.

1.3 To assess the antioxidant effect (*in vitro*) of hydrolysates of protein fractions derived from selenized chickpea sprouts.

Stage 2

1.4 To scale up the chickpea germination process in the presence of two concentrations of Na_2SeO_3 (23.5 mg/L and 47 mg/L, ratio 1:3 (chickpea: water)).

1.5 To compare the total isoflavones and phenolic content of chickpeas sprouts obtained at different germination times (0, 12, 24, and 48 h) and dried by freeze-drying (FD) or convection-drying (CD).

1.6 To quantify the total Se content from selenized chickpea flour germinated at 48 h and dried by FD or CD.

1.7 To assess the phenylalanine ammonia lyase (PAL) and β-glucosidase in critical steps of the germination and drying processes.

Stage 3

1.8 To formulate maize-germinated chickpea extrudates with different blends (blends of maize and 10, 20, 30, or 40% of germinated chickpea flour) and perform a sensorial analysis of the chickpea-maize extrudates.

1.9 To evaluate structural and functional properties of the extrudates including protein percentage and digestibility.

Stage 4

1.10 To validate, in an *in vivo* model, the potential of selenized chickpea germinated flour as an adjuvant of 5-FU chemotherapy.

1.11 To evaluate the GPx activity and TrxR in the liver of Nun/Nu mice treated with selenized chickpea germinated flour and 5-FU chemotherapy.

1.12 To assess the changes in the lipid and lipoprotein profile in blood plasma of Nun/Nu mice treated with selenized chickpea germinated flour and 5-FU chemotherapy.

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Chapter 2. Literature review on Se, chickpeas, and colorectal cancer

2.1 Colorectal cancer (CRC), epidemiology, and risk factors

Colorectal cancer (CRC) represents one of the main causes of death around the world. A combination of hereditary, lifestyle, environmental and dietary factors affect to more than 1.2 million new cases of CRC each year, generating large expenditures for the health sector (Clemente & Olias, 2017). In Mexico, CRC is the fourth most common reason of death causing 700 000 deaths per year (INSP, 2015).

Recently, scientists have been interested in the combinatory therapy that is the administration of one or more natural substances and conventional medications at a time (Rejhová *et al*., 2018). The aim is to intensify the antiproliferative effect of the medications administered with natural molecules to reduce the toxic consequences of high concentrations of chemotherapeutic agents (Rejhová *et al*., 2018).

CRC is a multifactorial disease that results from genetic, environmental, lifestyle and, dietary factors (Lin *et al*., 2020). Among the risk factors reported by epidemiological studies the advanced age, male sex, obesity, sedentary behavior, high-meat, high calorie, fat-rich, and fiber-deficient diet has been linked to increased CRC (Mafiana *et al*., 2018). It seems that CRC is an economic dependent disease because the major incidence rates belong to the first world countries such as Australia, New Zealand, Canada, the United States, and some parts of Europe. Conversely, the lowest risk includes China, India, and parts of Africa and South America (Marley & Nan, 2016). For this reason, in the past years, many scientists have focused their attention on the mechanisms behind these factors to prevent and detect early the disease.

CRC is a heterogeneous disease that occurs in the colon and rectum parts. The colon is divided in four parts: the ascending, transverse, descending, and sigmoid colon where most CRC arise (Recio-Boiles, 2021). Most of the carcinogenic tumors are originated from adenocarcinomas that are localized in the epithelial cells and often develop slowly (Recio-Boiles, 2021).

Chapter 3. Materials and methods

Recent studies have suggested that lipid metabolism disorders are a high risk in the carcinogenesis because they could cause abnormal expression of genes, proteins, and dysregulation of signaling pathways (Swinnen *et al*., 2020). Studies also have reported that higher levels of total cholesterol are associated with higher prevalence of CRC (Yang *et al*., 2020). Furthermore, lipids play an important role in the cancer cell growth and proliferation (Yang *et al*., 2020). Elevated plasma levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) have been reported in some types of cancers (Xie *et al*., 2019). During carcinogenesis the cancer cells demand high amounts of energy (Long *et al*., 2018) and the requirement of metabolic intermediates for macromolecule production in rapidly proliferating cancer cells is overwhelming (Luo *et al*., 2017). Survival and metastatic spreading of cancer cells also rely on exogenous cholesterol, lipoproteins, and fatty acids uptake and consumption, the latter through fatty acid β-oxidation (FAO) pathway, even in cells exhibiting high lipogenic activities (Yang *et al*., 2020).

Glucose metabolites, which are normally used for adenosin triphosphate (ATP) production, are redirected to be used in lipid synthesis via the pentose pathways. Both the metabolic rate and glucose uptake are increased to maintain the faster proliferation of cancer cells (Munir *et al*., 2019). This atypical metabolic process is known as the Warburg effect (Long *et al*., 2018). In addition, aerobic glycolysis and increased glutamine metabolism are crucial for cancer cells (Luo *et al*., 2017). Glutamine provides carbon and amino-nitrogen needed for amino-acid, nucleotide and lipid biosynthesis in cancer cells (Beloribi-Djefaftlia *et al*., 2016).

In CRC, lipid-related genes expression profiling such as ABCA1, ACSL1, AGPAT1, and stearoyl-CoA desaturase are overexpressed only in stage II CRC patients (Beloribi-Djefaftlia et al., 2016). LDL rich cancer cells have shown to be more resistant to chemotherapy (Beloribi-Djefaftlia *et al*., 2016). For that reason, lipid-lowering and anti-lipid per-oxidation treatment have advantages in comparison with other anti-cancer drugs (Long *et al*., 2018).

2.2 Mechanisms of chemotherapeutic agents

Currently, there are different treatment alternatives depending on CRC stage. The most common are the surgery, radiotherapy, and chemotherapy (Xie, 2020). Chemotherapy could be given in a systemic or regional regimen according to the CRC spreading. Besides, chemotherapeutics may be used as an adjuvant after surgery, neoadjuvant sometimes with radiation, and for advanced cancers (American Cancer Society, 2021).

Chemotherapeutics cause cell death or prevent cell growth, generally through inhibiting microtubule function, protein function, or DNA synthesis (Amjad et al., 2021). However, this does not just happen in malignant cells but also healthy ones. The non-specific action of these agents is responsible for the countless side effects (Rejhová *et al*., 2018).

The most common drug used in CRC treatment is the fluoropyrimidine 5-fluorouracil (5-FU) an antimetabolite drug which is a structural analog of the naturally occurring metabolites involved in DNA and RNA synthesis (Amjad *et al*., 2021). The main adverse effects of 5-FU are nausea and vomiting, loss of appetite, diarrhea, headaches, mouth sores, skin pruritus, alopecia, suppression of hematopoietic function, anemia, cardiotoxicity, photosensitivity, fatigue, depression, anxiety, and tumor resistance (American Cancer Society, 2021). In addition, Sommer et al. (2017) reported that 5-FU induced a significant increase of hepatocellular triglyceride levels that was due to an impairment of mitochondrial function and an increased expression of fatty acid acyl-CoA oxidase 1 (ACOX1), which catalyzes the initial step for peroxisomal *β*-oxidation. Likewise, 5-FU induced *in vivo* c-Jun N-terminal kinase (JNK) activation and the expression of pro-inflammatory genes IL-8 and ICAM-1. These mechanisms have been attributed to the 5-fluorouracil (5-FU)-induced steatohepatitis (Sommer *et al*., 2017).

Besides, according to the American Cancer Society (2021), the CRC treatment includes Capecitabine (Xeloda), Irinotecan (Camptosar), Oxaliplatin (Eloxatin), and Trifluridine-tipiracil (Lonsurf) as chemotherapeutics agents. Together, chemotherapeutic drugs affect the quality of patients' life and may affect the course, outcome, and the cost of the treatment (Rejhová *et al*., 2018).

To reduce the side effects and drug resistance of chemotherapy, researchers are betting on combinatory therapy. This new approach consists in the administration of low doses of drugs with one or more natural compounds. Thereby, contributes to improving the therapeutic response and the quality of patients' life (Rejhová *et al*., 2018).

These results showed that selenium-biofortified peptides are related with the antioxidant activity and reduction of lipid peroxidation. For that reason, peptides or proteins containing selenium could be a possibility to reduce the chemotherapeutic-induced steatopatitis (Guo *et al*., 2019).

2.3 Natural substances and combinatory therapy in cancer treatment

Bioactive substances present in plants, mushrooms, bacteria, or marine organisms generally protect the living being against external conditions mostly by antioxidant mechanisms (Vitale *et al*., 2020; Cui *et al*., 2020). These natural compounds are mostly pigments such as carotenoids, flavonoids, anthocyanins or terpenoids. In addition to these, some lipids, vitamins, and proteins, have been exerted chemo-preventive effect (Câmara *et al*., 2021). Although, for many natural compounds a precise mechanism of action is unknown or being investigated (Rejhová *et al*., 2018).

According to the National Cancer Institute (2021), the term chemoprevention is defined as the use of medication, vitamins, or other agents to reduce the risk, delay the progression, and avoid the recurrence of cancer. There is evidence that diets rich in folates, vitamin D and E, dietary fiber, garlic, minerals such as calcium and Se, spices, vegetables, and fruits could prevent CRC. The beneficial effect against colorectal cancer have been proven for several nutraceuticals (Table 1). The downregulated targets include receptors, inflammation factors, metastatic proteins, kinases, antiproliferative molecules, transcription factors, cell proliferative compounds, stem cell markers, and others. Those that are upregulated with the administration of nutraceuticals are the pro-apoptotic and tumor suppressor molecules, antioxidant enzymes, transcription factors, ligands, cell membrane compounds, among others (Calvani *et al*., 2020).

Table 1. *The most studied nutraceuticals in the CRC treatment.*

Nutraceutical	Administration	Cancer cells	Assays	Main results	Reference
Cyanidin-3-O-glucoside	Liposomes	Caco-2 cells	*In vitro*, the anticancer activity and morphology were evaluated.	Liposomes enhances the antioxidant activity and apoptosis rate. Besides, inhibited tumor cell proliferation.	Liang et al., 2017.
Manuka honey	Liposomes	HCT-116 cells	*In vitro,* cytotoxicity and viability assays	Manuka honey reduced the volume of spheroids, induce apoptosis	Cianciosi et al., 2020.
Asparagus saponins	Directed	HCT-116, HT-29 and Caco-2	*In vitro,* cytotoxicity activity	HTSAP-10 and PD were more cytotoxic than the other saponins tested.	Jaramillo-Carmona et al., 2018.
Brominated indole compounds from mollusc *Dicathais orbita*	Oral gavage administration	Cancer cells induced by azoxymethane (AOM)	*In vivo,* toxicity (hematology, blood biochemistry, and liver histopathology), and epithelial proliferation (immunohistochemical staining) were tested.	6-bromoisatin significantly enhancing apoptosis and reducing cell proliferation in the colonic crypts.	Esmaeelian et al., 2018.
Resveratrol (trans-3, 4', 5-trihydroxystilbene)	Directed	HeLa and MDA-MB-231 cells	Oxidative stress, biochemical mechanisms	Resveratrol blocks metastatic cells, deters cell growth and glycolysis, promotes ROS.	Rodríguez-Enríquez et al., 2019.
Pectin-free extract of *Lycium barbarum* (goji berry)	Directed	MCF7 and MDA-MB-231 breast cancer cells	*In vitro*, proliferation and molecular mechanisms.	Inhibition of growth breast cancer cells, increases the	Wawruszak et al., 2021.

				expression of p53, and activates.	
Gelatin nanogels system delivery curcumin	Directed	HeLa cells	*In vitro* cytotoxicity effect	Nanogels promising modality for efficient delivery in cancer treatment.	Nguyen-Tran et al., 2019.

Besides the natural therapy, some nutraceuticals or natural compounds have been used to enhance the anti-tumor effect. The combination of broccoli extract sulforaphane with 5-FU reduce cytotoxicity to normal cells, increased apoptosis, downregulation of Bcl2, and up-regulation of BAX (Elkahty *et al*., 2018).

Grifola frondose polysaccharide in combination with 5-FU demonstrated that the synergism enhanced the anti-tumor activity of 5-FU. The combination increased interleukin-2, tumor necrosis factor-alpha, and immune organs weight. Besides, the combination improved biochemical parameters deterioration, superoxide dismutase and MDA (Guang-Hua *et al*., 2019).

Rutin (glycoside from quercetin flavonoid) has shown antioxidant activity and in a combination with 5-FU enhanced apoptosis and p53 gene expression in PC3 cells. Besides, Bcl-2 decreased in the combination (Satari *et al*., 2019).

Aslam et al., 2021 reported that the combination of 5-FU and vitamin D3 showed the highest anti-cancer effects in *in vitro* and *in vivo* studies by upregulating several Ca+2 related molecules that are involved in tumor suppression. In addition, curcumin together 5-FU and oxaliplatin demonstrated significant inhibition in HCT-116 and HT-29 cells. This inhibition was associated with the decreased expression and activation of EGFR, HER-2, HER-3 (72–100%) and IGF-1R (67%) as well as their downstream effectors such as Akt and COX-2 (51–97%) (Patel *et al.,* 2007). Besides, a pre-treatment with curcumin followed by 5-FU evidenced a down-regulation of phospho-Akt, phospho-mTOR, phospho-AMPK, and phospho-ULK1 expressions. These results were validated in xenografted mice, in which the tumor growth was significantly suppressed with the combinatory treatment (Pan *et al*., 2017).

The combination of resveratrol and 5-FU modulate the TNF-β pathway, induced apoptosis, and suppressed NF-κB activation in cancer cells (Buhrmann *et al*., 2018). Other studies indicated that rosemary (*Rosmarinus officialis L.*) and oats (*Avena sativa L.)* enhance the chemotherapeutic effect of the 5-FU in Caco-2 cell line proved by enhancing the antiproliferative and cytotoxic effect of the chemotherapy (El-Burai *et al*., 2020).

Se became more popular in the treatment of CRC in combination with chemotherapeutics. it has been proven that Se in chickpea sprouts consumed by immune-suppressed mice xenografted with HT-29 cells exerted chemopreventive effects mediated by the activation of the intrinsic apoptotic pathway and Glutathione Peroxidase (GPx) activity (Guardado-Félix *et al*., 2019). The authors showed that a diet including Se-enriched chickpea sprouts with a high selenium content (2.29 μg Se/g $_{diet}$) significantly decreased the tumor growth.

2.4 Health benefits of chickpeas (Cicer arietinum L.)

Legumes are podded plants that contain large amounts of fiber and health-related compounds associated with chemo-preventive processes (Kumari & Chandra, 2021). Among the most studied legumes are soybeans, lentils, beans, peas, peanuts, and chickpeas. Chickpea (*Cicer arietinum L.)* is one of the most consumed legumes in the world, especially in the Mediterranean area as well in the Western world (Kumar-Gupta *et al*., 2017). Chickpea is ranked globally as a second important legume crop after dry beans. It is estimated that it is grown in 54 countries with nearly 90% of its area covered in developing countries (FAO, 2019). The leader in the production of chickpeas is India, followed by Australia, Myanmar, Pakistan, Turkey, Ethiopia, the Russian Federation, and Iran. Mexico was the ninth country in the world that stands out for a production of more than 121 thousand tons in 2016 (FAO, 2019).

Chickpea proteins have been related with strong anti-proliferative properties against HeLa (cervical cancer cells), MCF-7 (breast cancer cell), Saos (Sarcoma osteogenic cells) (Abedian *et al*., 2019). Other chemo-preventive compounds in

chickpeas include fiber, vitamin E, lignans, vitamin B, Bowman-Birk inhibitors (BBI), and isoflavones (Aranda-Olmedo & Rubio, 2020).

Isoflavones reduce heart diseases, menopausal transition, low-density lipoprotein-cholesterol content, and blood pressure; besides, there is evidence that they may improve endothelial function (Gómez-Zorita *et al*., 2020). In addition, it is well known that the isoflavone extract may be beneficial to diabetic individuals because it reduces glucose levels and the incidence of cataracts (Gómez-Zorita *et al*., 2020). Another reported beneficial result of the isoflavone intake is the anti-inflammatory effect in chronic illnesses (Wu *et al*. 2020) and the potential effect against osteoporosis (Zheng *et al*., 2016). Although isoflavones have been associated with the prevention of breast cancer, the reported mechanisms are inconclusive (Gómez-Zorita *et al*., 2020).

2.5 Germination in saline stress conditions

The germination processes have taken an unexpected turn due to the combination of different type of stress with the sprouting procedure. The goal with this combination is to increase the antioxidant molecules of plants and reduce some anti-nutrients factors of legumes, for instance (Guardado-Félix *et al*., 2017; Serrano-Sandoval *et al*., 2019)

Germination in presence of Se trigger sulfur (S) metabolic pathways due to their chemical similarity. Inorganic Se probably is transported into plants by diffusion or 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 may enter the S metabolic pathways where it would be converted in Se-amino acids. Once the inorganic Se enters the plastid 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). It is thought that selenite can be assimilated directly when it enters the plastid via SULTR3;1. 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). Further, Se^2 is incorporated into SeCys via cysteine synthase (CS) complex that consist in the enzyme serine acetyltransferase (SAT), its product, O-acetylserine (OAS), and the O-acetylserine thiol lyase (OASTL) enzyme (Schiavon & Pilon-Smits, 2016).

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, 2016). A mechanism that plants use to mitigate Se toxicity is the conversion of SeCys into Methyl-SeCys by selenocysteine methyltransferase (SMT) and then to volatile dimethyl(di)selenide DM(D)Se. Then, Se-cystathionine may be converted to selenohomocysteine (Se-homocysteine) by cystathione beta-lyase (CBL) (Schiavon & Pilon-Smits, 2016). 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-selenometionine (SeMM) via S-adenosyl-L-Met:Met-S-methyltransferase (MMT), and then the conversion to DMSe by methylmethionine hydrolase or via the synthesis of the intermediate molecule 3-dimethylselenoniopropionate (DMSeP) (Schiavon & Pilon-Smits, 2016). Se also can be incorporated into (seleno)glutathione, glucosinolates, and iron (Fe)-Se clusters. Se supplementation up-regulates the secondary metabolism of plants that involve enzymatic and non-enzymatic antioxidants. For instance, Se-enrichment enhanced health related compounds such as isoflavones in chickpea sprouts (Guardado-Félix *et al.,* 2017; Serrano-Sandoval *et al*., 2021).

2.6 Se against CRC

The interest of increasing the concentration of organic Se in foods through novel strategies is due to the importance of Se as an essential element in maintaining the health of humans and animals (Zheng *et al*., 2019). Se is an essential micronutrient for humans. It is involved in several biochemical networks, such as major metabolic pathways, protection against oxidative damage, and the

regulation of immune and reproduction systems (Kang *et al*., 2020). Se acts as a cofactor of selenoenzymes such as GPx and TrxR, which reduce the levels of reactive oxygen species and maintain the redox balance (Zhang *et al*., 2020). For that reason, Se has been classified as a chemopreventive agent due to its ability to reduce the risk, delay the progression, and avoid the recurrence of cancer (Guardado-Félix *et al*., 2019; Radomska *et al*., 2021). Additionally, it has been proved that Se plays an important role in the proper function of the brain, and it has been hypothesized that could prevent cardiovascular diseases (Benstoem *et al*., 2015).

The recommended dietary Se for humans is 55 µg per day (NIH, 2021). One billion of people worldwide have Se-deficient diets mainly due to the low Se concentrations in soil in some areas (Shreenath *et al*., 2021). The consumption of supra-nutritional levels of selenium (200 µg/day) reduces the risk of important diseases including CRC (Kieliszek *et al*., 2021). The antioxidant properties of Se are predominantly exerted by its incorporation into selenoproteins (Kang *et al*., 2020). Ultrafiltrated glutelin fraction hydrolysate of selenized chickpeas sprouts increased CAA (51.47%) compared with the control (Serrano-Sandoval *et al*. 2019).

Sonkusre, 2020 reported that *Bacillus licheniformis* derived biogenic Se nanoparticles (2 µg Se/mL) induce necroptosis in LNCaP-FGC cells. The analysis indicated an overexpression of TNF and interferon regulatory factor. Besides, decreased expression of androgen receptor and prostate antigen.

Se-hydroxyapatite nanoparticles induced apoptosis of bone cancer cells *in vitro* and bone tumors *in vivo*. The nanoparticles enhanced the caspase-dependent apoptosis and generation of ROS (Wang *et al*., 2016). Woo *et al*. (2021) reported that Se in combination with trastuzumab inhibits the growth of breast cancer (HER2-positive) via downregulation of Akt and beclin-1 that are related autophagy. Likewise, a study reported that inorganic selenium (Na_2SeO_3) diet provided only short-term delay tumor growth of mammary cancer. However, two organic diets based on MSA or SeMet exert more potent growth inhibition. Besides, the serum cytokine profile (interleukin-2, interleukin-6, interferon γ, and vascular endothelial growth factor) resulted elevated SeMet supplemeted mice (Chen *et al*., 2013).

Further, the effect of selenized chickpea sprouts was evaluated on the tumor growth of CCR in immunosuppressed mice. It was found that a diet rich in Se (2.29 mg/100g diet) increased the activities of GPx and TrxR in liver tissue, as well as cholesterol and low- and high-density lipoproteins (LDL, HDL). In addition, the tumor growth decreased significantly. It was also shown that the diet rich in selenium promotes the expression of genes related to programmed cell death (Guardado-Félix *et al.*, 2019). Other alternatives to treat cancer with Se are the selenized nanoparticles that trigger intracellular ROS overproduction, thus activates p53 and MAPKs pathways to promote apoptosis.

According to a meta-analysis the Se supplementation does not have protective effect on cancer risk. Although it decreases breast, lung, esophageal, gastric, and prostate cancer, no association was found with colorectal, bladder, and skin cancer (Cai *et al*., 2016). Due to the disagreement between studies, the results should be interpreted with caution due to Se-supplementation effects depends on the doses, diseases, diet, among others (Vincenti *et al*., 2018).

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Chapter 3. Materials and methods

3.1 Plant material

Chickpea seeds (*Cicer arietinum L.)* type Kabuli cultivar Blanco Sinaloa were collected from Angostura, Sinaloa, Mexico (25° 21´55" N 108° 09´ 44" O). The material was grown and harvested during the 2015 and 2016 season. The chickpea seeds were hand cleaned to remove dockage and all impurities including broken and diseased seeds and stored at -20°C for future germination.

3.2 Chickpea germination laboratory scale

Laboratory scale germination was performed according to the methodology described by Guardado-Félix et al. (2017). Before the germination, the seeds were disinfected for 3 min with a solution of sodium hypochlorite and the washed with distilled water three times. Then, chickpeas were hydrated for 6 h with a solution (85 mL) of sodium selenite (Na_2SeO_3) (Sigma Aldrich, St. Louis, MO, USA) in a concentration of 0, 1, or 2 mg per liter in the soaking water. The germination was carried out inside of an incubator in a tray (40 x 30 cm) at 24 ± 1 ºC and 80% of relative humidity. Chickpea germination continued by the spray of distilled water (10 mL) every 12 h. Chickpea sprouts were frozen at -80 ºC.

3.3 Chickpea flour production in laboratory scale

Frozen chickpea sprouts were freeze-dried at -50 ºC, 0.036 mbar for 72 h (LABCONCO, Kansas City, MO, USA). Then, the dried sprouts were milled and passed through a mesh (no. 60). The chickpea sprouts flour was stored at -20 ºC.

3.4 Total protein extraction

Hexane was used to defat the chickpea sprouts flours in a ratio of 1:4 w/v during 12 h in constant agitation. Then, the chickpea flour was mixed with distilled water in a ratio of 1:10 w/v, the pH was adjusted to 8.5 with NaOH (1 M) and stirred

for 2 h. After the agitation, the samples were centrifuged (10 000 x g, at 4 ºC, 20 min) (Eppendorf AG, Hamburg, Germany). The pellet was dissolved again with distilled water (1:5 w/v), stirred and centrifuged at the same conditions and the supernatant was stored at 4 ºC. The supernatants were pooled, and the pH was changed at 4.5 with HCl (1 M), then the solution was stirred and centrifuged at the same conditions and the final pellet was frozen at -80 ºC and freeze-dried.

3.5 Protein fractioning (Alb, Glo, Glu)

The fractions albumin (Alb), globulin (Glo), and glutelin (Glu) were extracted by the solubility's method described by Chang *et al*. (2011). The Alb fraction was extracted with distilled water (1:4 w/v), stirred by 2 h and centrifuged at 10 000 x g for 20 min. The pellet 1 was stored and the supernatant was precipitated with HCl (1 M) until reach pH 4.1 and centrifuged at the same conditions and the pellet represented the Alb fraction. Then, the pellet 1 was dissolved with NaCl (5%) stirred and centrifuged at the same conditions and the pellet 2 was stored and the supernatant was precipitated with HCl (1 M) lowering the pH to 4.3 and centrifugated at the same conditions to recover the pellet, Glo fraction. The pellet 2 was dissolved with 200 mL of NaOH (0.1 M) and centrifuged at the same conditions and the supernatant was precipitated with HCl (1 M) by reaching 4.8 of pH, then the Glu fraction was obtained of the pellet by centrifugation at same conditions. Albumin (Alb), globulin (Glo), and glutelin (Glu) fractions were frozen and freeze-dried.

3.6 Total soluble protein content

Total soluble protein content of all fractions was evaluated with the Coomassie dye ready-to-use (Coomassie Plus Reagent, Thermo Scientific, St. Louis, MO, USA). The determination was carried out following the specifications of the manufacturer adding 10 μL of the sample with 300 μL of the dye and then measuring the absorbance at 595 nm. Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) was used as a standard to build the curve (100-1500 μg/mL).

3.7 Total selenium (Se) content

Total Se determinations were performed following the method reported by Guardado-Félix *et al*. (2017) with minimum modifications. The digestion was carried out by adding HNO_3 (77%, 10 mL) in a system of microwave Mars 5 CEM (Matthews, NC, USA). The conditions used in the system were: increasing the temperature 180 ºC in a lapse of 15 min and then the temperature was maintained for 10 min, and finally the decreased to 50 ºC in 20 min. Then, the volume of the digested samples was adjusted to 20 mL with double distilled water and stored at -4 ºC. The quantification was performed in an inductively coupled plasma mass spectrometer (ICP-MS) (Thermo Scientific, NC, USA) with a Type C glass concentric nebulizer (Meinhard, MA, USA). Gases He (7%) in H_2 were used to avoid interferences. ^{165}Ho and ^{159}Tb were used as the internal standards, and He with 7% hydrogen was used as the reaction gas to avoid any possible interferences. The ion intensity at *m*/*z* 77 (^{77}Se) was monitored using time-resolved analysis software. Data were expressed as μg/g of protein in dry weight (dw).

3.8 SDS-PAGE and Tricine-SDS-PAGE gels

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Tricine (N-(2- Hydroxy-1,1-bis (hydroxymethyl) ethyl) glycine)-SDS-PAGE (Tricine-SDS-PAGE) were performed using a Mini-Protean Tetra Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). SDS-PAGE was performed according to the method described by López-Barrios et al. (2018). Gels consisted in 10% of polyacrylamide gel (pH 8.8) and 5% of staking gel (pH 6.8). Samples and molecular weight marker (15 μg protein/well) were loaded in Laemmli sample buffer (2X). The first voltage used was 60 V, then the voltage was 110 V. The composition of tricine-SDS-PAGE gels was 5% acrylamide, 3% bisacrylamide, and the resolving gel 18% acrylamide, 6% bisacrylamide (4.2 M urea) according to the reported by Jiang, Liu, Zhao, and Wu (2016). Samples (20 μg protein/well) and molecular marker were loaded in a reducing sample buffer (4X) as in Schägger (2006) reported. Initially, the running conditions were set at 60 V and then set at 140 V. The gels were stained and

destained following the method reported by Schägger (2006). The protein and peptides bands of the gels were identified with the GelAnalyzer™ 2010a software.

3.9 Enzymatic in vitro protein digestion

Protein extracts were digested following the protocol described by Lo and Li-Chan (2005). The pH of the total protein solution (5% w/v in distilled water, 0.02% sodium azide) was adjusted to pH 2.0 with HCl (2.0 M) before to add the first enzyme, pepsin (4% w/w). The solution was incubated for 1 h at 37 ºC and then the pH was set to 5.3 with $NaHCO_3$ (0.9 M). After the pH was adjusted to 7.5, pancreatin (4% w/w) was added. Then solution was incubated for 2 h at 37 ºC and finally the solution was immersed in boiling water to finish the digestion. Subsequently, the digested protein was centrifuged (16 000 x g, 10 min). The supernatant was collected and stored at 4 ºC for the further use.

3.10 Ultrafiltration of digested protein

The digested supernatants were ultrafiltered with 10 kDa filters (Centrifugal Filter Units, Amicon™ Ultra-15, Merck Millipore Ltd., Tullagreen, Ireland). The filtrates of less than 10k Da were recovered by centrifugation (5 000×g, 30 min).

3.11 Hydrolysates cellular antioxidant activity

Cellular antioxidant activity (CAA) was performed according to the method described by López-Barrios *et al*. (2018). Caco-2 cells were seeded (100 µL at 1×10^5 cells/well) in DMEM culture medium (Dulbecco´s modified Eagle´s medium) with 5% of fetal bovine serum and incubated at 37 ºC and 5% CO_2. Cells were washed with phosphate buffer solution (PBS) before the assay. After the washing, 100 µL of the hydrolysates (280 µg/mL, 1:1000 dilution) and 60 µM of dichloro-dihydro-fluorescein diacetate (DCFH-DA) (1:1 v/v) were incubated for 20 min. After the incubation, a rewashing was carried out and 100 µL of 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (100 mM) was added in order to induce oxidative stress. The fluorescence was measured at 538 nm with an excitation at 485 nm during 120

min with lapses of 2 min at 37 ºC. The positive control were the cells incubated with DCFH-DA (without protein hydrolysates) and AAPH. CAA was calculated with the following formula:

$$\%CAA = 100 - \left(\frac{AUC\ hydrolysates}{AUC\ positive\ control}\right) x\ 100$$

3.12 Up-scale chickpea germination

Chickpea germination was carried out at pilot plant level at Alimentos Lee™© (Apodaca, Nuevo León, Mexico). The cleaned and disinfected seeds were soaked 6 h with a solution of 0, 24, 48 or 96 mg of Na_2SeO_3 in a ratio of 1:3 (w/v) and then germinated at 22 ± 1 °C during 48 h and 80% of relative humidity. Samples were taken every 12 h according to the objectives of the study.

3.13 Up-scale chickpea flour production

Two drying methods were validated to reach less than 5% of humidity. The freeze-drying (FD) was carried out at -50 ºC during 72 h and 0.036 mbar (LABCONCO, Kansas City, MO, USA). The convection-drying (CD) was carried out at 55 ºC during 12 h in a convection oven (10 GN1/1; Electrolux, Stockholm, SE). After drying, the chickpea sprouts were milled, grinded (Standard Model no. 5 Wiley Mill; Swedesboro, NJ, USA), and pass through a mesh sieve no. 60.

3.14 Total phenolic and isoflavones extraction from germinated chickpea flour

The extraction of phenolic compounds and isoflavones were carried out by weighing 0.6 g of flour and 10 mL 80% methanol (VWR International, Radnor, PA, USA). After sonication for 10 min, the solution was centrifuged (10 000 x g, 20 min) and filtered with a Whatman filter no. 1 and then the volume was graded to 20 mL.

3.15 Total phenolic content evaluation

The phenolic extract was evaluated in a microplate using the method Folin-

Ciocalteu (Singleton *et al*., 1999) modified by Villela-Castrejón *et al*. (2017). Results were presented as mg of gallic acid/gram of flour (dw).

3.16 Phenylalanine ammonia-lyase (PAL) enzymatic activity

PAL enzyme was extracted following the method described by Guardado-Félix *et al*. (2017). The PAL extraction was carried out under ice. The chickpea flour (0.4 g) was mixed with 16 mL borate buffer (50 mM) containing 400 µL/L of 2-mercaptoethanol and final pH of 8.5, polyvinylpyrrolidone (PVP) (0.4 g), and homogenized in ice. Then, the enzymatic solution was centrifuged for 20 min at 4 ºC and 12 000 x g. PAL enzyme was the final supernatant. The reaction solution was prepared using the PAL enzyme (115 µL), the borate buffer (200 µL), and 100 mM L-phenylalanine substrate (35 µL) or distilled water for the control. The reaction solution was incubated at 37 ºC for 60 min and then the reaction was stopped with acetic acid (35 µL). The trans-cinnamic molecule was extracted by the addition of ethyl acetate (750 µL) in the solution, then ethyl acetate (600 µL) was evaporated (36 ºC, 25 min) and redissolved in methanol 50% (100 µL). The identification was carried out by HPLC-DAD. After injecting 5 µL, the chromatograms were acquired at 280 nm with a Luna-C18 (5µm) column at 30 ºC. The flow rate used was 1 mL/min and the mobile phase was 1% of acetic acid, 65% of methanol and the rest water. PAL activity was calculated as 1 mol of trans-cinnamic acid generated per mg of total protein and the standard curve (0.5-100 nmol/mL) was constructed with trans-cinnamic acid standard. The total protein was calculated using the Bradford method following the specifications of the manufacturer BioRad using bovine serum albumin as standard.

3.17 Isoflavones identification and quantification

Isoflavones from chickpea flours were identified and quantified according to the method described by Guardado-Félix *et al*. (2017) using an HPLC-DAD equipment and an HPLC-MS-TOF Agilent 1100. A reverse Zorbax Eclipse column (XDB-C_{18}; 3.0 mm ID X 150mm, 3.5 µm) was used for the separation and the chromatograms were acquired at 260 nm with a flow rate 0.4 mL/min with the same

gradient reported by Guardado-Félix *et al.* (2017) with 0.1% formic acid and 100% acetonitrile. Biochanin A, formononetin, and genistein standards were used to quantify the isoflavones aglycones and conjugates expressed in µg/g dw. The conditions of the HPLC-MS-TOF were followed as Guardado-Félix *et al*. (2017) reported.

3.18 β-glucosidase activity evaluation

β-glucosidase enzymatic activity was carried out according to Sanches de Lima and Iouko-Ida (2014) with some modifications. The extraction was performed by weighing 200 mg mixed with 3 mL of citrate buffer (0.05 M, pH 4.5, containing 0.1 M NaCl) and then homogenized every 15 min during 1 h. Subsequently, the samples were centrifuged (4º C, 10 000 x g, 10 min) and the supernatant was the enzyme. 0.4 mL of substrate, p-nitro-phenyl-β-D-glucopyranoside [p-NPG, 1 mM (Sigma Aldrich Co., St. Louis, MO, USA) in phosphate-citrate buffer (0.1 M, pH 6)], was incubated at 30 ºC for 10 min. After incubation, 0.2 mL of the enzyme solution was added, and the mixed solution was kept at 30 ºC for further 30 min. To finish the reaction, 1 mL of 0.5 M of sodium carbonate was added and then the mixture was centrifuged at 4 ºC, 6 800 x g during 10 min and the absorbance was read at 420 nm. The quantity of p-nitrophenol (p-NP) released by the reaction was measured by comparison to a calibration curve of p-NP (0.12 to 0.2 mM). One activity unit (AU) of β-glucosidase enzyme generates 1 mmol of p-NP/min under the experimental conditions.

3.19 Extrudates production

Germinated chickpeas without Se were dried at 55 ºC for 24 h in a gas forced convection oven 10 GN1/1 (Electrolux, STO, Sweden). Dried sprouts were milled in a grinder Standard Model no. 5 (Wiley Mill, PA, USA). Resulting flours passed a mesh with an opening of 180 µm. Germinated chickpea flours were stored in plastic bags at −20 °C until extrudates production. Maize mini grits (*Zea mays* L.) were purchased at Agroindustrias Integradas del Norte™ (Apodaca, Nuevo León,

México). Flour blends of maize with 10, 20, 30, or 40% (w/w) of germinated chickpea flour were prepared (MGCE-10, -20, -30 or -40), and two controls using only maize (ME) or only germinated chickpea (GCE). A twin-screw co-rotating extruder (BTSM-30, Bühler AG, Uzwil, Switzerland), with a shaft of 800 mm total length and a ratio L/D = 20 was used. The barrel of the extruder was composed of five blocks, and the last block was preheated at 90°C by a heat exchanger device (Tool Temp, Bühler AG, Uzwil, Switzerland). The solid and liquid dispenser flow rate was 24.70 kg/h and 2 kg/h, respectively. A die with a single circular 4 mm hole was used. The screws speeds were set at 381 rpm and round extrudates were obtained setting cutter at 404 rpm using three knives. The specific mechanical energy (SME), temperature in the end plate and final moisture were registered (Table 2).

Table 2. *Extrusion conditions of the maize-germinated chickpea flour.*

Extrudates	Specific mechanical energy (SME) (Wh/kg)	Temperature in the end plate (ºC)	Final moisture (%)
ME	210.44	90.1	6.6
GCE	121.14	153.0	23
MGCE-10	193.46	117.5	3.9
MGCE-20	197.14	130.6	7.1
MGCE-30	187.73	137.0	4.9
MGCE-40	141.13	140.0	5.0

ME= maize extrudates, GCE= germinated chickpea extrudates, MGCE-10, -20, -30 or -40= maize and germinated chickpea flour blends extrudates.

The expanded products were dried in a convection oven (Electrolux EOG Gas single oven X 601, Stockholm, Sweden) at 100 °C during 15 min and stored at room temperature for further analysis.

3.20 Resistant starch measurement

The hydrolysis and the quantification of resistant starch were performed with the AOAC Method 2002.02 and AACC Method 32-40.01. The resistant starch was measured to the whole and defatted extrudates. Samples were defatted using hexane during 12 h at 50ºC (Serrano-Sandoval *et al*., 2019). The moisture of the samples was calculated by the AOAC Method 925.10.

3.21 Fourier transform- infrared spectroscopy (FT-IR)

The spectral data of all materials were collected and corrected with the air background using a Perkin Elmer ATR-FTIR (Spectrum 1, Perkin Elmer, Norwalk, VA, USA) equipped with the Spectrum software (ver. 5.3.0). The spectra were generated in absorption mode at mid-IR (ca. 4000-800 cm^{-1}) with a resolution of 4 cm^{-1}. The regions of specific interest in this study included the heights of the carbohydrates zone (≈1000 cm^{-1}), protein amide I, II, and III groups. The spectral intensity of the α-helix (≈1650 cm^{-1}) and β-sheet (≈1635 cm^{-1}) secondary structures, as well as the intermolecular (Inter) (≈1616 cm^{-1}) and intramolecular (Intra) (1630 cm^{-1}) interactions, were acquired as described by Cameron and Moffatt (1984) and Cai and Singh (2004).

3.22 Expansion index

The expansion index of the extrudates was determined according to Ratankumar-Singh *et al*. (2014). The cross-sectional diameter of the extrudates was measured with a vernier caliper at three different positions. The expansion index was calculated as the cross-sectional diameter of the extrudate divided by the diameter of the die opening as shown in the following formula. The average of expansion index values was obtained from 100 random samples.

$$expansion\ index = \frac{extrudate\ diameter}{die\ diameter}$$

3.23 Texture analysis

Texture analyses were performed using a TA.XT plus texture analyzer (Stable Micro Systems Ltd, Surry GU7 1JG, England) with a Kramer shear cell of 100 cm^2. The cell was filled to its maximum with the extrudates. The tests were carried out with a 10 mm punch at a crosshead speed of 10 mm/s and a travel distance of 110 mm. Hardness, crunchiness, and crispiness were obtained from the force-time curve analyzed by the Texture Exponent 32 (Surrey, UK) program. According to Li et al. (2019) hardness (N) was defined as the peak positive force, crunchiness is the total count of positive peaks and crispiness (N.s) is the area under the curve. Four measurements were performed for each group.

3.24 Functional properties

The water absorption index (WAI), water solubility index (WSI), and swelling power (SP) were performed according to Rodríguez-Sandoval *et al*. (2012). Briefly, the extruded flour samples were passed through a mesh sieve no. 60. (W. S. Tyler, OH, USA) to normalize the sample size. The samples (0.5 g, dry weight) were mixed with 6 mL of distilled water and incubated at 30 ºC in a bath with shaking for 30 min. Then, the sample was centrifuged at 5 000 rpm for 20 min. The supernatant was decanted, and the volume was measured and filtered, then 2 mL of the filtrate was dried at 90 ºC for 4 h. The retained gel in the tubes was weighted. WAI, WSI, and SP were determined according to following equations. The tests were carried out in triplicate.

$$WAI = \frac{gel\ weight\ (g)}{sample\ weight\ (g)}$$

$$WSI\ (\%) = \frac{supernatant\ dry\ weight\ (g)}{sample\ weight\ (g)} x\ 100$$

$$SP = \frac{gel\ weight\ (g)}{sample\ weight\ (g) - supernatant\ dry\ weight\ (g)}$$

3.25 Total protein and in vitro protein digestibility (IVPD)

Total protein was analyzed according to AOAC method 960.52. The IVPD of extruded flours was assessed using the pH-drop method described by Lazo *et al*. (1998) and Hsu *et al*. (1977) with some modifications. Briefly, an enzymatic solution was prepared with 1.6 mg/mL of trypsin (Sigma-Aldrich, MO, USA) and 3.1 mg/mL of α-chymotrypsin from bovine pancreas (Sigma-Aldrich, MO, USA), and 1.3 mg/mL of a protease from *Streptomyces griseus* (Sigma-Aldrich MO, USA) and adjusted to pH 8. Then, a solution of 50 mL (6.25 mg of protein/mL) was adjusted to pH 8 and placed in a water bath at 37ºC with agitation. Subsequently, 5 mL of enzymatic solution was added to the sample solution and, after 10 min in the water bath, the pH was measured. The IVPD was calculated using following equation:

$$\%\ IVPD = 210.46 - 18.1\ x\ (pH\ in\ the 10\ )$$

3.26 Sensorial analysis

The sensorial analysis of the extruded samples was carried out by 100 panelists among 18 to 40 years that live in Monterrey, Nuevo León, Mexico. The participants signed the format of informed consent (Appendix A). Each sensorial attribute was evaluated on a 1 to 9 hedonic scale with 1 being dislike extremely, 5 being neither like nor dislike, and 9 being extremely like (Appendix B).

3.27 Cell culture

Human colorectal cancer cells expressing red fluorescence protein (HT-29-RFP) were maintained in McCoy medium (Gibco, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum (ATCC, Manassas, VA, USA), 100 U/mL of streptomycin and 100 U/mL of penicillium (Gibco, Grand Island, NY, USA). Cells were incubated at 37 ºC in an atmosphere of 5% of CO_2 and were sub-cultured when they reached 90% of confluence.

3.28 Diet preparation

Diets were formulated based on the established requirements by the American Institute of Nutrition for laboratory rodent´s diets (AIN-93G growth purified diet) (Table 3). Three types of diets were used to feed the rodents. The control diet was the standard AIN-93G, the experimental diets were those formulated from chickpea flour with and without Se. The percentage of the chickpea flour used in the formulation was 30% and the rest of the ingredients were adjusted according to the AIN-93G composition diet. The pellets were molded by hand with 25% of water, dried at 50 ºC for 6 h, sterilized (121 ºC for 7 min) and convection dried until the water content was less than 5%.

Table 3. *Diets composition according to American Institute of Nutrition for laboratory rodent´s diets according to the standard AIN-93-G.*

	Experimental diets		
	Control (AIN-93G)	**Germinated flour without Se**	**Germinated flour with Se**
Ingredients	g/100 g diet (dw)		
Germinated chickpea flour	0	30	30
Starch[1]	39.7	20.95	20.95
Casein[2]	20.0	11.8	11.8
Maltodextrin[3]	13.2	13.2	13.2
Sucrose[4]	10.0	10	10
Cellulose[5]	5.0	4.04	4.04
Oil[6]	7.0	4.9	4.9
Mineral mix[7]	3.5	3.5	0
Mineral mix without Se[8]	0	0	3.5
Vitamin mix[9]	1.0	1.0	1.0
L-cystein[10]	0.3	0.3	0.3

Choline birtrartrate[11]	0.25	0.25	0.25
t-Butylhidroquinone[12]	0.0014	0.0014	0.0014
Total Se per diet (μg/g diet, dw)	0.15	0.15	0.39 ± 0.04*

1: Starch (Food Ingredients, Westchester, IL, USA); 2: Purified casein (MP Biomedicals, Illkirch, France); 3: Maltodextrin (GPC, Salt Lake City, UT, USA); 4: Zulca™ sucrose; 5: Micro-cristalin cellulose (MP-Biomedicals); 6: Nutrioli™ soy oil; 7: Mineral mix AIN-76 (MP Biomedicals, Illkirch, France); 8: Mineral mix Se free AIN-76 (MP Biomedicals, Illkirch, France); 9: Vitamin mix AIN-76 (MP Biomedicals, Illkirch, France ; 10: L-cysteine (Sigma, St. Louis, MO, USA); 11: Choline bitartrate (Sigma, St. Louis, MO, USA); 12: t-Butylhydroquinone (Sigma, St. Louis, MO, USA). *Total Se content in the pellets after drying and sterilization processes.

3.29 Animal model

The animal protocol was approved by the Institutional Committee for the Security and Use of Laboratory Animals of the Medicine School of Tecnologico de Monterrey (ID number 2020-002). Eighty-eight immunosuppressed male Nu/Nu mice aged 6-8 weeks and weighing 19.9-22.5 g (UPEAL-CINVESTAV) were maintained under specific pathogen-free conditions in vinyl cages with laminar air flow at 23 ºC and relative humidity of 50% ± 5%, with a 12 h light/dark cycle. Mice were acclimated for 2 weeks, during this period they were fed with AIN-93G standard diet. During the study, antibiotic prophylaxis (sulfamethoxazole and trimethoprim (Bactrim™, Roche Mexico)) was administered in the water in a concentration of 1 mg/mL and 0.2 mg/mL, respectively. After the acclimatization, the mice were tagged and randomized into 8 groups (n=11) (Table 4). The experimental diets were provided for 63 days. The weight of the mice and the consumed diet food were registered weekly. The experimental diets, the water, and the box were sterilized (121 ºC, 15 psi, 15 min) and changed weekly (Figure 1).

Table 4*.Treatments designed to study and compare the effects of the combination of 5-FU with Se contained in sprouted chickpea flour in immunocompromised animals with colon cancer xenografts (n = 11).*

Group	Tumor	Chemotherapy (5-FU mg/kg)	Total Se (μg/g diet, dw)	Diet

Basal	No-xenografted	0	0.14	AIN-93G
NC	Xenografted	0	0.14	AIN-93G
C15	Xenografted	15	0.14	AIN-93G
C30	Xenografted	30	0.14	AIN-93G
C60	Xenografted	60	0.14	AIN-93G
C15NSe	Xenografted	15	0.14	Germinated chickpea without Se
C15Se	Xenografted	15	0.39 ± 0.04	Germinated chickpea-Se
C30Se	Xenografted	30	0.39 ± 0.04	Germinated chickpea-Se

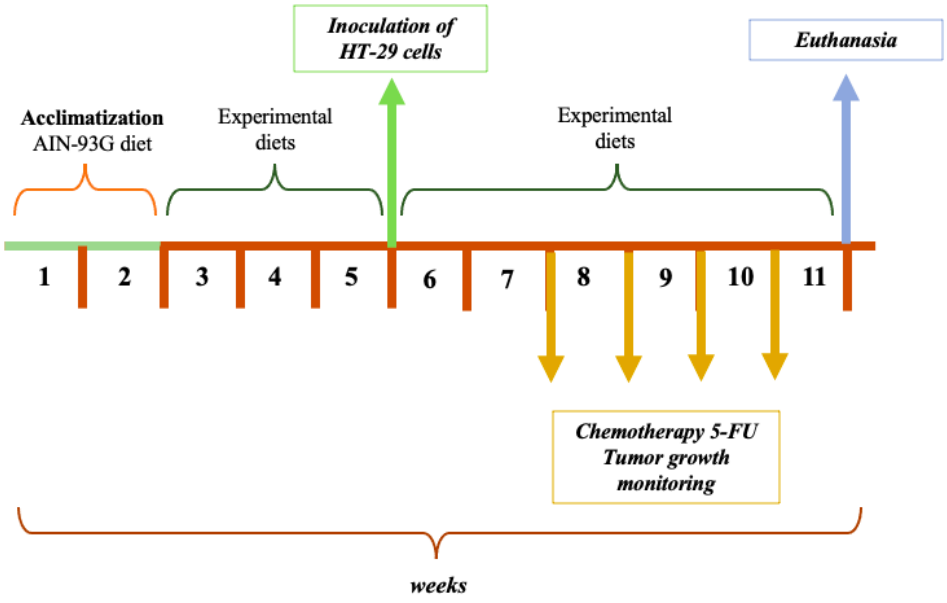

Figure 1. Experimental strategy of the in vivo protocol.

3.30 Animal welfare

Animal welfare was achieved by meeting the physiological (adequate box, litter, water, and food ad libitum) and psychological requirements (environmental enrichment: cardboard tunnels and/or swings) to have the mice in an optimal quality of life during the study. In addition, if the animal showed abnormalities, the humanitarian end point was determined to avoid suffering, pain and/or stress for the animal. The abnormalities were detected through changes in the animal's behavior, as well as changes in the eyes, ears, nose, vibrissae, and cheeks, taking as reference the code of facial expressions of pain in the laboratory mouse (Langford et al., 2010). In addition, physiological parameters such as body temperature (35-39 ºC), type of stool, respiration (84-280 / min) and weight loss (≥ 20% in a period of 2 weeks) were monitored in the physiological station.

3.31 Inoculation of HT-29 RFP cancer cells

After 21 days of feeding the experimental diets, the mice were xenografted by subcutaneous injection in the dorsal part with $1x10^6$ HT-29 RFP fluorescent cells suspended in 200 µL of McCoy medium (Gibco, Grand Island, NY, USA). The mice were taken and placed on the grid allowing it to hold onto it with its front paws while the skin was lifted. The needle was inserted at the base of the skin area parallel to the body to avoid inoculating in layers below the skin. It was aspirated lightly, and the sample volume was injected at a moderate speed. The needle was removed, and the skin was pressed to prevent fluid from leaking through the punctured skin site.

3.32 Tumor growth monitoring

Seven days after inoculation, tumor growth was monitored every four days using an iBOX Scientia animal imaging system (UVP LLC, Upland, CA, USA) which measures relative fluorescence of tumor. The mice were anesthetized with sevoflurane (Induction 4%; Maintenance 3.5%) to allow reading within the iBOXScientia equipment.

3.33 Modeling tumor growth

The data of the tumor volume was used to modeling tumor growth by fitting the values to the Gompertz function in Python Software®. The function contains four-parameters, and the model can be expressed as:

$$W(t) = B + (A - B)\exp\left(-\exp\left(-kG(t - Ti)\right)\right)$$

Where, *A* is the upper asymptote, *B* the lower asymptote, k_G the growth rate coefficient, and *Ti* a parameter that shifts the growth curve horizontally called inflection point.

Each experimental unit was modeled, and *A*, k_G, and *Ti* parameters were obtained individually, and the means and standard deviations were calculated with those that were in the range of observation time.

3.34 5-FU chemotherapy

Mice of some experimental groups were injected with 5-FU in a physiological saline solution after two weeks of being inoculated. Doses of 15, 30 or 60 mg/kg of body weight were administered intraperitoneally every week 14 days after inoculation.

3.35 Euthanasia and tissue removal

The animals were euthanized after 42 days of inoculation of the cancer cells. All animals were anesthetized with sevoflurane and euthanized by cardiac puncture to collect blood. The blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and centrifuged at 3000 rpm for 15 min at 20 °C to obtain the supernatant (serum) and it was immediately stored at -80 ° C for further analysis. The four limbs of the animal are fixed to the worktable with the belly in a supine position and an incision was made in the animal's skin from the hypogastrium to the epigastrium and the same cut was made in the peritoneum to expose intraperitoneal organs and the liver and tumor were carefully removed. The tumor and the liver were identified, and the adhered tissue was

removed by cutting with a scalpel to try to recover them in their entirety. The weight of the organs was recorded, and they were frozen with liquid nitrogen and later stored at -80 ° C for future analysis. The strategy model of the *in vivo* protocol is presented in Figure 1.

3.36 Lipid and lipoprotein evaluation

The concentration of total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and triglycerides in plasma were analyzed with the Cobas 111 equipment (Roche, Basel, Switzerland).

3.37 Determination of glutathione peroxidase and thioredoxin reductase activities

The glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) activities were measured with the GPx MTP (ALPCO, Bensheim, Germany) and colorimetric TrxR (Cayman Chemical Company, Ann Arbor, MI, USA) kits, respectively. The required reagents were prepared according to the manual provided by the kits. Briefly, liver proteins were extracted using cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT for GPX and 50 mM potassium phosphate, pH 7.4 and 1 mM EDTA for TrxR) in a ratio of 1:10 w/v, homogenized and centrifuged at 10,000 rpm for 15 min at 4 ° C (Eppendorf, Hamburg, Germany). The assays were performed according to the manufacturer specifications. The units of enzymatic activity of GPx were defined as the activity that causes the formation of 1 mmol of $NADPH^{+}$ of NADPH per min at pH 8 and 25 °C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide. And the enzymatic units of TrxR were defined as the NADPH-dependent production of 2 µmol of 2-nitro-5thiobenzoate per min at 22 °C. Both enzymatic activities were expressed as milligrams of protein contained in the supernatant. The protein content was determined in the supernatant fractions by the BCA method (Thermo Scientific, Rockford, IL, USA) according to the provided instructions.

3.38 Statistical analysis

All the experiments were made three times. The results were expressed as mean with the standard deviation. R-Studio, Minitab, and JMP software were used. ANOVA´s followed by a post-hoc test (Tukey´s HSD test) with 95% of confidence level were performed. Likewise, Pearson correlations were obtained in some analysis with a 95% of confidence. General linear model (*glm*) and Fisher´s LSD test were used in the *in vivo* study. The type of the analysis depends on the objective of the study.

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Chapter 4. Digestibility and antioxidant capacity of protein hydrolysates of chickpea sprouts.

Manuscript: Changes in digestibility of proteins from chickpeas (*Cicer arietinum L.)* germinated in presence of selenium and antioxidant capacity of hydrolysates

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4.1 Background

It is well known that chickpea seeds have relatively high protein content (24.6%) and the main storage proteins are albumins (Alb) (12%), globulins (Glo) (56%), and glutelins (Glu) (18.1%) (Chang *et al*., 2011). Liu *et al*. (2015) reported that Se is accumulated in proteins and peptides such as selenomethionine (SeMet), selenocysteine (SeCys), selenodiglutathione (GS-Se-SG), selenomethylselenocycteine (SeMeSeC), and selenium-binding proteins.

Se has a key role in the antioxidant defensive mechanism as it is involved in the proper function of different enzymes and selenoproteins. Among 25 known selenocysteine-containing proteins in mammals, some of the most significant selenoproteins include gluthatione peroxidases (GPx's), and thioredoxin reductases (TrxR's), which are needed to maintain cellular redox homeostasis (Silvestrini *et al*., 2020).

Despite the potential of selenized germinated chickpea flour, very few studies have been carried out to elucidate where the Se is accumulated. The objectives of this study were 1) to evaluate the changes in the protein profile of chickpea during germination in the presence of Se, 2) to identify the protein fraction with the highest

quantity of selenium and 3) to assess the cellular antioxidant activity of hydrolysates from selenized chickpea sprouts protein fractions.

4.2 Results and discussion

4.2.1 Changes in protein profile of germinated chickpeas

Convicilin (68 - 72 kDa), vicilin major subunit (48 - 50 kDa), legumin α (40 - 44 kDa), vicilin minor subunit (25 - 28, 30, 34 - 37 kDa), legumin β (20 kDa), and lectin β (<20 kDa) bands were the main proteins observed in the germinated chickpeas (Figure 2A). Alb, Glo, and Glu represent approximately 75% of the total protein content in chickpea (Rumiyati *et al*., 2012). Vicilin major subunits (48 - 50 kDa) reduced their intensity during the germination process (Figure 2). The expression of protein bands of 34-37 kDa decreased after two germination days in control samples. The highest intensity of protein bands in the range of 30-35 kDa was observed in control samples germinated for two or three days (Figure 2A). The intensity of the protein bands between 34 and 37 kDa remained constant during the four germination days when chickpeas were pre-treated with 2 mg of Na_2SeO_3/ 100 g. On the other hand, these bands had lower intensity in control chickpeas germinated for more than one day. Meanwhile in chickpeas pre-treated with 1 mg Na_2SeO_3/ 100 g, the intensity reduction was observed after three germination days. The Se inhibitory effects on proteolysis had been previously reported in animal flesh (Wang *et al*., 2018; Calvo *et al*., 2016) and in this is the first study that demonstrates that Se retards proteolysis in germinated seeds.

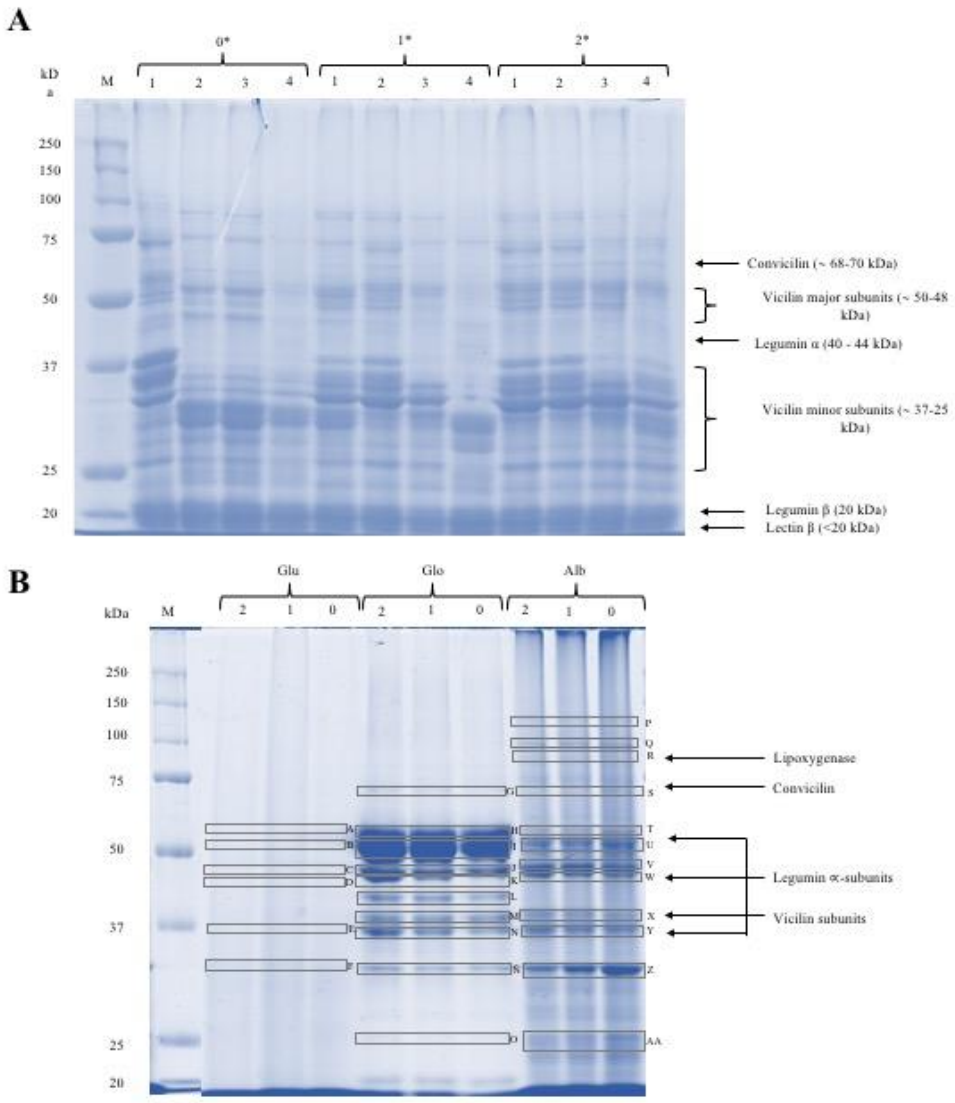

Figure 2. Protein profiles from chickpea germinated with selenium. A) Total protein (TP) profiles. Number lanes 1-4: Germination days. 0*, 1*, and 2*: mg Na2SeO3/ 100 g of seeds. B) Protein fraction (PF) profile of fourth day of germination. Number lanes 2*,1* and 0*: mg Na_2SeO_3/ 100 g of seeds. Glu: Glutelins, Glo: Globulins, and Alb: Albumins. M: protein molecular mass marker. Letters from A to AA in black boxes indicate the position of main bands found in the gel.

During the germination process, the storage proteins break down to act as a source of amino acids, nitrogen, and carbon for bio-molecules synthesis. It was

reported that the storage proteins might be degraded mainly through protease pathway in rice and soybean (He *et al*., 2007). These changes have been observed previously in the germination of lupin (Rumiyati *et al*., 2012), black beans (López-Barrios *et al*., 2016), brown rice (Liu *et al*., 2011) and chickpea (Portari *et al*., 2005).

As expected, vicilin minor subunits were mostly found in Glo-fraction (M, N) and Alb-fraction (X, Y) but nothing in the Glu-fraction (Figure 2B). Protein profiles were very similar to those previously reported for Alb, Glo, and Glu chickpea fractions (Rumiyati *et al*., 2011). Vicilin minor subunits at 35 and 32.5 kDa (E), glutelin at 55 kDa (A), and legumin ∝-subunits at 40 and 43 kDa (C, D) were recognized in Glu-fraction. Furthermore, lipoxygenase band was found in the Alb-fraction at 93 kDa (R); likewise, convicilin at 67 kDa (S, G), legumin ∝-subunits at 40 and 43 kDa (V-W, J-K), vicilin major subunits at 50 kDa (U, I), and vicilin minor subunits at 35 and 32.5 kDa (X-Y, M-N) were found in the Alb- and Glo-fraction (Figure 1B).

4.2.2 Se content in protein extracts

Se level in TP control (0.3 µg Se/g of TP extract) was similar to that reported by Jahreis *et al*. (2015). The Se contained in the total protein extract of germinated chickpea pre-treated with 1 mg, or 2 mg of Na_2SeO_3/ 100 g increased 17 to 29-fold in comparison to the control (Table 5). Guardado-Félix *et al.* (2017) reported Se content increases from 59 to 115-fold on the fourth day of chickpea germination in the presence of Na_2SeO_3. Chickpea proteins incorporated Se since the first germination day and there was no increase with germination days.

Table 5. Se content Se content in total protein (TP) during different germination days (1, 2, 3, and 4 days).

Germination day	µg Se/g of TP		
	0*	1*	2*
1	0.361 ± 0.07^{c}	5.762 ± 0.06^{b}	9.050 ± 0.18^{a}
2	0.314 ± 0.06^{c}	5.553 ± 0.10^{b}	9.679 ± 0.19^{a}
3	0.313 ± 0.01^{c}	5.244 ± 0.12^{b}	9.200 ± 0.19^{a}
4	0.296 ± 0.02^{c}	5.555 ± 0.13^{b}	9.406 ± 0.40^{a}

Mean ± standard deviation of three replicates. Mean values in each row sharing the same letter were not significantly different ($p < 0.05$). TP: Total Protein. *0, 1*, and 2*: mg Na_2SeO_3/100 g seeds.

Some researchers have demonstrated that Se accumulates in proteins. Hu *et al.* (2014) showed that the content of selenoproteins increased up to 18 times compared with control. Besides, Liu *et al.* (2015) identified four Se compounds, selenocysteine (SeCys2), methylselenocysteine (MeSeCys), Se (IV), and selenomethionine (SeMet) as the principal organic Se species in the protein extracts Se-enriched brown rice. In cereals, Bianga *et al*. (2013) evidenced that Se substituted about 4% of sulfur (S) in methionine (3% as SeMet). On the other hand, in chickpeas, Se has incorporated in the form of SeMet (Zhang *et al*., 2012).

The Glu-fraction of germinated chickpeas pre-treated with 2 mg Na_2SeO_3/ 100 g showed the highest Se accumulation followed by Alb-fraction and Glo-fraction (Table 6). Fang *et al*. (2010) also found the highest amount of Se in the Glu fraction from Se-enriched rice. These results agree with recent research that found the major content of organic Se was found in Glu in the form of SeMet in Se-enriched rice grains (Gong *et al*., 2018).

In contrast, independently of Na_2SeO_3 concentration, the Se content in Glo fraction of germinated chickpeas increased 3-fold in comparison to control. Similarly, in adzuki beans, the lowest accumulation of Se was found in the Glo fraction (Oliveira

et al., 2017). Alb fraction in Se-enriched brown rice also included a considerable amount of inorganic Se, which was soluble in water (Liu *et al.,* 2011).

Table 6. *Selenium content in albumins (Alb), globulins (Glo), and glutelins (Glu) of four days germinated chickpeas.*

	µg Se/g of PF		
PF	**0***	**1***	**2***
Alb	0.841 ± 0.034^{e}	4.820 ± 0.270^{d}	10.369 ± 0.230^{b}
Glo	1.337 ± 0.064^{e}	4.253 ± 0.054^{d}	4.595 ± 0.166^{d}
Glu	0.987 ± 0.063^{e}	7.762 ± 0.190^{c}	17.142 ± 0.660^{a}

Mean ± standard deviation of three replicates. Mean values in each row sharing the same letter were not significantly different ($p < 0.05$). PF: Protein Fraction. Alb: Albumins, Glo: Globulins, and Glu: Glutelins. *0, 1*, and 2*: mg Na_2SeO_3/100 g seeds.

4.2.3 Protein hydrolysates and cellular antioxidant activity

The bands around 10 kDa were the most intense in the hydrolysates. The bands in the TP extract mainly corresponded to the Alb hydrolysates (Figure 3). After *in vitro* digestion, the proteins bands were more intense in the Glo fraction of the ungerminated grain and the germinated control. Glo fraction had the lowest Se content and therefore the digestibility was facilitated. As mentioned earlier, Se has proteolysis inhibitory effects (Wang *et al*., 2018; Calvo *et al*., 2016) and in consequence had a significant effect on protein digestibility. Further experiments are required to analyze the regulatory effects of proteolytic enzymes depending on protein amino acid composition and Se concentration. Liu *et al*., (2008) reported that Se increased the Glo susceptibility to proteolytic hydrolysis.

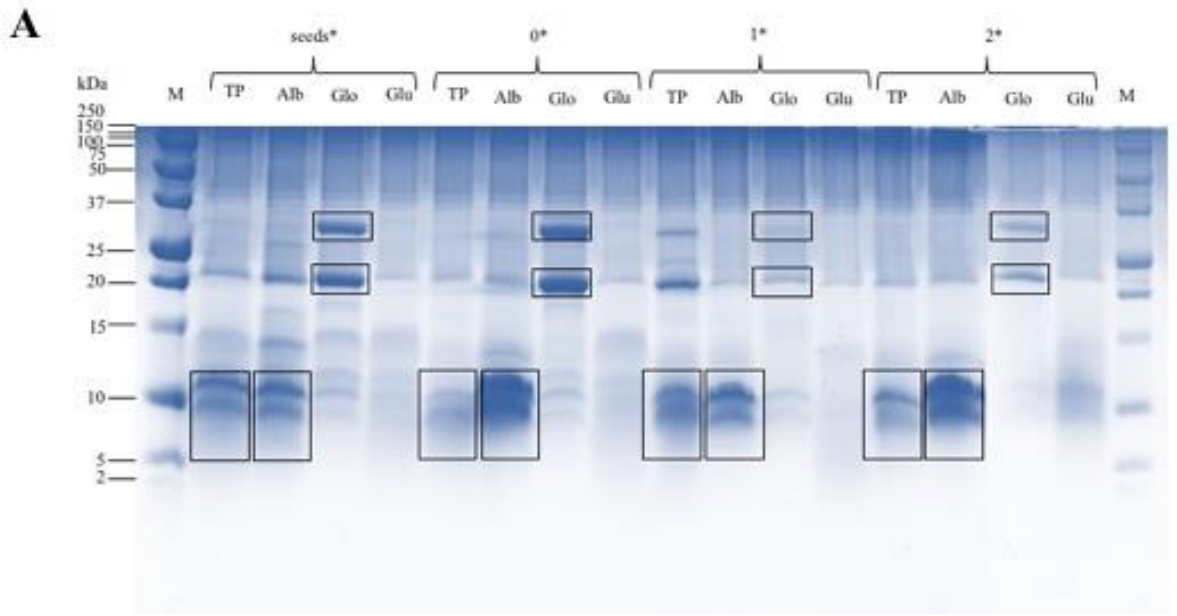

Figure 3. Peptide profile (Tricine-SDS-PAGE) after in vitro digestion of the fractions at fourth day of germination. M: Protein molecular mass marker. TP: total protein, Alb: Albumin, Glo: Globulin, and Glu: Glutelin. 0*, 1*, and 2*: mg Na_2SeO_3/ 100 g of seeds. Black boxes indicate the position of main differences found in the gel.

Peptides <10 kDa had higher cellular antioxidant activity than the protein hydrolysates of >10 kDa. Similar findings have previously been reported by Liu *et al.* (2015); fractions with lower molecular weight showed higher antioxidant activity (DPPH radical scavenging activity). Glu fractions had higher CAA than the Alb. A significant increase in CAA was observed in the protein fractions from germinated chickpeas pre-treated with 2 mg Na_2SeO_3/ 100 g. The highest contrast was observed in the antioxidant peptides obtained from the Glu fraction when chickpea was pre-treated with Na_2SeO_3. The %CAA of the Glu fraction <10 kDa increased from 39.022 ± 2.335 to 59.108 ± 2.060 with the Se supplementation. In addition, it is proven that peptides from a germinated chickpea in the presence of Se (2 mg of Na_2SeO_3/ 100 g of seeds) could have equivalent CAA as SeMet, SeCys, and Na_2SeO_3 (Table 7).

Table 7. Cellular antioxidant activity (CAA) of protein hydrolysates recovered at 180 min of pepsin-pancreatin digestion.

PF	Treatment	Fraction	%CAA
Alb	0*	<10kDa	37.385 ± 0.646^{c}
		>10kDa	21.046 ± 1.249^{e}
		Total	21.236 ± 2.169^{e}
	2*	<10kDa	41.375 ± 1.672^{c}
		>10kDa	24.559 ± 3.429^{e}
		Total	35.749 ± 1.775^{cd}
Glu	0*	<10kDa	39.022 ± 2.335^{c}
		>10kDa	28.863 ± 1.683^{de}
		Total	35.047 ± 3.584^{cd}
	2*	<10kDa	59.108 ± 2.060^{ab}
		>10kDa	52.194 ± 2.158^{b}
		Total	56.216 ± 3.644^{ab}
SeMet	--	--	56.868 ± 2.804^{ab}
SeCys	--	--	62.170 ± 0.190^{a}
Na_2SO_3	--	--	59.482 ± 3.344^{ab}

Mean ± standard deviation of three replicates. Mean values in each row sharing the same letter were not significantly different ($p < 0.05$). PF: Protein fraction. Alb: Albumins. Glu: Glutelins. *0, 1*, and 2*: mg Na_2SeO_3/100 g seeds.

Hu *et al.* (2014) also demonstrated that the antioxidant activity of selenoproteins from Se-enriched soybean increased up to 4-fold compared with proteins from the control. Antioxidant activities of selenoproteins from brown rice were evaluated by different assays (such as the hydroxyl radical-, superoxide anion-

, and 1,1- diphenyl-2-picrylhydrazyl-scavenging assays) showing significant increases compared to control (Liu *et al*., 2012). Further, Zhao *et al.* (2013) showed that the antioxidant activities of egg white proteins were remarkably enhanced by selenization process.

In addition, it is known that the enhanced hydroxyl-radical scavenging activity of selenoproteins can be partially attributed to its metal chelating ability (Can-Peng *et al*., 2014). In the antioxidant assay, Se-species enter to the cells (Caco-2) at a supranutritional dose and carry out several mechanisms that counteract the oxidative stress. The effects of Se in the cells are mainly related to the redox cycling: cell signaling, cell cycle checkpoint genes and proliferation, DNA stability, caspase-mediated apoptosis, reduced inflammatory response, angiogenesis, and osteoclast inactivation (Zang *et al*., 2013). The improvement of the antioxidant activity of selenoproteins quantitatively depended on their Se content. Although the specific mechanism by which Se enhances the antioxidant activity of the protein is still uncertain, when the protein is combined with Se, its antioxidant activity is remarkably improved (Hu *et al*., 2014).

4.3 Conclusions

This study demonstrated that the germination process in the presence of 1 mg and 2 mg of Na_2SeO_3/ 100 g of seeds increased the Se content by 17 and 29-fold in total protein compared to the control chickpea sprouts. The Se accumulation during the germination process modified the protein profile of chickpea sprouts. The Se content was mainly discovered in the Glu fraction that resulted in the most antioxidant peptides (<10 kDa) after the *in vitro* digestion, particularly when chickpeas were pre-treated with 2 mg Na_2SeO_3/100 g seeds. Besides, it is demonstrated that the Se has regulatory effects on proteolysis, reducing degradation of storage proteins during germination and affecting the digestibility of Glo fraction.

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Chapter 5. Up-scale of chickpea germination.

Manuscript: Deglycosylation of isoflavones in selenized germinated chickpea flours due to convection drying.

Authors: Sayra N. Serrano-Sandoval, Daniela Guardado-Félix, Janet A. Gutiérrez-Uribe

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Impact factor (2021): 4.952, Q1.

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5.1 Background

It is reported that chickpeas contain isoflavones that have a very important role in the redox balance, cancer, cardiovascular disease, obesity, and diabetes (Zaheer *et al*., 2017). Besides, it has been reported that the Se-enrichment during germination of chickpeas (Serrano-Sandoval *et al*., 2019; Zhang *et al*., 2012), rice (Gong *et al*., 2018), and adzuki beans (de Oliveira *et al*., 2017) allows the accumulation of the mineral into proteins and increase the quantity of other phytochemicals such as isoflavones (Gao *et al*., 2015; Guardado-Félix *et al*., 2017; Lazo-Vélez *et al*., 2018). The increase of isoflavones during germination in presence of Se has been related to the overexpression of genes related to the biosynthesis of polyphenolic compounds (D´Amato *et al*., 2018; Hawrylak-Nowak *et al*., 2018; Mimmo *et al*., 2017), and the increase of phenylalanine ammonia-lyase (PAL) activity, the first enzyme in the phenylpropanoid pathway (Wang *et al*., 2016; Li *et al*., 2019). On the other hand, Se also has reported toxicity in some plants and legumes that depends on the tolerance of the plant (Fioritti-Corbo *et al*., 2018; Zhao *et al*., 2019; Romero *et al*., 2019). It is reported that Se toxicity inhibits plant growth, and it is detectable through some indicators such as the low content of some compounds that are present during growing (Fioritti-Corbo *et al*., 2018), and the radicle size (Zhao *et al*., 2019).

Due to the promising results of selenization of chickpea through germination in our research group (Guardado-Félix *et al*., 2017, Guardado-Félix *et al*., 2019; Serrano-Sandoval *et al*., 2019), there is an interest in adding these sprouts to different foods. Additionally, for the large-scale production of Se-enriched germinated chickpeas flour, it is crucial to find an economic drying method that can retain the Se concentrations and isoflavones.

Freeze- and convection-drying (FD, CD) methods are the most studied procedures but there are few reports about the effect of these methods on the Se content and the isoflavones profile. The objective of this research was to increase isoflavones and total phenolic due to Se-enrichment in germinated chickpea obtained at large-scale production using different Se concentrations and evaluate the effect of freeze- and convection- drying on the retention of Se and isoflavones. Besides, evaluate the β-glucosidases and PAL activities in dried samples and during the germination process.

5.2 Results and discussion

5.2.1 Total Se concentration

Total Se content augmented 4.33-, 6.79-, and 29.51-fold in the FD Se-germinated chickpea flours when Na_2SeO_3 was used in the soaking water at concentrations of 24, 48, and 96 mg/L, respectively, compared to the control germinated chickpea flour. Se retention was higher in the flours obtained from germinated seeds of the highest dose of Na_2SeO_3, while the lowest retention was obtained in those with the lowest dose (Table 8).

Table 8. Total selenium (Se) content (µg/g of flour, drying weight (dw)) and Se retention (%) in flours made with chickpeas soaked in water with different concentrations of Se (0, 24, 48, and 96 mg of Na_2SeO_3/L) and then germinated by 48h and dried by convection-drying (CD) or freeze-drying (FD).

mg of Na_2SeO_3/L in the soaking water	Drying type	Se content (µg/g of flour, dw)	Se retention (%)
0	CD	0.774±0.052 e	-
	FD	0.884±0.002 e	
24	CD	3.299±0.056 d	8.21 ± 0.38 e
	FD	3.825±0.143 d	9.12 ± 0.01 de
48	CD	5.403±0.247 c	10.00 ± 0.17 cd
	FD	6.001 ±0.005 c	11.59 ± 0.43 c
96	CD	22.780 ±0.265 b	17.31 ± 0.28 b
	FD	26.089 ±0.751 a	19.82 ± 0.81 a

Means ± standard deviation of three experiments and three replicates. The significantly different mean values are labeled with different letters in each column according to the results of the Tukey's HSD post hoc test with 95% of confidence.

CD process reduced by 12.7% the concentration of Se in germinated chickpea, when 96 mg of Na_2SeO_3/L were used in the soaking water, in comparison to FD. No significant changes due to CD were observed in the Se content of flours obtained with 24 or 48 mg of Na_2SeO_3/L. It has been observed that when Se enters the cell, it is capable of intercalating with sulfur and thus embedding itself in proteins (Schiavon & Pilon-Smits, 2016). On the other hand, volatilization of Se has been observed when it is found at toxic concentrations for plants (Gupta & Gupta, 2017; Schiavon & Pilon-Smits, 2016).

The highest level of Na_2SeO_3 used in this study was found toxic for chickpea germination since a significant decrease of 64% in the radicle size was observed in the germinated chickpea soaked with 96 mg of Na_2SeO_3/L related to the control (Table 9). Radicle size is an indicator of toxicity in plants (Luo *et al*., 2017) and therefore volatilization was observed.

***Table 9**. Radicle size in centimeters of the germinated chickpea control and germinated chickpea soaking with 96 mg of Na_2SeO_3/ at 48h of germination.*

Sample	Radicle size (cm)
Germinated chickpea control	2.73 ± 0.59 a
Germinated chickpea soaking with 96 mg of Na_2SeO_3/L	0.99 ± 0.59 b

Mean of 100 measurements of different seeds of the three experiments and standard deviation. Tukey's HSD assessment establish that different letters indicate that there are significant differences among the values.

Although the Se content accumulated as the Na_2SeO_3 increased in the soaking water, the concentrations obtained in a previous study (Guardado-Félix *et al*., 2017) carried out at laboratory scale were not reached. Guardado-Félix *et al*. (2017) used 24 mg of Na_2SeO_3/L in the soaking water and reached a Se concentration almost 2-fold higher than the obtained with 96 mg of Na_2SeO_3/L in this study. Due to the machinery used for soaking at industry, it was necessary to use 352% more water than in the laboratory scale. Therefore, strategies are required for the optimization of seeds hydration for large-scale production to reduce the amount of water to obtain chickpea germinates with higher Se retention.

5.2.2 Total phenolic and PAL enzymatic activity in chickpea flours

The highest content of phenolics was found in the 48 h germinated chickpeas soaked with 24 or 48 mg Na_2SeO_3/L. Increases of 24 and 22% in the total phenolics content were observed at 48 h germinated chickpeas soaked with 24 or 48 mg Na_2SeO_3/L, respectively, when compared with control. Se treatment enhanced 106 and 103% the PAL activity in 48 h germinated chickpeas soaked with 24 and 48 mg Na_2SeO_3/L, respectively, in comparison to the control. The coefficient of determination of the correlation of the PAL activity and the total phenolic content in FD, germinated chickpea was 0.7647 (Figure 4).

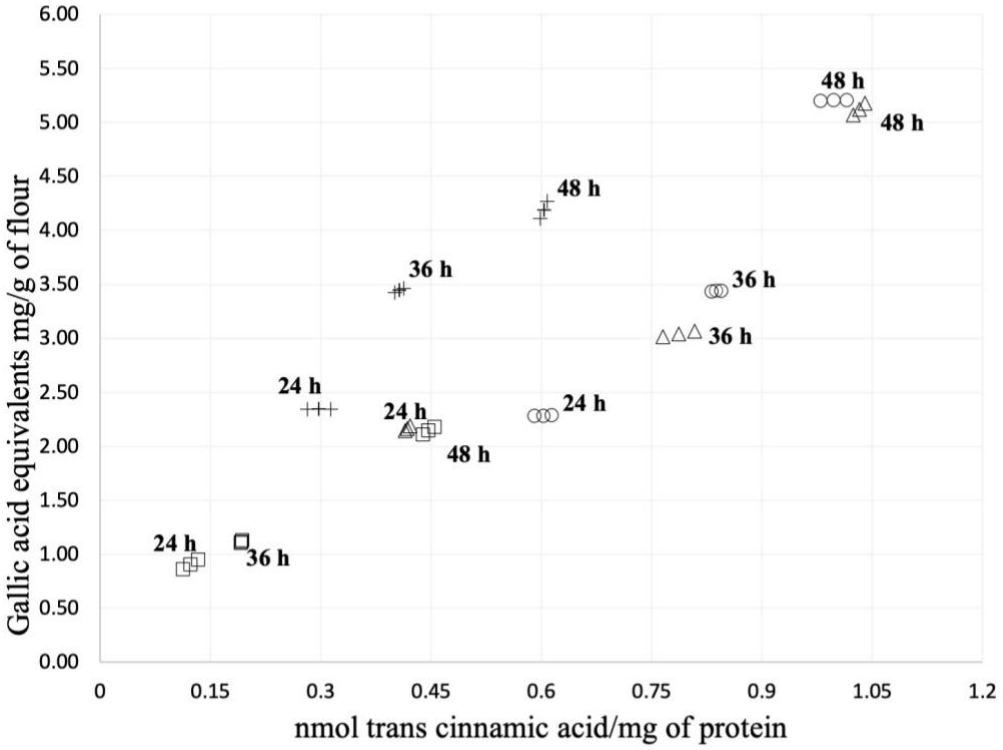


Figure 4. Dispersion graph of total phenolic content indicated by gallic acid equivalents per gram of flour (GAE/g of flour) and PAL activity as nmol of trans-cinnamic acid per milligram of protein of germinated chickpea in presence of different selenium (Se) concentrations (0, 24, 48, or 96 Na_2SeO_3/L) obtained at 24, 48 or 48 h of germination dried by freeze-drying. The cross, circle, triangle, and square represents the groups of 0, 24, 48, and 96 mg of Na_2SeO_3/L, respectively.

Se supplementation in soaking water at 24 or 48 mg Na_2SeO_3/L allowed the increase of PAL activity and consequently the total phenolics in the germinated chickpeas. Wang *et al.* (2016) reported that the polyphenolic compounds accumulation in peanuts that grew in seleniferous soil was due to the phenylpropanoid pathway activation. Later, Guardado-Félix *et al.* (2017) corroborated the increase of isoflavonoids during the germination of chickpea in presence of Se mainly due by the activation of PAL. Besides, other studies have reported the augment of other bioactive compounds in addition to phenolics that increased under Se biofortification such as flavonoids, vitamin C, anthocyanin,

chlorophyll, and carotenoid (Mohammad *et al*., 2020). An induction of genes related to phenolic biosynthesis has been observed due to Se treatment (Hawrylak-Nowak *et al*., 2018). Se-enriched lamb´s lettuce also accumulated significantly more phenolic compounds under normal temperature (Hawrylak-Nowak *et al.*, 2018). As well, the Se supplementation induced an accumulation of flavonoid and polyphenol compounds in *Fragaria x ananassa* plant (Mimmo *et al*., 2017). D´Amato *et al*. (2018) reported that moderate concentrations of Se (i.e., not higher than 45 mg/L) are the best treatments to obtain high phenolic compounds content in rice (*Oryza sativa* L.).

It is known that Se can act as an abiotic elicitor activating the phenylpropanoid pathway and other pathways but with certain limitations. Fioritti-Corbo *et al*. (2018) reported that common bean showed toxicity to sodium selenate (Na_2SeO_4) at 5000 g/ha (Fioritti-Corbo *et al*., 2018). Additionally, low doses (≤ 2 mg/L) of selenite could promote the growth of *C. pyrenoidosa*, but doses higher than 5 mg/L inhibited it (Zhao *et al*., 2019). Romero *et al*. (2019) evaluated the citotoxicity and mitochondrial alterations that carry out the inorganic Se species in the *Tetrahymena thermophila* growing. In this study, it was observed that 96 mg Na_2SeO_3/L treatment was a toxic concentration for chickpeas sprouts because of the volatilization of Se, the inhibition of growing and PAL activity, and consequently, the depletion of total phenolic compounds concentration.

5.2.3 Isoflavone profile and β-glucosidase activity of germinated chickpea flour

A significant increase in all isoflavones was observed through germination time ($p \leq 0.05$) (Figure 5, Table 10). Se caused the reduction of some isoflavones, particularly in the FD treatment at 48 h of germination. Isoformononetin glycoside had reductions of 13.72, and 84.65%, related to the control, in the 24 and 48 mg of Na_2SeO_3/L treatments, respectively. Likewise, malonylated formononetin glycoside was reduced 19.24, 58.74, and 69.49% related to the control in the treatments of 24, 48, and 96 mg of Na_2SeO_3/L. Biochanin A glycoside showed reductions of 17.82 and 89.94% in the 24 and 48 mg of Na_2SeO_3/L treatments related to the control. Isoflavone malonylated biochanin A glycoside reported reductions up to 19.39,

42.85, and 82.24% related to the control in the treatments of 24, 48, and 96 mg of Na_2SeO_3/L, respectively. Aglycones formononetin and biochanin A isoflavones showed reductions up to 10.64 and 73.16% and 16.51 and 67.16% in the 24 and 48 mg of Na_2SeO_3/L, respectively. The isoflavones glycosides content was reduced in the CD treatments in comparison to FD, particularly decrements of 67.63, 50.36, 84.04, and 57.17% were observed in the samples obtained after 48 h of germination in the isoformononetin glycoside, malonylated formononetin glycoside, biochanin A glycoside, and malonylated biochanin A glycoside, respectively. In contrast, formononetin and biochanin A aglycones content in 48 h germinated chickpeas increased by 118.98 and 156.13% in the CD related to FD.

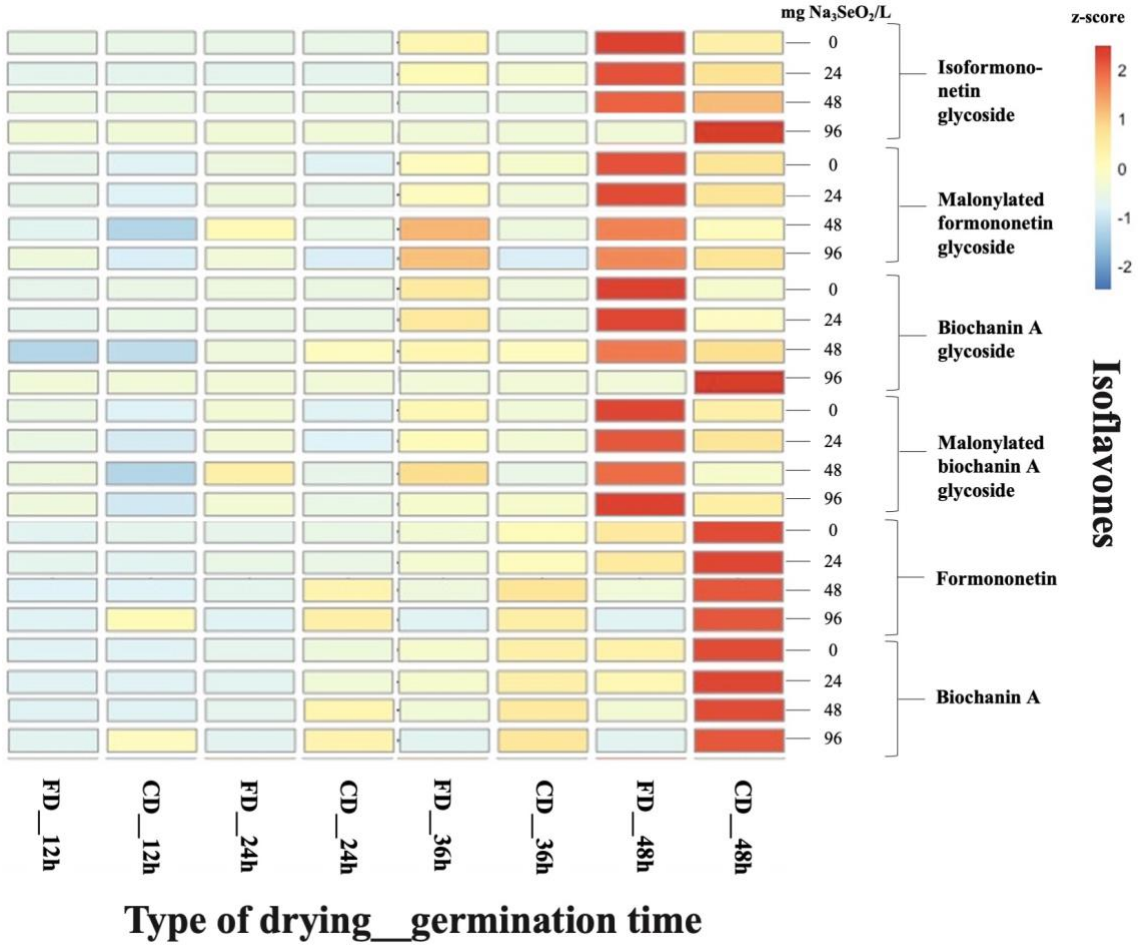


Figure 5. Heatmap of the isoflavones (µg/g dry weight (dw) profile affected by Se (mg Na_2SeO_3/L in soaking water), germination time, and drying type (convention-drying: CD, freeze-drying: FD).

Table 10. Individual values of the isoflavones (μg/g dry weight (dw)) profile affected by selenium (mg Na_2SeO_3/L in soaking water), germination time, and drying type (convention-drying: CD, freeze-drying: FD).

		Type of drying							
	mg Na_3SeO_2/L	FD 12h	CD 12h	FD 24h	CD 24h	FD 36h	CD 36h	FD 48h	CD 48h
Isoformononetin glycoside	0	ND	ND	ND	ND	173.36 ± 8.23 e	ND	624.13 ± 2.93 a	202.04 ± 1.51 d
	24	ND	ND	ND	ND	152.88 ± 1.64 f	80.80 ± 0.55 h	538.47 ± 4.30 b	262.71 ± 4.77 c
	48	ND	ND	ND	ND	ND	ND	95.81 ± 0.49 g	65.44 ± 2.00 i
	96	ND	ND	ND	ND	ND	ND	ND	38.90 ± 0.08 j
Malonylated formononetin glycoside	0	45.41 ± 0.73 m	ND	97.51 ± 2.25 k	ND	232. 97 ± 11.62 g	161.97 ± 4.57 j	881.31 ± 15.56 a	437.54 ± 9.91 c
	24	46.67 ± 0.33 m	ND	87.16 ± 2.67 kl	47.32 ± 0.68 m	189.62 ± 3.56 hi	103.14 ± 0.50 k	711.69 ± 8.23 b	356.92 ± 5.69 d
	48	61.62 ± 0.05 lm	ND	168.96 ± 3.08 ij	80.61 ± 1.10 kl	300.93 ± 7.15 e	93.35 ± 0.48 k	363.66 ± 1.95 d	157.44 ± 3.02 j
	96	48.52 ± 0.16 m	ND	52.46 ± 0.07 m	ND	214.85 ± 1.00 gh	ND	268.87 ± 1.82 f	163.12 ± 0.15 ij
Biochanin A glycoside	0	7.46 ± 1.29 m-o	10.39 ± 0.41 k-n	22.77 ± 0.65 f-i	15.14 ± 0.23 i-m	118.71 ± 7.01 c	25.97 ± 0.12 fg	294.55 ± 0.12 a	47.00 ± 1.03 e
	24	8.67 ± 0.60 l-n	16.79 ± 0.11 h-k	17.82 ± 0.17 h-k	19.81 ± 0.32 g-j	106.36 ± 1.96 d	24.44 ± 0.01 f-h	242.03 ± 1.19 b	53.32 ± 0.74 e
	48	3.65 ± 0.12 no	4.55 ± 0.04 no	10.50 ± 0.22 k-n	14.33 ± 0.61 j-m	16.29 ± 0.46 i-l	14.08 ± 0.03 j-m	29.62 ± 0.31 f	20.97 ± 0.49 g-j
	96	ND	ND	ND	ND	ND	ND	ND	7.14 ± 0.19 m-o
Malonylated biochanin A glycoside	0	58.97 ± 4.00 h-j	29.71 ± 1.17 k	84.17 ± 3.39 g	35.93 ± 0.73 jk	136.00 ± 8.23 ef	80.08 ± 14.70 gh	367.15 ± 5.70 a	157.24 ± 7.37 de
	24	63.91 ± 1.51 g-i	25.45 ± 0.01k	79.41 ± 1.72 gh	36.36 ± 1.05 jk	116.47 ± 3.24 f	81.03 ± 3.11 gh	295.95 ± 2.99 b	169.63 ± 3.52 d
	48	65.20 ± 1.29 g-i	17.40 ± 0.42 k	116.83 ± 2.25 f	55.32 ± 1.62 ij	143.72 ± 5.85 e	58.31 ± 0.33 hij	209.82 ± 0.61 c	84.73 ± 2.21 g
	96	27.09 ± 0.54 k	19.82 ± 0.11 k	28.51 ± 0.08 k	25.56 ± 0.65 k	29.97 ± 0.14 k	30.71 ± 0.14 k	65.15 ± 0.60 g-i	39.01 ± 0.50 jk
Formononetin	0	29.03 ± 0.19 o-q	37.93 ± 0.65 n-p	46.41 ± 0.55 m-p	64.71 ± 0.95 k-n	117.96 ± 0.00 i	203.33 ± 2.15 fg	312.52 ± 5.77 d	684.36 ± 22.92 a
	24	32.27 ± 0.18 op	26.30 ± 0.18 o-q	50.84 ± 2.00 l-p	57.27 ± 0.25 k-o	113.74 ± 3.86 ij	173.60 ± 2.91 gh	279.27 ± 0.50 e	649.84 ± 16.58 b
	48	23.43 ± 0.17 pq	24.19 ± 0.23 pq	43.48 ± 0.13 m-p	168.87 ± 6.27 h	71.38 ± 1.59 k-m	219.80 ± 1.50 f	83.86 ± 0.37 jk	405.76 ± 2.87 c
	96	ND	24.88 ± 0.22 pq	ND	31.11 ± 0.24 o-q	ND	32.35 ± 0.13 op	ND	80.55 ± 1.76 kl
Biochanin A	0	17.12 ± 0.15 i-k	17.15 ± 0.36 i-k	36.96 ± 0.39 hi	71.11 ± 1.26 fg	97.14 ± 1.48 e	189.65 ± 3.08 c	178.77 ± 4.58 c	457.88 ± 13.30 a
	24	16.54 ± 0.13 i-k	16.25 ± 0.11i-k	23.69 ± 0.02 i-k	69.70 ± 0.04 fg	88.31 ± 2.92 ef	177.36 ± 5.26 c	149.20 ± 0.17 d	437.16 ± 13.30 a
	48	20.57 ± 0.11 i-k	21.76 ± 0.49 i-k	29.00 ± 0.75 ij	104.41 ± 0.84 e	53.02 ± 0.33 gh	131.86 ± 4.72 d	58.69 ± 0.25 gh	271.10 ± 1.52 b
	96	ND	5.91 ± 0.17 jk	ND	8.48 ± 0.07 jk	ND	11.45 ± 0.42 jk	ND	24.39 ± 0.82 ij

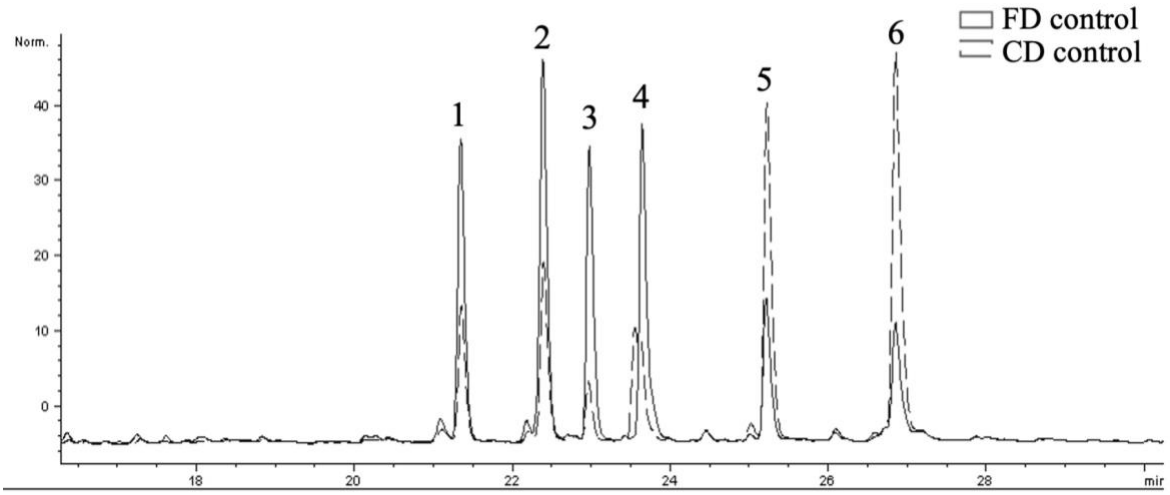
Means ± standard deviation of three experiments and three replicates. The significantly different mean values are labeled with different letters in each column according to the results of the Tukey's HSD post hoc test with 95% of confidence.

Compared to the β-glucosidase activity of FD control sample, the CD germinated chickpea flour without Se supplementation had 60.5% more activity but when 96 mg Na_2SeO_3/L were used the activity increased only 8.8%. In general, the β-glucosidase activity was higher in the flours obtained by CD (Table 11, Figure 6).

Table 11. Delta percentage of the increase of β-glucosidase activity in the samples of 48 h of germination due to the convection-drying (CD) in comparison to freeze-drying (FD).

mg Na_2SeO_3/L	**Delta percentage of the increase of β-glucosidase activity (%)***
0	60.50 ± 16.2 a
24	38.02 ± 9.19 ab
48	27.13 ± 6.77 bc
96	8.78 ± 0.41 c

*The delta percentage was calculated by the subtraction of the CD value minus FD value divided by the FD value of the samples multiplied by 100. FD and CD values correspond to means of three experiments and three replicates.



Peak order	Compunds	UV λ max (nm)	$[M+H]^+$
1	Isoformononetin glycoside	253, 305	431
2	Malonylated formononetin glycoside	256, 306	517
3	Biochanin A glycoside	259, 331	447
4	Malonylated biochanin A glycoside	259, 323	533
5	Formononetin	249, 304	269
6	Biachanin A	259, 329	285

Figure 6. Change from glycosidic isoflavones to aglycones due to convection-drying (CD) in comparison to freeze-drying (FD) is shown in the chromatogram at 260 nm of control chickpea germinated at 48 h.

According to Niamnuy *et al.* (2012), malonyl- and acetyl- groups were converted to glucosides and these into aglycones when the temperature and time of drying increased in soy. Similarly, Swinny and Ryan (2005) stated that air-drying produced aglycones of formononetin and biochanin A in the isoflavone extracts of red clover (*Trifolium pratense* L.) due to β-glucosidases. Additionally, other authors reported the transformation of malonyl-glucosides into glucosides or aglycones under a heat treatment (oven-drying and roasting) (Lee & Lee, 2009; Aguilar *et al.*, 2012). Likewise, a conversion of daidzin and genistin into their aglycones (daidzein

and genistein, respectively) was found in a thermal drying (50 ºC) of okara (Muliterno *et al*., 2017). Similarly, a predominance of aglycones was found in the okara when heating drying methods were used. The authors explained that isoflavones are more vulnerable to transformation than degradation during heating (Guimarães *et al*., 2020). On the other hand, Swinny and Ryan (2005) declared that freeze-drying (FD) prevented the enzymatic hydrolysis of red clover tissue that contains predominantly malonyl glucosides. From the point of view of health, isoflavones aglycones are more desired due to their greater absorption and availability and therefore they are more bioactive than glycosides (Valderrain-Rodríguez *et al*., 2014). The reduction in the increase of β-glucosidase activity at higher doses of Na_2SeO_3 was related to the lower activity of this enzyme in FD germinated chickpea flours (data not shown).

Despite the increase of total isoflavones during germination time (Figure 7), the different doses of Na_2SeO_3 in soaking water did not favor the increase of total isoflavones related to the control. Guardado-Félix *et al.* (2017) published that the germination of chickpea in presence of Se raised isoflavones mainly formononetin and its glycosylated forms with a treatment of 2 mg of Na_2SeO_3/100 g of seeds. Likewise, malonylated genistein and daidzein glycosides increased in Se-enriched germinated soybean (Lazo-Vélez *et al*., 2018). However, it is well known that most of the glycosides are highly water soluble (Belščak-Cvitanović *et al*., 2018) and lost in the water used for soaking and spraying process in the industry (Sanches de Lima *et al*., 2014). Consequently, it is necessary to improve the soaking conditions to avoid the leaching of compounds such as isoflavones, loss of Se and reduce the water use. One improved soaking method was described by Salces *et al*. (2019) that consists in a controlled hydration (2 h) in a rotating soaking basket. This process minimized the leaching of soluble components and reduce water waste during the process.

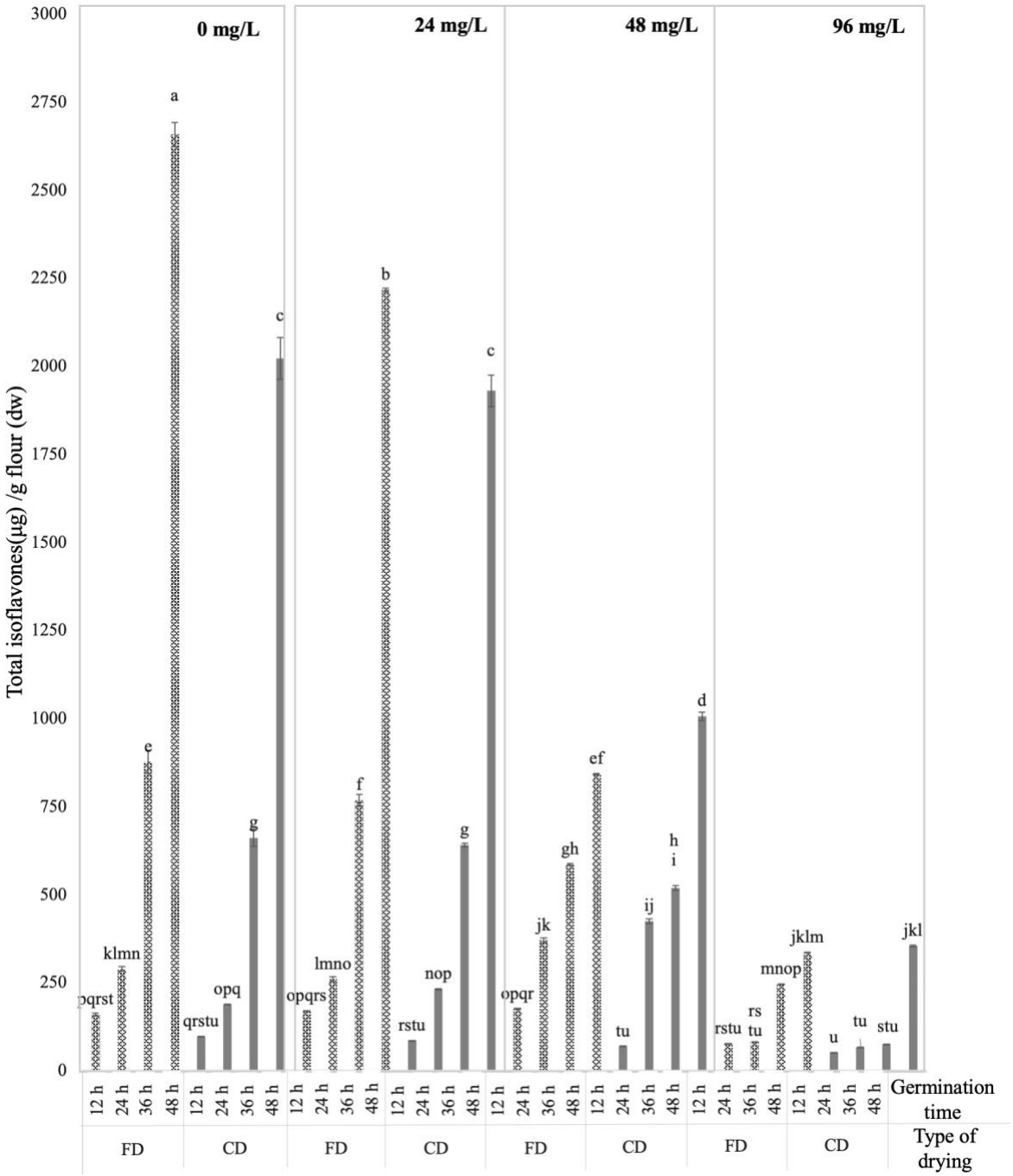


Figure 7. Total isoflavone contents (µg/g dw) of freeze- (FD) or convection dried (CD) germinated chickpea flours obtained at different germination times and using sodium selenite concentrations of 0, 24, 48 or 96 mg/L in soaking water. Mean ± standard deviation of three replicates. Mean values in each bar sharing the same letter were not significantly different ($p < 0.05$).

5.3 Conclusions

The best conditions to produce a germinated selenized chickpea flour at up-scale condition with the highest Se retention and isoflavones content (3.299±0.056 and 1930±45µg/g of flour, respectively) were obtained when 24 mg of Na_2SeO_3/L were used during soaking and germinated for 48h. The biosynthesis of phenolics was corroborated by the increase of PAL activity. The CD is an economic drying method that allowed the conversion of isoflavones glycosides into their aglycones due to the activation of β-glucosidase enzyme and without any effect on the Se concentration when low or intermediate doses (24 or 48 mg/L) of Na_2SeO_3 were used. Soaking with 96 mg of Na_2SeO_3/L showed toxicity in chickpeas sprouts that were visible in the radicle size, low PAL enzyme activity, and for consequence low phenolics and isoflavone content. It is necessary to improve the soaking method to avoid the possible leaching of water-soluble compounds and to reduce the waste of water in the industrial scale.

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Chapter 6. Chickpea sprouts extrudates: functional, physicochemical, and sensorial properties.

Manuscript: Germinated chickpea-maize extrudates: Starch-lipid interactions, functional, physicochemical, and sensorial properties.

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6.1 Background

In Mexico, it is estimated that only 14% of the population have no nutritional health issues (del Castillo Negrete, 2013), 70% of the population is overweight, and almost one third suffers from obesity (ISSSTE, 2016). In fact, obesity and overweight are becoming a threat to the Mexican Health System (Secretaría de Salud, 2013) and it is taking over malnutrition (Ibarrola-Rivas & Galicia, 2017). It is thought that the high consumption of processed, and snacks foods has contributed to the increase in obesity and diabetes worldwide, (del Castillo Negrete, 2013) and approximately 25% of the total daily caloric intake of children is made up of snack foods (Hegazy *et al*., 2017).

Extrusion processing is commonly used in the food industry to produce breakfast cereals, pasta, snacks, pet foods, animal feed, cereal baby foods, among other products (Wang *et al*., 2019a; Singha *et al*., 2018). The high temperatures and shear of extrusion process have demonstrated to increase the protein digestibility and resistant starch content, inactivate undesirable enzymes and decrease toxins and antinutritional factors, generating a healthy food (Singha *et al*., 2018; Wang *et al*., 2019 a,b). Maize snacks are the most popular in the Mexican market of the

extruded products (Hegazy *et al*., 2017; Singha et al., 2018). However, they contain a high percentage of starch (56-74%), tend to be low in protein, and have high glycemic index (Singha *et al*., 2018; Shevkani *et al*., 2019).

Legumes have been used successfully as partial substitution of maize flour producing extruded food with high protein quality, fiber, vitamins, phenolic compounds, and minerals such as potassium, iron, zinc, calcium, and magnesium. Besides, legumes are rich in lysine and contain all the essential amino acids except cysteine and methionine which are limiting, whereas cereal grains are rich and limiting in the opposite (Hegazy *et al*., 2017; Shevkani *et al*., 2019; Wang *et al*., 2019a). Additionally, sprouted legume flours are a good source of antioxidant compounds, as well as protein, and have been used in the formulation of extruded products (Serrano-Sandoval *et al*., 2019; Gong *et al*., 2018). Cruz-Ortiz *et al*. (2020) obtained extrudates with a low glycemic index and high resistant starch content when used 44% of germinated soybean flour and 56% of maize starch. Hegazy *et al*. (2017) reported that the addition of germinated chickpea and tomato to maize snack improved the nutritional value and antioxidant activity of the extrudates due to the decrease of phytic acid and tannic acid and higher protein digestibility. Hence the supplementation with chickpea flour to maize extrudates improves the protein quality (Wang *et al*., 2019a). However, the taste and general acceptability are affected by a high percentage of chickpea flour supplementation (Singh *et al*., 2017).

It is well known that higher amounts of lipids confer resistance to starch toward α-amylolysis due to chemical interactions (Annor-Amponsah *et al*., 2013). For that reason, the consumption of amylose-lipid complexes reduced the glucose spikes in blood (Annor-Amponsah *et al*., 2013). The increase in resistant starch content has been proposed as an instrument in the management of obesity and type 2 diabetes. Besides, it is known that resistant starch improves gut health because it can be processed in the colon by some probiotic bacteria that produce several short-chain fatty acids (Gutiérrez & Tovar, 2021; von Borries-Medrano *et al*., 2018; Wang *et al*., 2020). The objective of this study was to formulate healthy snacks of maize

grits supplemented with germinated chickpea flour that were acceptable by consumers and with satisfactory structural and functional properties.

6.2 Results and discussion

6.2.1 Resistant starch, starch-lipid interactions, and protein digestibility of the extrudates

It is known that the extrusion process promotes several molecular and structural changes in starch fractions due to the harsh thermo-mechanical conditions, that include the loss of crystalline structure, gelatinization, and dextrinization. In addition, pressure, mechanical shear, temperature, and moisture favor new molecular interactions, as the formation of starch-lipid complexes (Wang *et al*., 2020). For these reasons it was important to understand the nature and causes of the changes in the starch digestibility of our samples; thus, to stablish the influence of the chemical composition, we analyzed the extrudates with and without fat. In defatted samples, the supplementation with germinated chickpea flour increased the resistant starch content from 2.52 g/100g (ME) to 3.81 g/100g (MGCE-40), this could be related with the enhanced thermal resistance of legume starches, that has been reported in other studies (Chávez-Murillo *et al*., 2018; Yniestra *et al*., 2019). In fact, GCE had the highest content of resistant starch in defatted and non-defatted samples (Table 12). When the extrudates were analyzed without defatting, they showed even higher RS contents ($p=0.0001$). This increase was more remarkable in ME (194.18%) than in those made of 100% germinated chickpea flour (82.70%), demonstrating that maize starch interacted with lipids and proteins when blended with germinated chickpea flour. Interestingly, the RS increase was more remarkable when high proportions of germinated chickpea flour were used, particularly, the MGCE-10 samples increased by ≈28% and MGCE-40 by ≈54% compared with defatted samples. This could be explained from a molecular perspective, where these complexes are commonly formed by amylose-lipid conglomerates that can be

ordered as crystalline form (Wang *et al*., 2020; von Borries-Medrano *et al*., 2018) or by the combination of amylose and long branch chains of amylopectin form single-helical complexes with fatty acids (Calvo-López & Martínez-Bustos, 2017).

As starch is the principal component of maize (72-73%) (Paraginski *et al*., 2014) in the ME there was more amylose content available to form resistant starch V complexes with lipids. Meanwhile in the blends of maize and germinated chickpea flour, the increase in resistant starch was mainly due to the increase in the amount of protein (Table 12). According to previous reports, proteins reduce the digestibility of corn starch due to starch granules encapsulation, interaction between protein filaments and three-dimensional network of corn starch, hydrogen bonds between hydrophilic groups of proteins and interaction of proteins with digestive enzymes (Chen *et al*., 2017).

Total protein content of extrudates proportionally increased with the percentage of germinated chickpea flour supplemented in comparison to ME (Table 12). Wang et al. (2019a) reported an increase of 100.70% in the maize extrudates supplemented with 50% of chickpea flour. Contrary to the resistant starch results, the maize-germinated chickpea flour blends did not affect the protein digestibility. The IVPD that was found in the GCE (79.16%) increased by the extrusion process (raw chickpea: 75.08%, data not shown) as in previous reports (Rathod & Annapure, 2016; Patil *et al*., 2016; Ali *et al*., 2017). There was not significant difference among the IVPD of the GCE, MGCE-20, -30, and -40. The IVPD of MGCE-10 was not significantly different to the observed in ME, indicating that maize extrudates with improved IVPD required more than 10% of germinated chickpea flour. Patil *et al*. (2016) reported that 5% of chickpea in combination with wheat flour increased the protein digestibility from 59.26 to 61.44% and when 15% of chickpea flour was used, it increased to 62.46%. Wang *et al*. (2019a) observed that there were no significant differences among the blends supplemented with 50 to 80% of chickpea flour but little was known about the functionality of the extrudates.

Table 12. Resistant starch content (g/100 g dwb= dried weight basis) of the extrudates before and after defatting, total protein content (%) and in vitro protein digestibility (IVPD).

	Resistant starch g/100g dwb		%Total	%Protein
Extrudates	**Defatted**	**Non-defatted**	**protein**	**digestibility**
ME	2.52 ±0.02j	7.40±0.37b	6.54 ± 0.02d	76.28 ± 0.24c
GCE	4.32 ±0.06e	7.89±0.00a	21.85 ± 0.17a	79.16 ± 0.03a
MGCE-10	3.03 ±0.04i	3.88±0.00f	8.03 ± 0.00cd	77. 58 ± 0.80bc
MGCE-20	3.43 ±0.05h	4.42±0.38e	9.89 ± 0.00c	77.90 ± 0.10ab
MGCE-30	3.52 ±0.06gh	5.94±0.09d	9.97 ± 0.92c	78.52 ± 0.24ab
MGCE-40	3.81 ±0.00fg	6.99±0.01c	13.51 ± 0.41b	78. 98 ± 0.23ab

ME= maize extrudates, GCE= germinated chickpea extrudates, MGCE-10, -20, -30 or -40= maize and germinated chickpea flour blends extrudates. Mean ± standard deviation of three replicates, the same letter was not significantly different ($p \leq 0.05$).

6.2.2 FTIR spectroscopy analysis of extrudates

The spectral intensity of extruded flours is shown in Figure 8 The carbohydrates region was characterized by different bands typical in this macromolecule. Strong absorption was observed in the region 1000-990 cm^{-1} due to C-O stretching vibrations. Another strong absorption was observed in the region 1020-1023 cm^{-1} due to C-O-C skeletal vibration. Medium intensity bands were observed in the region 1083-1079 cm^{-1} and 1157-1154 cm^{-1} due to C-OH skeletal vibration and C-O asymmetric binding, respectively. A weak signal was observed in the 947-938 cm^{-1} due to β-glycosidic C-H deformation (Figure 8). Krishnan *et al*. (2020) reported that in the region of amide I (≈1750 cm^{-1}) the strong ester (C=O) band show the formation of fatty acids ester complexes that confirmed the formation of lipid-protein complexes, this effect can be observed in the Figure 8. According to Krishnan *et al*. (2020), the structures resulted in V-type starch that are an impediment in the union of the enzyme-substrate system that in consequence

reduce the glycemic index of the starch. The protein zone showed strongest bands due to amide I, II, and III (1748-1649 cm^{-1}) which was common in all extrudates groups but more intense in GCE.

The extrusion conditions arranged amylose-lipid conglomerates in different orientation, and these were influenced by the thermal resistance of pulse starches that are prone to retrogradation at a higher extent than cereals (Wang *et al*., 2020). Notable differences were found in the deconvoluted carbohydrates zone (1000-1060 cm^{-1}) due to the inclusion of germinated chickpea flour (Figure 8B). The amorphous starch component (1022 cm^{-1}), related to disorganized or fragmented starch molecules, showed important differences between MGCE-10 and MGCE-40. (Figure 8B). On the other hand, the remnant crystalline (1047 cm^{-1}) dominion of starch molecules in ME contrasted with that in GCE due to the previously reported thermo-mechanical resistance of chickpea. Interestingly MGCE-10 had a similar intensity to ME while MGCE-40 was similar to GCE, demonstrating the strong effect of maize starch when low doses of germinated chickpea flour were used and chickpea protein interaction when high doses were used. Previous reports had shown that random arrangements might occur among legume flour components that, depending on the extrusion conditions, may weaken their crystalline structure (Chávez-Murillo *et al*., 2018).

The pronounced differences corresponding to the protein structural conformation are shown in the deconvoluted protein zone (Figure 8C). The protein secondary structures showed a band broadening in the α-helix signal (1635 cm^{-1}), being related to higher water and oil interactions, as well as in the β-sheet (≈1645 cm^{-1}), related to improved stability and resistance to starch and protein digestion in 100% maize extrudates. Previous studies have shown that α-helix and β-sheet protein secondary structures are the responsible for modulating the protein digestion (Chávez-Murillo *et al*., 2018). The protein secondary structures (α-helix and β-sheet) changed their proportions, particularly in MGCE-10 that compared to MGCE-40 had lower IVPD. These changes in the materials molecular conformation could result in

different digestion performance in the final products (Cai & Singh, 2004, Chávez-Murillo *et al*., 2021). Intra-molecular (1625 cm^{-1}) interactions were slightly broader when chickpea was used in the extrudates, which has been related to enhanced interaction (Figure 8C) (Chávez-Murillo *et al*., 2021). Also, C-N stretching vibration, attributed to Maillard´s reaction products, resulted by heating and induced protein and starch interactions (Ochoa-Yepes *et al*., 2019).

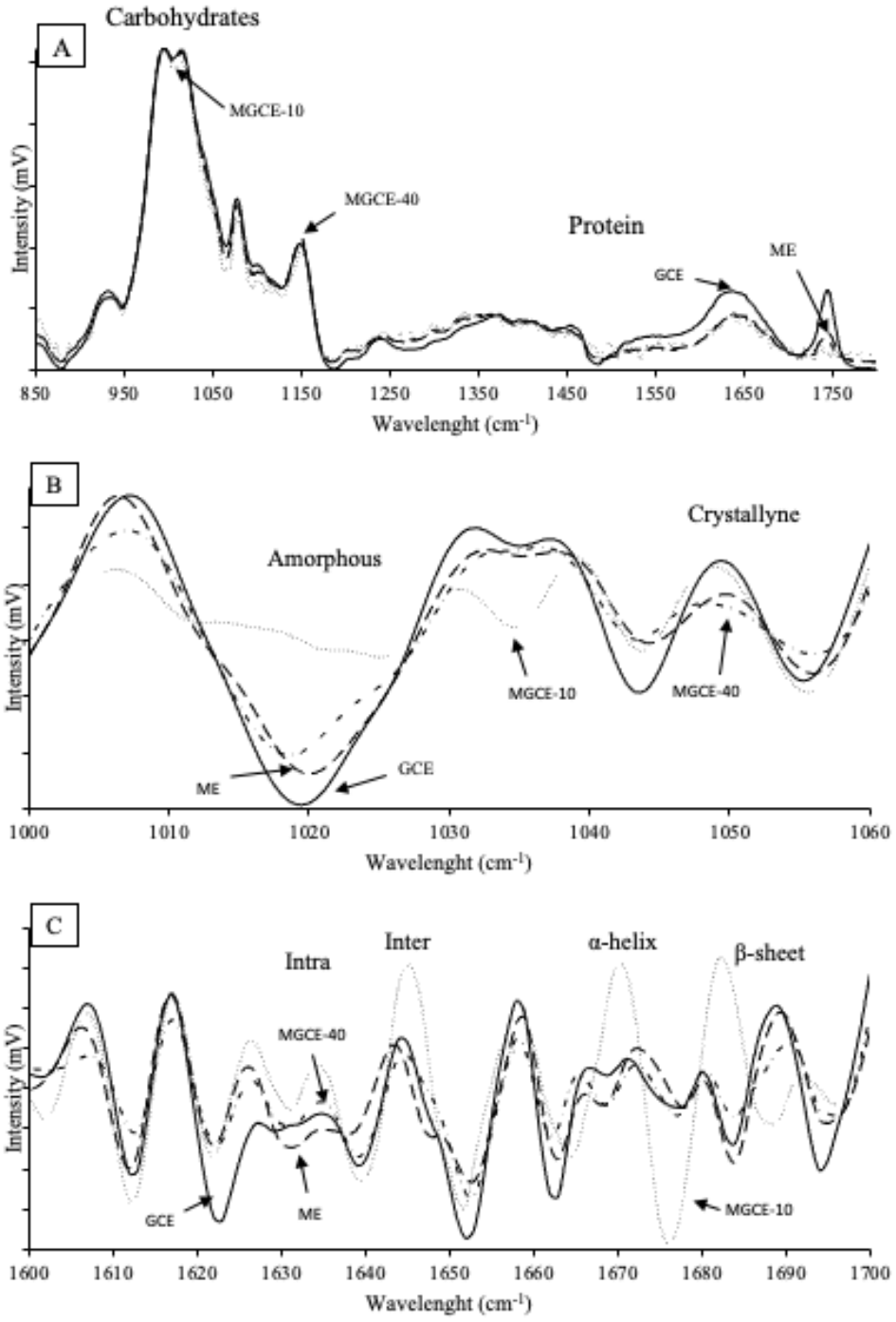

Figure 8. Full FTIR spectra of maize extrudates (ME), germinated chickpea extrudates (GCE), and maize extrudates supplemented with 10 and 40% of germinated chickpea flour (MGCE-10 and MGCE-40). B) Deconvoluted carbohydrates zone, C) Deconvoluted protein zone.

6.2.3 Expansion index

Expansion index is an important characteristic in puffed products that also affect textural parameters and consumer acceptability (Shevkani *et al*., 2019; Hegazy *et al*., 2017). ME had higher expansion index compared to GCE (Table 13). The highest expansion index of the mixtures was observed MGCE-30 and MGCE-40 even than ME. These results are contrasting to Hegazy *et al*. (2017) that reported that the expansion is higher in maize extrudates mainly due to the starch content. According to Day and Swanson (2013), the starch and proteins interact affecting extrudates' expansion since proteins adjust the water distribution and contribute to the extensive covalent and nonbonding networking that take place during extrusion. Singha *et al*. (2018) affirmed that an increase of protein to the starch ratio affects the starch gelatinization in extrudates supplemented with chickpea flour. According to Li *et al*. (2005), an increase of the expansion index was found in the maize extrudates fortified with soybean flour (≤ 40%) that contains high content of protein. Proteins do not suffer much thermal damage in the extrusion process and result in an effective expansion (Silvestre-De-León *et al*., 2020).

6.2.4 Texture analysis

There were no significant differences among the extrudates in the crunchiness parameter, while ME had the highest hardness (Table 13). Lee *et al*. (2006) reported that ME have high hardness since their protein content mainly is constituted by zeins. A decrease in hardness and improved porous texture and crispness resulted in maize extrudates supplemented with soybean flour (Li *et al*., 2005). Wang *et al*. (2019b) reported higher hardness values in maize extrudates supplemented with 20% chickpea flour but in this research, we used germinated chickpea. As expected, the crispness followed the same tendency as the hardness.

A significant negative correlation (-0.755, p=0.001) between extrudates crispiness and germinated chickpea flour addition was observed. As expected, this negative correlation was also observed for hardness (-0.729, p=0.002). But when

40% of germinated chickpea flour, stronger interactions between starch increased the hardness and crispness compared to the extrudates produced with only 30%. Yovchev *et al*. (2017) reported that significant negative correlation was found between hardness and expansion ratio for chickpea and barley extrudates.

6.2.5 Functional properties (WAI, WSI, and SP)

The lowest water absorption index (WAI) and the highest water solubility index (WSI) were found in the GCE (Table 13). Due to the interactions between starch and protein, the solubility of the germinated chickpea proteins was reduced as it was observed in all the extrudates obtained with the combination of germinated chickpea flour and maize grits in comparison to GCE (Day & Swanson, 2013). On the other hand, the swelling power (SP) did not have significant differences among the extrudates indicating that the water holding capacity of starch was the same in the extrudates.

Table 13. Structural and functional properties extruded from maize-chickpea flours.

Extruded	Expansion Index	Texture			WAI	WSI	SP
		Crunchiness	Hardness (N)	Crispiness (N.sec)			
ME	3.80±0.14b	90.33± 3.79a	274.20±2.85a	7455 ± 193a	6.22±0.20ab	22.74±4.94b	8.07±0.26a
GCE	2.35±0.05d	NA	NA	NA	4.60±0.26c	42.21±1.97a	7.96±0.18a
MGCE-10	3.56±0.11c	76.67± 7.64a	203.38±12.27b	5705.7±97.2b	6.70±0.03a	20.88±0.83b	8.47±0.12a
MGCE-20	3.59±0.10c	89.33±11.85a	176.73±4.64c	4907.9±105.3c	6.56±0.64ab	20.12±1.12b	8.21±0.81a
MGCE-30	4.03±0.10a	83.67±0.58a	121.64±4.30d	3614.0±25.8d	6.35±0.09ab	20.61±0.62b	8.00±0.17a
MGCE-40	3.96±0.09a	87.33±4.16a	187.58±5.41b	5154.6±157.6c	5.78±0.29b	25.08±0.90b	7.72±0.39a

WAI: water absorption index; WSI: water solubility index; SP: swelling power. ME= maize extrudates, GCE= germinated chickpea extrudates, MGCE-10, -20, -30 or -40= maize and germinated chickpea flour blends extrudates. Mean ± standard deviation of three replicates, the same letter was not significantly different ($p < 0.05$). NA=It was not possible to measure the hardness of the control chickpea due to their expansion ratio (2.35 ± 0.70) and the gaps of the Kramer cell.

6.2.6 Sensory analysis

ME, MGCE-10, -20, and -30 did not have significant differences in the overall acceptability (Figure 9). GCE had the lowest score (4.93; $p<0.05$). Regarding the chewiness parameter of MGCE-10, -20, -30, and -40 obtained the highest values (6.46, 6.19, 6.52, and 6.11, respectively) without significant differences among them ($p<0.05$). On the other hand, the ME and GCE had the lowest values, being 5.41 and 5.77, respectively. The crunchiness parameter had no significant differences among the extrudates ($p<0.05$). The ME and MGCE-10, -20, -30 got values between 5-6 for the taste parameter without significant differences. The GCE was rated with 4.16 in the taste/flavor, which was significantly lower than others. In fact, 37.5% of the comments in the survey affirmed that the extrudates had a good taste and

suggested adding some flavor to the extrudates (Figure 10). According to Singh *et al*. (2017) the appearance, flavor, and the overall acceptability scores dropped each time the chickpea flour increased to the maize extrudates. Contrary to these results, in this study a great acceptability was observed in the MGCE-10, -20, -30 and -40 even higher than those of ME. In contrast to previous reports, the great acceptability was attributed to chickpea germination.

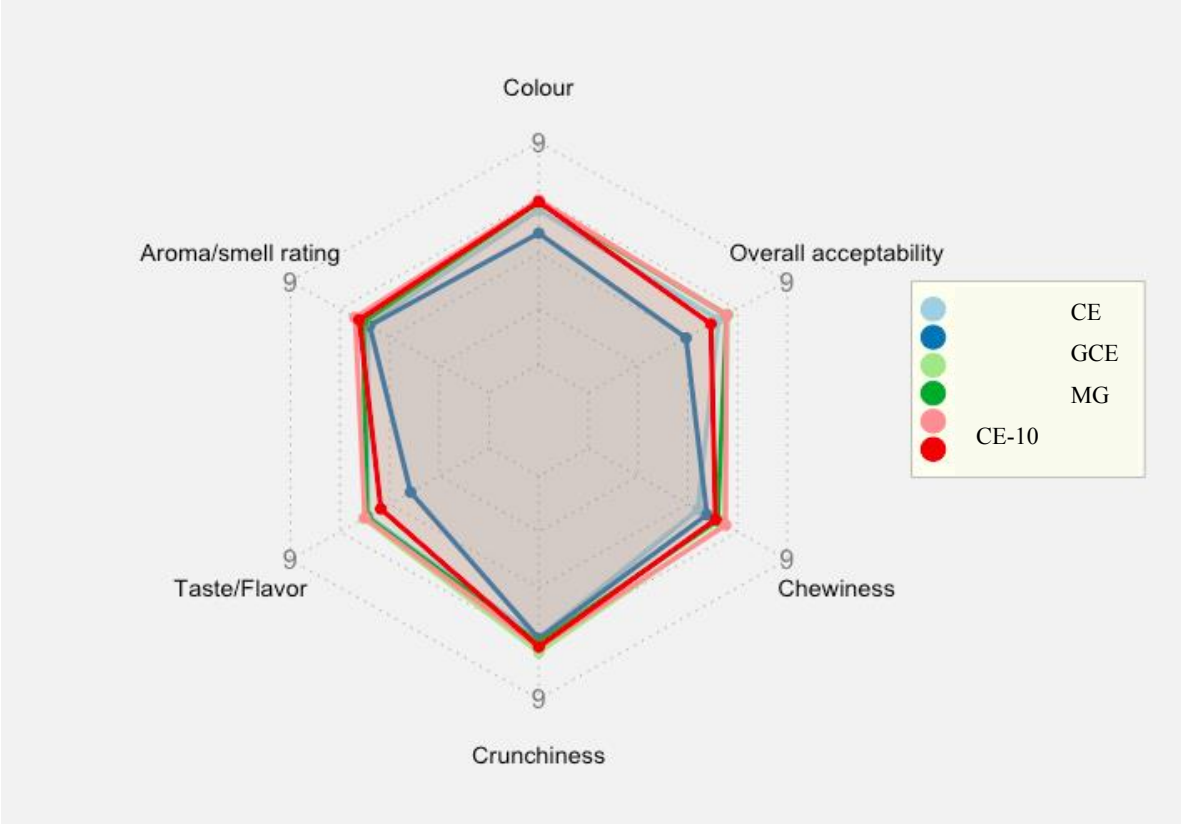

Figure 9. Spider chart of the sensory evaluation of ME= maize extrudates, GCE= germinated chickpea extrudates, MGCE-10, -20, -30 or -40= maize and germinated chickpea flour blends extrudates.

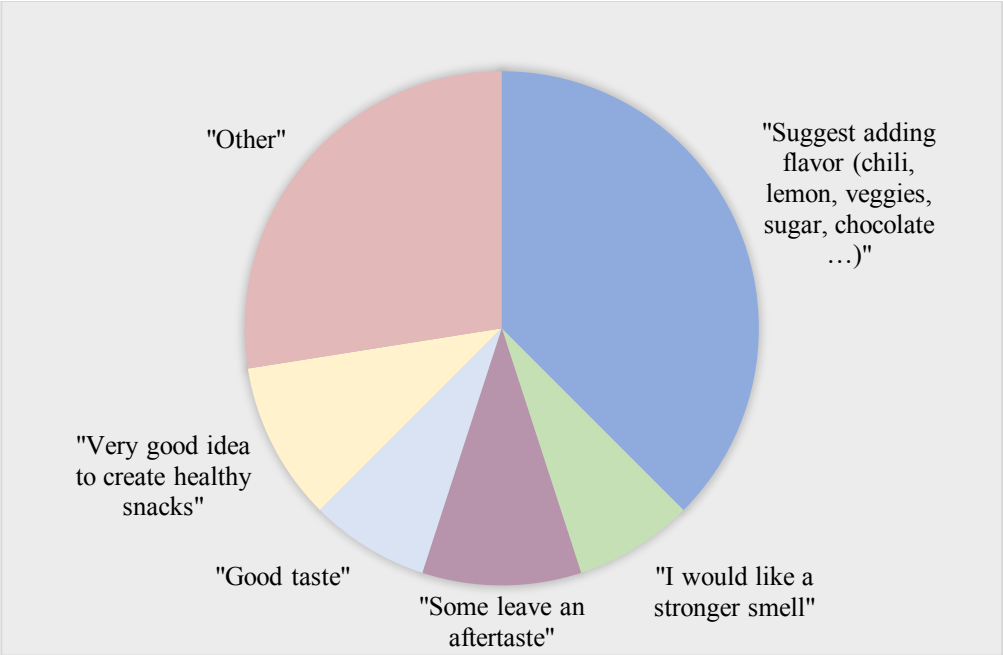

Figure 10. Commentaries of the sensorial test of maize-chickpea extrudates.

6.3 Conclusions

Extrusion process was used as a method to produce snacks with higher content of protein and resistant starch combining maize grits and different amounts of germinated chickpea flour. Interactions between starch, protein, and lipids were corroborated and affected the starch but not the protein digestibility. These interactions also affected the WAI and WSI and at low doses, these parameters were correlated with the amount of germinated chickpea flour used to formulate the extrudate. The textural results did not have an influence in the consumers overall acceptability. However, the expansion index and the chickpea content were important factors in the sensorial analysis when the highest dose of germinated chickpea flour was used (40%). Then, incorporating 30% of germinated chickpea flour to a maize extrudate increased the protein content without negative effects on protein digestibility but improved the expansion index and had an overall acceptability similar to that of a ME.

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Chapter 7. Se-enrichment diet as a chemotherapy adjuvant in a xenografted colorectal cancer Nu/Nu mice model.

7.1 Background

Cancer is one of the most common causes of death worldwide, almost 20 million of cases are reported each year with almost 60% of mortality (WHO, 2021). In Mexico, colorectal cancer (CRC) is the fourth most common reason of death causing 700 000 deaths per year (INSP, 2015). Conventional treatments depend on where the cancer is and the progress of it, but mainly are radiotherapy and chemotherapy (Hashiguchi *et al*., 2020). The use of chemotherapeutic compounds such as 5-fluorouracil (5-FU) in patients with CRC has been a very effective strategy in the control of tumor growth and proliferation.

Recently, scientists have been interested in the combinatory therapy that is the administration of one or more natural substances and conventional medications at a time (Rejhová *et al*., 2018). The aim is to intensify the antiproliferative effect of the medications administered with natural molecules to reduce the toxic consequences of high concentrations of chemotherapeutic agents (Rejhová *et al*., 2018). It has been known that many natural compounds suppress proliferation of cancer cells by inducing cell cycle arrest or induce apoptosis resulting in the inhibition of tumor growth.

Jesus *et al*., (2020) report that an ohmic extract of vine pruning residue causes sensitivity response of 5-FU and help to reduce tumor growth. A study evaluated the synergistic effects of Manuka honey and 5-FU. The authors reported that the combination enhanced the anticancer effects of the therapy on colon cancer by reducing cell proliferation, promoting apoptosis, and suppressing metastasis (Afrin *et al*., 2018). Mao *et al*. (2018) reported that Se-enriched *Grifola frondose* polysaccharide and 5-FU increase the antitumor activity, enhance the immune functions, and could reduce the toxicity of the chemotherapy. Black raspberry

anthocyanins combined with 5-FU or Celecoxib enhance the chemotherapy efficacy (Li *et al*., 2021).

Besides, combined therapies with Se in some types of cancer were reported. Se (10μM) with docetaxel (DTX) at 500 pM (Park *et al*., 2015; Ertilav *et al*., 2019), Na2SeO3 (200 nM) and cisplatin (40 μM) (Sakallı *et al*., 2016; Wu *et al*., 2017), Na2SeO3 (3 mg/kg) and Adriamycin prodrug (10 mg/kg) (Wu *et al*., 2019); 50 μM Paclitaxel and 5 μM Na2SeO3 (Guler & Ovey 2020). The authors concluded that these combined therapies stopped cancer progression and tumor growth by different mechanisms depending on the dose and the Se form.

Guardado-Félix *et al*. 2019 evaluated a diet based on selenized chickpea sprouts flour on immune-suppressed mice xenografted with human colorectal cancer (HT-29RFP) cells. The authors showed that a diet with high Se content (2.29 μg Se/g $_{diet}$) significantly decreased the tumor growth mainly mediated by the activation of the intrinsic apoptotic pathway and the antioxidant protection of lipids and through GPx and TrxR activity. Errant lipid metabolism is recognized as one of the main hallmarks of cancer cells because proliferation requires an important increase of lipid biosynthesis and because it has been shown that lipid catabolism generates signal molecules that can regulate cancer metastasis and progression (Huang & Freter, 2015). However, there are no evidence of a diet based on selenized chickpea sprouts flour as adjuvant of a chemotherapy 5-FU in an *in vivo* model. The objectives of this study were to evaluate the tumor growth, the serum lipid and lipoprotein levels, and hepatic GPx and TrxR enzymatic to elucidate some mechanisms of a combinatory therapy in a xenografted Nu/Nu mice model with a diet based on selenized chickpea sprouts and low doses of chemotherapy (5-FU).

7.2 Results and discussion

7.2.1 Change of body weight

It is noticed that significant differences in the change of body weight were observed only in the sixth, seventh, and eighth week (Figure 11). The sixth week was one week after inoculation and in this week the basal and C15Se have significant differences. In the seventh week, a significant loss was noticed in the C15Se group in comparison with basal, NC, and C15. Besides, a significant loss was noticed in the week eight in C15Se group in comparison with basal, NC, C15, C30, and C60. No significant gains or losses were observed in all other weeks. According to Guardado-Félix *et al*. (2019) mice begin to lose weight when 5-FU was administered due to the chemotherapy effect. Mutschler *et al*. (2018) reported that a 24.8% of cancer patients present weight changes, 11.1% lost, and 17.3% gained weight because of the chemotherapy. Conversely, Jung *et al*. (2020) reported that there were no changes in body weight, body mass index, or body composition during chemotherapy. Similarly, Hsu *et al*. (2020) demonstrated that a supplementation made of beef ameliorated the weight lose induced by 5-FU, because in the 5-FU group a significant decrease was observed in the weight of muscles and fat.

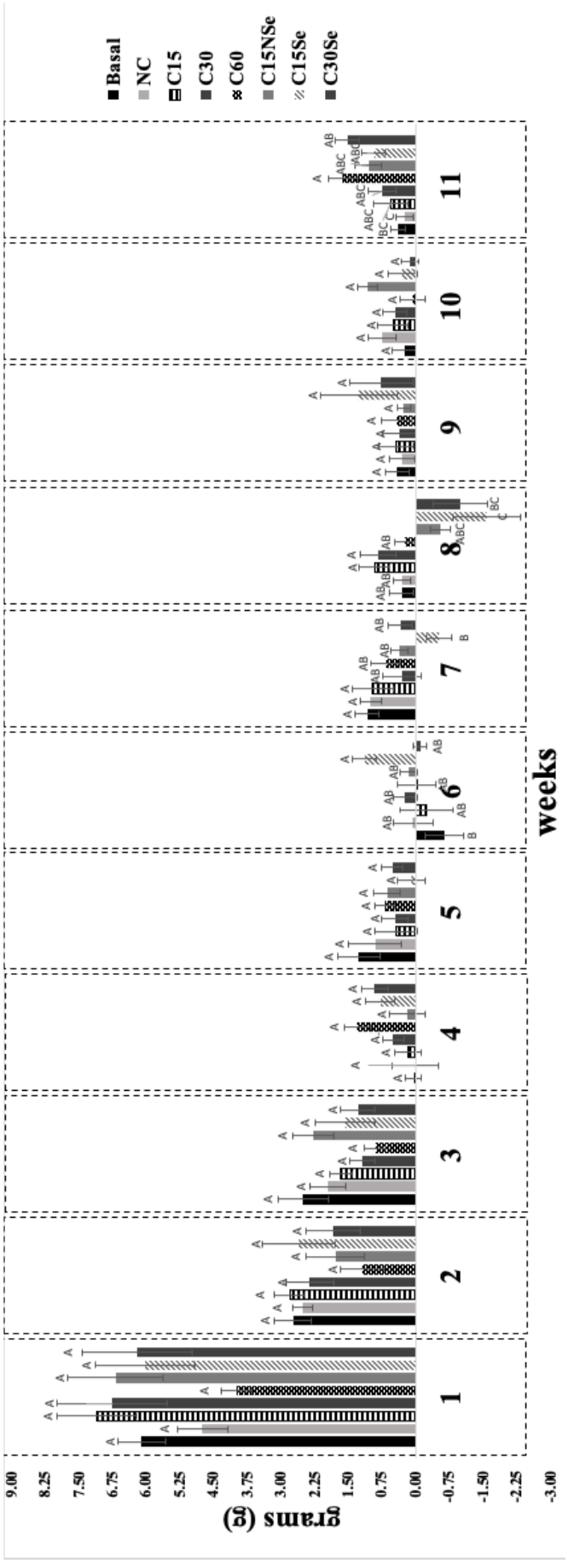

Figure 11. *Change of the body weight weekly. Groups: No chemotherapy= NC; Chemotherapy (5-FU) 15 mg/kg weight= C15; Chemotherapy (5-FU) 30 mg/kg weight= C30; Chemotherapy (5-FU) 60 mg/kg weight= C60; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet without Se= C15NSe; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet with Se= C15Se; Chemotherapy (5-FU) 30 mg/kg weight and chickpea diet with Se= C30Se.*

7.2.2 *Tumor growth monitoring*

The fluorescence intensity of the tumors of NC and C15 groups was higher than in the rest of the treatments due to the accumulation of a higher number of fluorescent cells (Figure 12). In contrast, the tumor images of C30, C60, and C15NSe group; showed the center of their tumors with less fluorescence intensity, indicating that the tumor mass was more flattened. This represents a bias in the tumor volume calculation since the program considers a formula for volume based on the fluorescent area but not the intensity as an indicator of tumor depth.

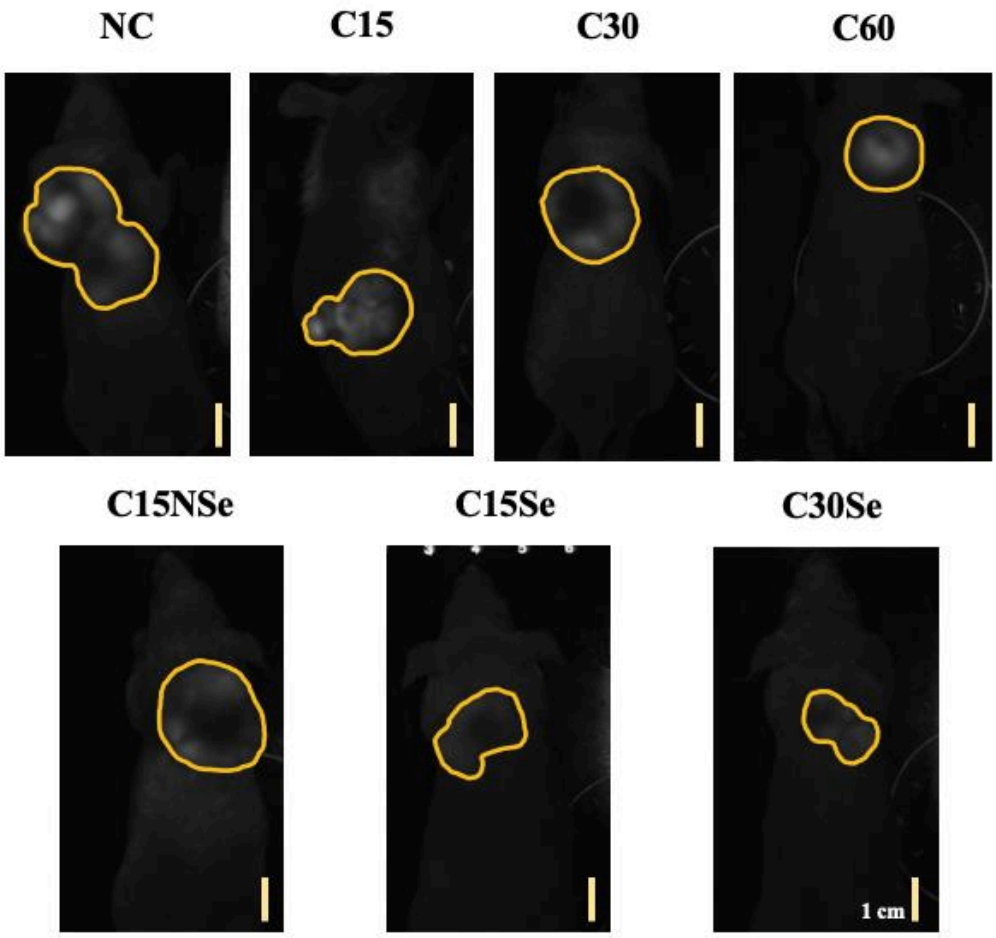

Figure 12. iBox images of tumor growth in the final of the study after 6 weeks of inoculation of all experimental groups. No chemotherapy= NC; Chemotherapy (5-FU) 15 mg/kg weight= C15; Chemotherapy (5-FU) 30 mg/kg weight= C30; Chemotherapy (5-FU) 60 mg/kg weight= C60; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet without Se= C15NSe; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet with Se= C15Se; Chemotherapy (5-FU) 30 mg/kg weight and chickpea diet with Se= C30Se.

Figure 13A shows the fitting of the experimental groups to the Gompertz model. The curves show the tendency of the growth rate. For NC group, the tendency shows that the curve is in the first step of the sigmoidal and that it will

continue growing. Likewise, the curve of the group C15NSe tend to increase. The curve C30 shows a reduction in the growth rate but not a clear asymptote as the observed for C15Se, C60, C15 or C30Se.

It can be observed in the 3D graph (Figure 13B) that the upper asymptote (*A*) and the inflection time (*Ti*) of NC group was higher than the others. The other groups showed a similar *A* and *Ti*. Figure 13C shows the average of the three parameters modeled for each experimental unit with the Gompertz function. The upper asymptote (*A)* of the C15 and C30 groups showed the highest values, and they were significantly different from C60 group. No significant differences were found in the NC, C15, C30, C15NSe, C15Se, and C30Se. The k_G parameter, the tumor growth rate, was significantly higher in NC group in comparison to C15, C30, C15NSe, C15Se, and C30Se groups. The k_G was reduced by 81.3% in the C30 group in comparison to NC. The inflection time (*Ti*) was reduced with the chemotherapies and selenized treatments. C60 group obtained lower value of *Ti* in comparison to the obtained by the NC group. Besides, the *Ti* of NC group was significantly higher than the observed in the selenized C15Se and C30Se groups by 1.26-fold.

It has been reported that the combination of a chemotherapeutic with Se increase the prognosis of cancer. The combination of docetaxel (500 pM) and Se (10 μM) was evaluated in breast cancer cells. After 72 h of incubation decreased cell growth by 15% and increased apoptosis by 63% (Park et al., 2015). Likewise, Ertilav et al. (2019) exposed glioblastoma cells to docetaxel (10 nM) for 10 h and 11 μM Se for 10 h. The combination inhibited cell proliferation and viability. Sakallı et al. (2016) showed that a therapy with Na_2SeO_3 (200 nM) and a chemotherapeutic cisplatin (40 μM) on breast cancer cells (MCF-7). The combination showed an apoptotic effect. The combination of Se molecules with cisplatin decreased the cell viability inducing apoptosis (Wu et al. 2017).

Later, Wu et al. (2019) combine and evaluate a therapy that include Na_2SeO_3 (3 mg/kg) and Adriamycin prodrug Ac-Phe-Lys-PABCADM (PADM) (10 mg/kg) in an *in vivo* model of gastric cancer cells. This combined therapy promoted apoptosis. Paclitaxel (50 μM) combined with Na_2SeO_3 (5 μM) have shown anticancer activities in MCF-7 cancer cells (Guler and Ovey 2020).

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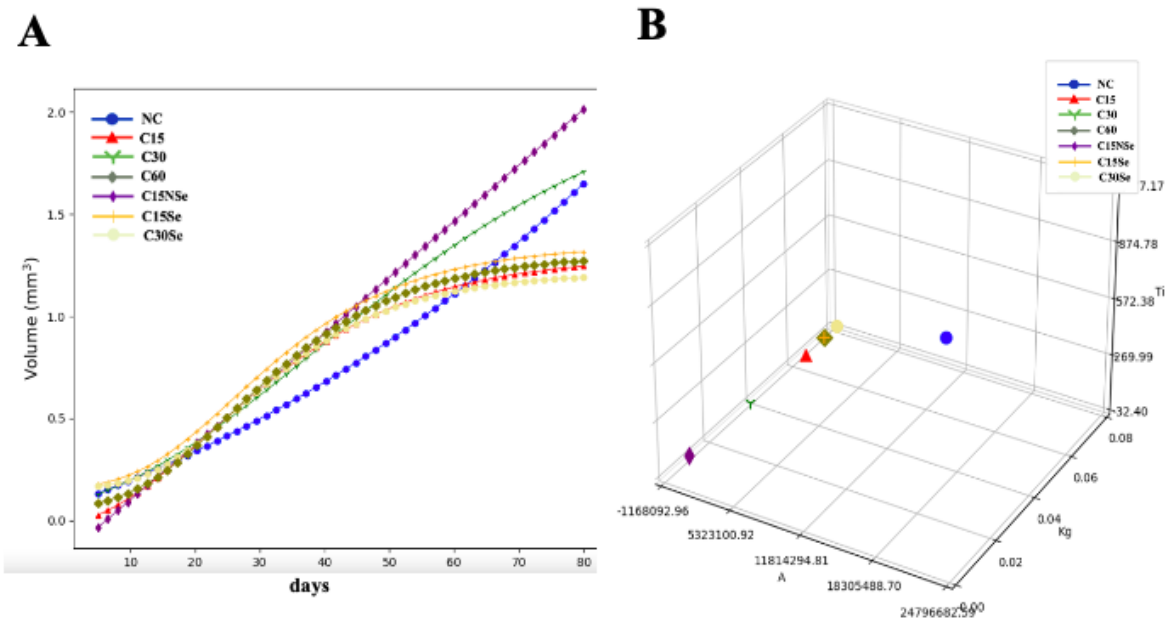
The highest dose of 5FU showed the lowest final tumor volume although the growth rate was higher than the obtained for the rest of the treatments since the inflection time was significantly lower. The inflection times for C15Se and C30Se were similar indicating that lower doses of chemotherapy were required to reduce the tumor growth even during the exponential phase. The growing rate was lower in C15Se and C30Se than the obtained with a higher dose of chemotherapy (C60). It is important to point out that the final volume of the tumors observed in C15Se and C30Se were overrated considering the lower intensity of fluorescence despite a similar area to other treatments.



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Group	*A*	k_G	*Ti*
NC	12162.4 ± 6858.6 ab	0.16 ± 0.09 a	30.49 ± 6.03 a
C15	16648.6 ± 12538.63 a	0.05 ± 0.04 c	26.29 ± 3.24 abc
C30	16970.1 ± 11025.68 a	0.03 ± 0.04 c	29.62 ± 6.31 ab
C60	7595.7 ± 2238.34 b	0.11 ± 0.08 ab	23.73 ± 3.95 c
C15NSe	11551.8 ± 5889.06 ab	0.07 ± 0.04 bc	29.83 ± 5.14 ab
C15Se	11565 ± 3740.4 ab	0.06 ± 0.04 bc	24.25 ± 5.17 bc
C30Se	10403 ± 3667.6 ab	0.07 ± 0.01 bc	24.24 ± 4.41 bc

Mean ± standard deviation of the parameters of the experimental units that fell within the range of the observation time, the same letter was not significantly different ($p < 0.05$).

Figure 13. A) Gompertz model of the experimental groups; X axis= time (days); Y axis= tumor volume (mm^3). B) 3D parameter distribution of Gompertz constants; X axis (A)= upper asymptote; Y axis (k_G)= growth-rate; Z axis (Ti)= inflection time. C) Mean ± standard deviation of the Gompertz constants. Groups: No chemotherapy= NC; Chemotherapy (5-FU) 15 mg/kg weight= C15; Chemotherapy (5-FU) 30 mg/kg weight= C30; Chemotherapy (5-FU) 60 mg/kg weight= C60; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet without Se= C15NSe; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet with Se= C15Se; Chemotherapy (5-FU) 30 mg/kg weight and chickpea diet with Se= C30Se.

7.2.3 Serum lipids and lipoproteins evaluation

No significant differences between the experimental groups and the basal group in serum total cholesterol that were observed (Table 14). It is important to highlight that there was a considerable high standard deviation. The only significant differences found in the HDL-C in serum were observed between the C30Se and NC and between C30 and C30Se. C15NSe was 1.4- and 1.95-fold significant higher in LDL-C than NC and basal group, respectively. Triglycerides of basal group was significantly higher than those measured in C15, C30, C15NSe, and C30Se group. The reductions were in a range of 22 to 28% with the experimental groups.

Although Kush & Khosrow (2021) reported that oncogenes can trigger the cholesterol biosynthesis as well cholesterol can increase oncogenic pathways, in this study no significant differences were found in cholesterol levels. On the other hand, Kush & Khosrow (2021) stated that only high HDL-C in serum reduces ROS, cellular viability, migration, and invasion of cancer cells. This could be observed in the C30Se group that was significantly higher in HDL-C compared to NC and C30.

In contrast to the observed in C60 and C30Se, Lofterød *et al*. (2018) reported that high triglycerides and HDL-C promote tumor growth and progression. C15NSe did not show inhibitory effect on the tumor growth and could be related to the LDL-C levels that contrast to those observed in C60, C15Se, and C30Se. High lipoprotein LDL-C and cholesterol levels have been associated with a worse survival and could be potential prognostic markers of cancer progression (Maran, Hamid, and Sahul-Hamid, 2021; Lin *et al.*, 2020). Likewise, Shyamananda *et al*., 2021 stated that high cholesterol level and LDL-C in blood is significantly correlated with the risk of progression of cancer because they support the rapid proliferation of cancer cells.

Another clinical study of CRC patients in stage III showed that there was a significant association between triglycerides and CRC progression (Chen *et al*., 2020). Triglyceride levels of basal group were significantly higher than those measured in C15, C30, C15NSe, and C30Se group. Long *et al.* (2018) stated that

lipid-lowering and anti-lipid per-oxidation treatment have advantages in comparison with other anti-cancer drugs.

Table 14. Serum lipids and lipoproteins (mg/dL) after euthanasia of all experimental groups.

Groups	Cholesterol	HDL-C	LDL-C	Triglycerides
Basal	86.1 ± 4.9 a	65.1 ± 2.6 ab	4.6 ± 1.3 c	101.7 ± 21.9 a
NC	82.7 ± 4.7 a	61.9 ± 3.5 bc	6.4 ± 1.4 bc	83.2 ± 32.9 ab
C15	91.1 ± 14.8 a	62.8 ± 8.3 abc	6.3 ± 0.6 abc	79.7 ± 25.4 b
C30	84.2 ± 15.9 a	59.6 ± 5.6 c	6.9 ± 1.1 abc	79.4 ± 18.68 b
C60	90.0 ± 15.4 a	65.3 ± 4.2 ab	6.4 ± 1.5 abc	93.5 ± 8.5 ab
C15NSe	86.8 ± 8.6 a	62.1 ± 5.8 abc	9.0 ± 2.4 a	73.4 ± 23.2 b
C15Se	92.8 ± 10.7 a	65.8 ± 3.8 ab	6.5 ± 1.1 abc	84.5 ± 23.8 ab
C30Se	89.3 ± 16.5 a	66.8 ± 5.7a	7.3 ± 2.9 ab	79.8 ±26.3 b

Low density lipoprotein cholesterol= LDL-C; High density lipoprotein cholesterol= HDL-C; No chemotherapy= NC; Chemotherapy (5-FU) 15 mg/kg weight= C15; Chemotherapy (5-FU) 30 mg/kg weight= C30; Chemotherapy (5-FU) 60 mg/kg weight= C60; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet without Se= C15NSe; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet with Se= C15Se; Chemotherapy (5-FU) 30 mg/kg weight and chickpea diet with Se= C30Se. The values are the means ± standard deviation, the same letter indicates that there no significant differences ($p < 0.05$).

7.2.4 TrxR and GPx enzymatic evaluation

Increases of 2-, 1.6-, and 1.9-fold were found in in GPx enzymatic activity from mice fed with selenized treatments in comparison to basal, NC, and C15, respectively (Table 15). GPx activity increased in the selenized groups (C15Se and C30Se) and in mice treated with the highest dose of chemotherapy (C60). It is well known that supplementation of Se trigger the overexpression and the increase of enzymatic activity of GPx mainly because Se is incorporated into selenoenzymes, one of the most important is GPx (Razaghi *et al*., 2021). Chickpeas sprouts enriched with Se (> 2 µg/g) increased GPx and TrxR activities (Guardado-Félix *et al*., 2019). Besides selenonanoparticles, SeNPs, (1.8 µM) enhanced the increase of GPx and TrxR (Liu et al., 2020). Other study showed that Se-enriched (1 g Se/g diet)

mushroom diet showed a significant increase of GPx (Maseko *et al*., 2014). Besides, Se-enriched *Saccharomyces cerevisiae* supplementation (>200 µg/kg weight) to broilers showed a significant increase in GPx and TrxR (Hou *et al*., 2020). In our study, no significant differences were observed in the TrxR enzyme activity due to the high standard deviation.

Likewise, in this study the experimental group with the highest dose of chemotherapy showed an increase in the GPx due to the ROS increase (Zhang *et al*., 2020). Besides, chickpea without Se enhanced the enzymatic activity (C15NSe group) in comparison to the chemotherapy alone C15. Low GPx expression was correlated with a worse survival rate in patients with pancreatic adenocarcinoma who received chemotherapy (Meng *et al*., 2018).

Table 15. GPx and TrxR enzymatic activities after the in vivo study.

Group	GPx (µmol/min/mL/mg protein)	TrxR (nmol/min/mL/mg protein)
Basal	11.8 ± 2.3 d	114.6 ± 31.2 a
NC	15.1 ± 4.6 cd	137.5 ± 67.1 a
C15	12.6 ± 4.4 cd	97.2 ± 90.5 a
C30	16.6 ± 4.4 bc	94.4 ± 70.4 a
C60	20.1 ± 3.6 ab	99.1 ± 40.1 a
C15NSe	20.7 ± 4.4 ab	62 ± 55.8 a
C15Se	23.8 ± 6.7 a	68.4 ± 35.8 a
C30Se	21.7 ± 6.1 a	68.8 ± 40.6 a

One unit of glutathione peroxidase (GPx) is defined as the amount of enzyme that caused the oxidation of 1.0 µmol of NADPH to $NADP^+$ per minute per mg of protein at 25 ºC. One unit of thioredoxin reductase (TrxR) is defined as the NADPH-dependent production of 2 nmol of 2-nitro-5-thiobenzoate per minute at 22 ºC. No chemotherapy= NQ; Chemotherapy (5-FU) 15 mg/kg weight= C15; Chemotherapy (5-FU) 30 mg/kg weight= C30; Chemotherapy (5-FU) 60 mg/kg weight= C60; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet

without Se= C15NSe; Chemotherapy (5-FU) 15 mg/kg weight and chickpea diet with Se= C15Se; Chemotherapy (5-FU) 30 mg/kg weight and chickpea diet with Se= C30Se. The values are the means ± standard deviation, the same letter indicates that there were no significant differences ($p < 0.05$).

7.3 Conclusions

According to the Gompertz model, the k_G predicts that the tumor growth rate was significantly higher in the NC group in comparison to almost all the treatments. The inflection time (*Ti*) of the tumor growth observed for NC group was significantly higher than the observed in the selenized C15Se and C30Se groups by 1.26-fold. HDL-C in serum showed that there were significant differences between the C30Se group and NC and C30 groups by 1.07- and 1.12-fold. C15NSe did not show inhibitory effect on the tumor growth and could be related to the observed in the LDL-C levels in contrast to observed in C60, C15Se, and C30Se. Triglycerides of basal group were significantly higher than those measured in C15, C30, C15NSe, and C30Se group; the reductions were in a range of 22 to 28% with the experimental groups. As a result, C30Se showed the best significant outcomes in the *in vivo* study: lower *Ti* compared to NC, higher HDL-C compared to NC and C30; lower triglycerides compared to basal group; and higher GPx activity compared to basal, NC, C15, and C30 groups.

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Chapter 7. Se-enrichment diet as a chemotherapy adjuvant in a xenografted colorectal cancer Nu/Nu mice model.

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Chapter 8. Conclusions and recommendations

8.1 Conclusions

This study demonstrated that germination in presence of Se augmented the mineral, isoflavones content, and health-related properties. Se was mainly stored in the Glu fraction and it had a regulatory effect on proteolysis reducing degradation of proteins. Besides, this study demonstrated that CD is an economic drying that permits the Se and isoflavones retention and the conversion of glycosides into aglycones isoflavones due to activation of β-glucosidase enzyme. High Se concentrations (≥ 96 mg Na_2SeO_3/L) trigger toxic effects in chickpeas sprouts that could be detected by the detrimental of isoflavones, phenolics, and PAL activity and the radicle size.

Extrusion was used to produce a food based on germinated chickpea sprouts and maize with high content of protein and resistant starch. It is observed that the lipid, protein, and starch interactions affected the starch but not the protein digestibility. Chickpea flour affected other parameters (WAI, WSI). The textural results did not have an influence in the consumers overall acceptability. Incorporating 30% of germinated chickpea flour to a maize extrudate increased the protein content without negative effects on protein digestibility.

The tumor growing evaluated by the Gompertz model showed that the *Ti* of NC group was significantly higher than the observed in the selenized C15Se and C30Se groups. HDL-C in serum showed that there were significant differences between the C30Se group and NC and C30. LDL-C was significantly higher in C15NSe in comparison to basal and NC group. Triglycerides of basal group was significantly higher than those measured in C15, C30, C15NSe, and C30Se group; the reductions were in a range of 22 to 28% with the experimental groups. Almost an increase of 2-, 1.6-, and 1.9-fold were found in the GPx groups in selenized treatments in comparison to basal, NC, and C15 groups, respectively. As a result, C30Se showed the best significant outcomes in the *in vivo* study: lower *Ti* compared

to NC, higher HDL-C compared to NC and C30; lower triglycerides compared to basal group; and higher GPx activity compared to basal, NC, C15, and C30 groups.

8.2 Recommendations

Further studies are needed to evaluate the molecular mechanisms implicated in this *in vivo* study. Undoubtedly, germination in the presence of Se is a technique to enrich food. Although it was shown that hydrolyzed peptides had high antioxidant capacity *in vitro*, there is still a gap between knowing the species and dose of Se that has the desired effect. Therefore, research should be directed to studying which species of Se are formed during germination and the doses that can have a health-related effect. Besides, it is important to study the steatopatitis produced by 5FU and the Se effect to reduce it. The research also could be focus in the change of microbiota in CRC *in vivo* models due to Se ingestion.

Appendix A

CONSENTIMIENTO INFORMADO PARA PARTICIPAR EN UN PROYECTO DE INVESTIGACIÓN

Nombre del participante:_____

Edad: _____

Introducción

En el presente estudio se evaluará el nivel de preferencia de un producto elaborado a base de harina de garbanzo y maíz mediante una prueba sensorial hedónica. Este estudio va dirigido a adultos de ambos sexos en edad de 18-55 años, de la ciudad de Monterrey, Nuevo León; México. Es importante que sí usted presenta cualquier tipo de intolerancia a los ingredientes del producto no participe en la prueba.

Resumen del estudio

Se darán 5 muestras de 100 mL de la bebida preparada con el bagazo de mango. Se solicitará que llenen la escala de la prueba hedónica, la cual indicará el nivel de preferencia del producto.

Riesgos del estudio

Dada la naturaleza del estudio, **NO EXISTE** riesgo alguno que atente contra la seguridad o salud del participante. Las muestras han sido preparadas en estricto apego a las prácticas de higiene para la preparación de extruídos NOM-187-SSA1/SCFI-2002. La información recabada del estudio es estrictamente confidencial de acuerdo con los lineamientos estipulados por el Reglamento de la Ley General de Salud en materia de investigación para la salud (Artículos 13 y 16), Ley de Información Estadística y Geográfica (Artículos 38 y 42) y del comité de bioética de la la Universidad Autónoma de Nuevo León.

Beneficios del estudio

1. La información que se reúna será utilizada para fines del conocimiento del nivel de preferencia del estudio.
2. Agradecemos de antemano su autorización y consentimiento. Se le proporcionara una copia si lo requiere del presente documento para posibles futuras aclaraciones.

Al firmar este documento acepto participar en la prueba.

Firma del participante_____

Fecha_____

_____	_____
Dra. Daniela Guardado Félix	M.C. Sayra Nayely Serrano Sandoval
Investigador Responsable	Estudiante Responsable

Appendix B

Edad: _____ **Género:** _____ **Folio:** _____

INSTRUCCIONES

Frente a usted se encuentran seis muestras distintas marcadas con diferentes números. Por favor coma un poco de galleta y agua entre cada muestra y califique su respuesta a partir de la siguiente guía:

¿Cuánto te gusta o disgusta el COLOR de la botana?

525	
245	
378	
123	
698	
423	

¿Cuánto te gusta o disgusta el OLOR de la botana?

525	
245	
378	
123	
698	
423	

¿Cuánto te gusta o disgusta el SABOR de la botana?

525	
245	
378	
123	
698	
423	

¿Cuánto te gusta o disgusta la textura (CRUJENCIA) de la botana?

525	
245	
378	
123	
698	
423	

Si percibe usted al masticar la botana algo de GOMOSIDAD ¿cuánto te gusta o disgusta esa sensación?

525	
245	
378	
123	
698	

AGRADO GENERAL, tomando en cuenta las características evaluadas ¿qué tanto te gusta o disgusta?

525	
245	
378	
123	
698	

Comentarios o sugerencias:

_____

¡MUCHAS GRACIAS POR TU VALIOSA PARTICIPACIÓN!

Published papers

Serrano-Sandoval, S. N., Guardado-Félix, D., & Gutiérrez-Uribe, J. A. (2019). Changes in digestibility of proteins from chickpeas (*Cicer arietinum L.)* germinated in presence of selenium and antioxidant capacity of hydrolysates. *Food Chemistry, 285,* 290-295. https://doi.org/10.1016/j.foodchem.2019.01.137.

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