UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

FACULTAD DE CIENCIAS QUÍMICAS E INGENIERÍA

MAESTRÍA Y DOCTORADO EN CIENCIAS E INGENIERÍA

EVALUACIÓN DE INGREDIENTE FUNCIONAL EN LA ELABORACIÓN DE PAN DE CAJA PARA SU POTENCIAL USO COMO CARDIOPROTECTOR Y ANTIGENOTÓXICO.

T E S I S

Que para obtener el grado de

MAESTRO EN CIENCIAS

Presenta:

Q.F.B. ALDO LEYVA SOTO

DIRECTORA DE TESIS:

Dra. Rocío Alejandra Chávez Santoscoy

CO-DIRECTORA DE TESIS:

Dra. Aracely Serrano Medina

TIJUANA, BAJA CALIFORNIA. JUNIO 2019.

HOJA DE APROBACIÓN

"En tiempos de cambio, quienes estén abiertos al aprendizaje se adueñarán del futuro, mientras que aquellos que creen saberlo todo estarán bien equipados para un mundo que ya no existe…"

ÍNDICE GENERAL

		Página
Hoja de aprobación		II
Lista de tablas		VIII
Lista de figuras		IX
Agradecimientos		X
Resumen		XIII
CHAPTER I	**INTRODUCTION**	
1.1	Background	2
1.2	Hypothesis	3
1.3	Justification	3
1.4	Overall objective	4
1.5	Specific objectives	4
1.6	The flavonoids	5
1.6.1	Structure and source of flavonoids	5
1.7	The metabolism of flavonoids in the human body	7
1.8	Antioxidant activity of flavonoids	9
1.8.1	Chemo preventive effect by Antioxidant activity	11
1.9	Effect on cancer and inflammatory diseases	14
1.10	Effects on Metabolic Syndrome	17
1.11	Effect on Diabetes	19
1.12	Cardiovascular diseases and vascular effects	23
1.13	Conclusions	25

CHAPTER II		**FIRST INTERVENTION WITH FUNCTIONAL FOOD**	
2.1		Introduction	28
2.2		Results	29
	2.2.1	Study Population	29
	2.2.2	Determination of Total Phenolic, Flavonoids Content and Antioxidant Capacity	30
	2.2.3	Changes Caused by Daily consumption of Chocolate	31
	2.2.4	Frequency of Nuclear Abnormalities in Buccal Epithelial Cells	31
2.3		Discussion	34
	2.3.1	Determination of Total Phenolic and Flavonoids Content	34
	2.3.2	Characteristics of Study Population	34
	2.3.3	Determination of Total Phenolic and Flavonoids Content	35
	2.3.4	Changes in Measured Parameters by Daily Dark Chocolate Consumption	37
	2.3.5	Frequency of Nuclear Abnormalities in Buccal Epithelial Cells	38
2.4		Materials and Methods	39
	2.4.1	Study Design and Participants	39
	2.4.2	Chocolate Consumption (Independent Variable)	40
	2.4.3	Determination of Total Phenolic Content	40
	2.4.4	Determination of Flavonoids Content	41
	2.4.5	Antioxidant Capacity	41
	2.4.6	Epithelial Genotoxicity, Biochemical and Anthropometric Parameters Measure (Dependent Variables)	41
2.5		Statistical Analyses	42
2.6		Conclusions	42

CHAPTER III **SECOND INTERVENTION WITH FUNCTIONAL FOOD**

3.1		Introduction	44
3.2		Materials and Methods	45
	3.2.1	Bread making	45
	3.2.2	Sensory Analysis and color of bread crumb	46
	3.2.3	Concentration of (-)-epicatechin and quercetin in bread samples	46
	3.2.4	Bread Shelf life	46
	3.2.5	Relative humidity (RH) and water activity (Aw)	46
	3.2.6	Microbiological study	47
	3.2.7	Study Design and biochemical and anthropometric parameters measure (dependent variables) in participants	47
	3.2.8	Genotoxicity in buccal epithelium cells	49
	3.2.9	Protective effect of (-)-epicatechin and quercetin in Caco-2 cells	49
3.3		Statistical analyses	50
3.4		Results	51
	3.4.1	Effect of adding (-)-epicatechin and quercetin (1:1) on baking performance and bread features	51
	3.4.2	Flavonoid content and shelf life of breads	52
	3.4.3	Microbiological study.	53
	3.4.4	Changes in measured parameters in the participants who consumed the enriched bread (BF).	55
	3.4.5	Frequency in nuclear abnormalities in buccal epithelial cells	57
	3.4.6	Protective effect of (-)-epicatechin and quercetin in Caco-2 cells	59
3.5		Discussion	60
	3.5.1	Effect of added flavonoids on baking performance and bread features	60
	3.5.2	Effect of flavonoids in its consumption in enriched bread and on *in-vitro* bioactivity	61
3.6		Conclusions	62

CHAPTER IV	**FINAL CONCLUSIONS**	64
CHAPTER V	**FUTURE WORK**	67
CHAPTER VI	**REFERENCES**	69
CHAPTER VII	**ANNEXES**	81

LIST OF TABLES

CONTENT		Page
Table 1.	The flavonoids and their food sources and concentration	7
Table 2.	Mechanisms and sources of flavonoids with antidiabetic potential.	23
Table 3.	Characteristics of study population at recruitment.	30
Table 4.	Phenolic and Flavonoid content in used dark mil chocolates for the study.	31
Table 5.	Dietary, anthropometric and biochemical variables determined prior and after the dietary intervention with either flavonoid-rich chocolate or milk chocolate for 6 months.	36
Table 6.	Characteristics of study population at recruitment.	48
Table 7.	Effect of the addition of (-)-epicatechin and quercetin on baking properties and physical characteristics of the bread.	51
Table 8.	Microbiological effect of storage temperature on bread samples	54
Table 9.	Dietary, anthropometric and biochemical variables before and after 3 months of consumption of control bread or bread enriched with (-)-epicatechin and quercetin.	56

LIST OF FIGURES

CONTENT **Page**

Figure 1.	The basic skeleton structure of flavonoids and their classes.	6
Figure 2.	Overview of flavonoid absorption and post-absorptive metabolism.	9
Figure 3.	Possible interaction sites of the flavonoids with the carcinogenic process.	16
Figure 4.	Frequency in nuclear abnormalities in buccal epithelial cells during consumption.	32
Figure 5.	Correlation study through Pearson's linear regression analysis in the beginning of the study at the 84 participants.	33
Figure 6.	Appearance of (a) Control whole wheat bread (CN); (b) bread enriched with 0.05% of a mixture of (-)-epicatechin and quercetin in a proportion of 1:1 (BF).	52
Figure 7.	Flavonoid content, relative humidity (RH) and water activity (Aw) were measured during 15 days of storage.	53
Figure 8.	Effect of 3 storage temperatures (4°C, 25°C and 40°C) in breads enriched with 0.05% of a mixture of (-)-epicatechin and quercetin in 1:1 proportion.	55
Figure 9.	Frequency in nuclear abnormalities in buccal epithelial cells and its correlation with biochemical parameters.	57
Figure 10.	Comparison of relevant variables and nuclear abnormalities of buccal epithelial cells.	58
Figure 11.	Intracellular calcium (Ca^{+2}) behavior in Caco-2 cells after stimulus with 10 μM of quercetin (Que), (-)-epicatechin (Epi) or the mixture of both flavonoids in 1:1 proportion	59

// AGRADECIMIENTOS

Cuando al final del camino, vemos el conocimiento reflejado en nuestras acciones, en la labor que hacemos a diario, es en ese valioso momento cuando nos damos cuenta que el mérito de llegar a la meta no es solo tuyo. Nosotros mismos somos el reflejo de un grupo de personas que se esforzaron por brindarnos todo su apoyo, su conocimiento, su experiencia y su paciencia. Mi más grande agradecimiento es para mis padres Mara Berenice Soto Graciano y Aldo Leyva Cota, que a pesar de todas las adversidades que hemos enfrentado como familia, nunca me ha faltado su apoyo, agradezco su paciencia, su fuerza, su amor y su voluntad para alentarme y preocuparse por que sea una persona de bien.

Agradezco infinitamente a la Dra. Rocío Alejandra Chávez Santoscoy, por todo su tiempo, su confianza, pero sobretodo, por el apoyo, su profesionalismo y su amistad a través de éstos años.

Dra. Aracely Serrano Medina, gracias por guiarme por el buen camino, por su profesionalismo y por su amistad, por enseñarme a separar lo personal de lo profesional, para que se cumplan los objetivos de éste proyecto.

Dr. Omar Porras, Miltha Hidalgo, y a todo el equipo del laboratorio de Biología Celular, agradezco todo el apoyo y todo el amor brindado en la estancia internacional realizada en el Instituto de Nutrición y Tecnología de los Alimentos de la Universidad de Chile, todo lo aprendido y el apoyo por cumplir con las metas establecidas.

Gracias abuelos Manuel Leyva y Andrea Cota, ustedes son las personas que después de mis padres, más se preocupan por mí, sus canas son sinónimo de sabiduría, me han enseñado tanto, y el amor que me han brindado me mantiene fuerte y vivo.

Agradezco a mis amistades, Perla Castillo, Javier Zavala, Olivia Inzunza, Brenda González, Oswvaldo Martínez, Miguel Angulo, Germán Sánchez y Noemí Arellano, por todo el apoyo emocional, por ayudarme a levantarme todos los días, por acompañarme en momentos buenos y no tan buenos, por alentarme a cumplir mis metas y gracias por formar parte de éste bonito proyecto.

A mis hermanos Mariela y Emanuel, a mis tías, y en general, a toda mi familia que han estado de manera incondicional brindándome su amor y siendo partícipes de todos mis proyectos.

A todos los académicos que han inculcado valores en mí, que me han aconsejado y dejaron algo positivo en mi vida, para afrontar las adversidades y retos que siguen.

Se cierra un ciclo, pero inicia otro, hoy por hoy, esa transmisión de un saber vale mucho más que todo lo que podremos obtener, a todas estas personas, solo se les puede dedicar un pequeño, pero muy sincero y afectuoso agradecimiento.

Por último, solo nos resta esforzarnos por ser personas que demuestren esta misma enseñanza y calidad, porque el conocimiento es la mejor herencia que podemos recibir y también la que podemos dejar.

AGRADECIMIENTOS INSTITUCIONALES

Agradezco de forma infinita a mi alma máter, la Universidad Autónoma de Baja California (U.A.B.C), por brindarme la oportunidad de realizar el estudio de posgrado en este caso la maestría en ciencias; de igual forma a la Facultad de Ciencias Químicas e Ingeniería (F.C.Q.I), a la Escuela de Ciencias de la Salud (ECiSalud) y al programa de posgrado (MyDCI), por las instalaciones y los medios para la realización de este proyecto. A todo el personal de U.A.B.C. que ha sido parte importante para la realización de este trabajo.

Agradezco profundamente el apoyo brindado por el Consejo Nacional de Ciencia y Tecnología (Conacyt), por el apoyo brindado durante estos dos años en el programa de maestría y el apoyo brindado durante la estancia en Santiago de Chile.

De igual forma, agradezco a la Universidad de Chile, específicamente al Instituto de Nutrición y Tecnología de los Alimentos donde se llevaron a cabo todos los experimentos de cultivo celular.

RESUMEN

Flavonoids are the most abundant polyphenolic compounds present in fruits, vegetables and some cereals and legumes. They have long been recognized to develop many health beneficial activities such as antioxidant, anti-inflammatory, hepatoprotective, among others. Furthermore, flavonoids have been associated with the decrease of total cholesterol levels, the inhibition of LDL lipid peroxidation and the increase of HDL, which is related to cardiovascular protection. Depending on the glycosylation of the molecule the bioactivity, bioavailability and pharmacokinetic will change. In recent years, the incidence of atherosclerotic cardiovascular disease, obesity, and diabetes has increased largely worldwide. In a first intervention, we evaluated the genoprotective effect of the consumption of flavonoids-rich chocolate on 84 young volunteers. Biochemical indicators related to the prevention and treatment of cardiovascular risk and metabolic syndrome were also determined. A randomized, placebo-controlled, double-blind study was performed in the Autonomous University of Baja California. The treatments comprised the daily consumption of either 2 g of dark chocolate containing 70% cocoa, or 2 g of milk chocolate, for 6 months. The total amount of phenolic compounds and flavonoids was determined in both types of chocolate. Anthropometrical and Biochemical parameters were recorded prior to and after the study. The evaluation of the genotoxicity in buccal epithelial cells was performed throughout the duration of the study. Flavonoids from cocoa in dark chocolate significantly prevented DNA damage, and improved the nucleus integrity of cells. In a second intervention, we did a randomized, double blind placebo-controlled study (n = 156) was conducted to determine the effect of consuming a bread enriched with 0.05% of a 1:1 mixture of (−)-epicatechin and quercetin on anthropometric, and biochemical parameters of the participants. Genoprotective effect in buccal epithelium cells was evaluated by the micronucleus test. Total cholesterol, LDL-cholesterol, total triglycerides and fasting plasma glucose, significantly decreased after 3 months of daily enriched bread consumption. Nuclear abnormalities also decreased (15.8 ± 3.2 % down to 8.3 ± 1.0%). Finally, in-vitro protective effect of this flavonoids was evaluated by monitoring intracellular calcium concentration in Caco-2 cells stimulated with H_2O_2. Both flavonoids together could synergistically prevent intracellular Ca^{2+} overload due to H_2O_2.

CAPÍTULO I

INTRODUCTION

1.1 BACKGROUND.

According to the World Health Organization, CVD (Cardiovascular Diseases) are the leading cause of death worldwide. Every year more people die from CVD than from any other cause [1]. They account for almost 30% of all deaths, and around 50% of deaths from non-communicable diseases [2].

Diets rich in whole grains and legumes have been linked to lower concentrations of lipids. The natural phytochemicals present in these plant foods can have synergistic effects and attenuate hyperlipidemia by regulating some genes involved in lipid metabolism. Beans are complete foods, rich in complex carbohydrates, proteins, dietary fiber and starch [3]. On the other hand, corn is also rich in dietary fiber and some phenolic acids. The possibility of decreasing the concentration of serum lipids interfering with the absorption of cholesterol and bile acids has been extensively investigated using compounds extracted from these staples (for example, gel-forming fiber, resistant starch, phytosterols and saponins) [4].

The frequency of micronuclei (MN) in peripheral blood lymphocytes blocked by cytokinesis (PBL) has become one of the best-established biomarkers for studying DNA damage that occurs in vivo in humans. The application of this method in population biomonitoring studies requires a deep understanding of how lifestyle and common host variables can influence MN frequency in PBL [4-5].

The term "nutraceuticals" is coined in 1979 by Stephen DeFelice [6]. It is defined as "foods or parts of foods that provide medical or health benefits, including the prevention and treatment of the disease." These can range from isolated nutrients, dietary supplements and diets to genetically engineered foods, herbal products, and processed products such as cereals, soups and beverages.

1.2 Hypothesis.

The intake of functional bread rich in flavonoids will help reduce the number of cellular abnormalities in the oral epithelium due to its celluloprotective and antioxidant effect. In addition, it will improve the main clinical biochemical parameters in patients with lifestyles that can trigger cardiovascular diseases.

1.3 Justification.

Currently, the lifestyles of the state of Baja California, and in general, its population, place the state in the first places in mortality, in addition its multicultural influence by fast and different food, make this region very vulnerable to suffer and trigger heart disease and other food syndromes.

In 2008, CVD caused 17 million estimated deaths and led to 151 million DALY's (years of healthy life lost), which represent 10% of the DALY's in total in that year. Behavioral risk factors such as physical inactivity, tobacco use and unhealthy diets account for almost 80% of the burden of cardiovascular disease [5]. While it is estimated that 17.5 million people died in this case in 2012, which represents 31% of all deaths recorded in the world. Of these deaths, 7.4 million were due to coronary heart disease, and 6.7 million due to stroke. In the world every 4 seconds a heart attack occurs, in the United States of America every 26 seconds and in Mexico it is estimated every 3 minutes [4]. Factors such as unhealthy food, excess alcohol, physical inactivity and tobacco use contribute directly to the onset of chronic cardiovascular and respiratory diseases, as well as cancer and diabetes [6].

The elaboration of functional foods rich in flavonoids, can be used to prevent genotoxic damage and as cardioprotectors improving biochemical parameters, such as cholesterol and triglycerides, also for the population to feel motivated to consume it in order to change their type of diet and with this to have a better quality of life.

1.4 Overall objective.

Evaluate the antigenotoxic effect in buccal epithelial cells and the possible protective effects of the bioactive flavonoids contained in functional bread in people with risk factors for heart diseases and life habits related to chronic diseases.

1.5 Specific objectives.

1. Make a whole grain bread, generating a functional food, incorporating quercetin and epicatechin, and evaluate its texture, smell, color and taste.

2. Evaluate the microbiological quality of the functional food, according to the Official Mexican Standard 111.

3. To evaluate the effect of bioactive compounds on intestinal epithelial cells and their redox impact on the intracellular environment.

4. To analyze the celluloprotective effects of a mixture of flavonoids (quercetin and epicatechin) on those in buccal epithelial cells in people with risk factors for cardiovascular diseases, observing the behavior in the number of cellular abnormalities found in buccal epithelium.

5. Determine and observe the changes in the main clinical biochemical parameters such as triglycerides, cholesterol, and glucose, before, during and after the ingestion of the case bread enriched with quercetin and epicatechin.

6. Correlate the effect of the consumption of bioactive flavonoids (quercetin and epicatechin) in buccal epithelial cells and the Cardiac Risk Index (CRI).

1.6 The flavonoids.

Some studies support that the consumption of diets based on foods that come from vegetables has a close relationship with the improvement of health. Naturally, humans do not produce these natural compounds [1], they are obtained through the intake of foods rich in these, or through food supplements. As a component of the diet, flavonoids have been associated to health-promoting properties, some of these effects are attributed to its structure, such as its antioxidant capacity and the scavenging and elimination of free radicals [2]. Other features are exerted to their interaction with proteins and specific enzymes, for example, xanthine oxidase, cyclooxygenase, lipoxygenase, AMPK and phosphoinositide 3-kinase [3]. Some studies suggest that flavonoids protect against many infectious diseases, and those related to age, also have beneficial effects in the prevention and treatment of chronic and degenerative diseases such as cancer, diabetes and cardiovascular disorders [4]. In humans, they represent essential compounds as the non-energetic part of the diet. Moreover, during the last years, it has been of great interest to be able to report the health-promoting effects that several flavonoids have. These nutrients, known to be excellent antioxidants, such as catechins, others are known to act in cardiovascular diseases such as hesperidin and quercetin, on the other hand, genistein and luteolin have neurological and hepatic action [5]. However, the most critical objective is to actively support the evidence that leads us to understand the mechanism of action by which, flavonoids exert their effect.

The primary purpose of this review is to provide a critical evaluation of the state of the art related with the effects of flavonoids in several diseases and disorders with worldwide importance, such as diabetes, metabolic syndrome, cardiovascular diseases, and cancer among others. Also, we will describe the mechanism of action by which they could help in the prevention and treatment of these diseases based on the reported evidence.

1.6.1 Structure and source of flavonoids.

The flavonoids, concerning their chemical structure, have some phenolic hydroxyl groups, which can vary, in addition to having excellent properties as chelators of iron and other

transition metals, which gives them a high antioxidant capacity. The basic skeleton of flavonoids consists of 1-benzopyran. It is a C6-C3-C6 system, where the aromatic rings, which we will call A and B, are connected by a ring C, forming a central pyran. Generally, flavonoids contain hydroxyl groups (-OH) at 2, 3, 5, 7, 3 ', 4' and 5 ' positions. Ring "A" has mostly hydroxyl groups at position 5 and 7, on the other hand, ring B at position 4 ', if it is a hydroxyl group, at position 3' and 4 ', 3', 4 'and 5' [6]. Flavonoids might be in the form of aglycones or β-glucosides [2]. It should be noticed that the antioxidant capacity of the flavonoids depends on the oxide-reduction properties of their hydroxyphenyl groups and of the chemical groups attached to the main skeleton. Flavonoids are classified as chalcones, flavones, flavonols, anthocyanins, isoflavones, flavanols [3]. The differences in their chemical groups attached to the basic skeleton determine the classification of each flavonoid (Figure 1).

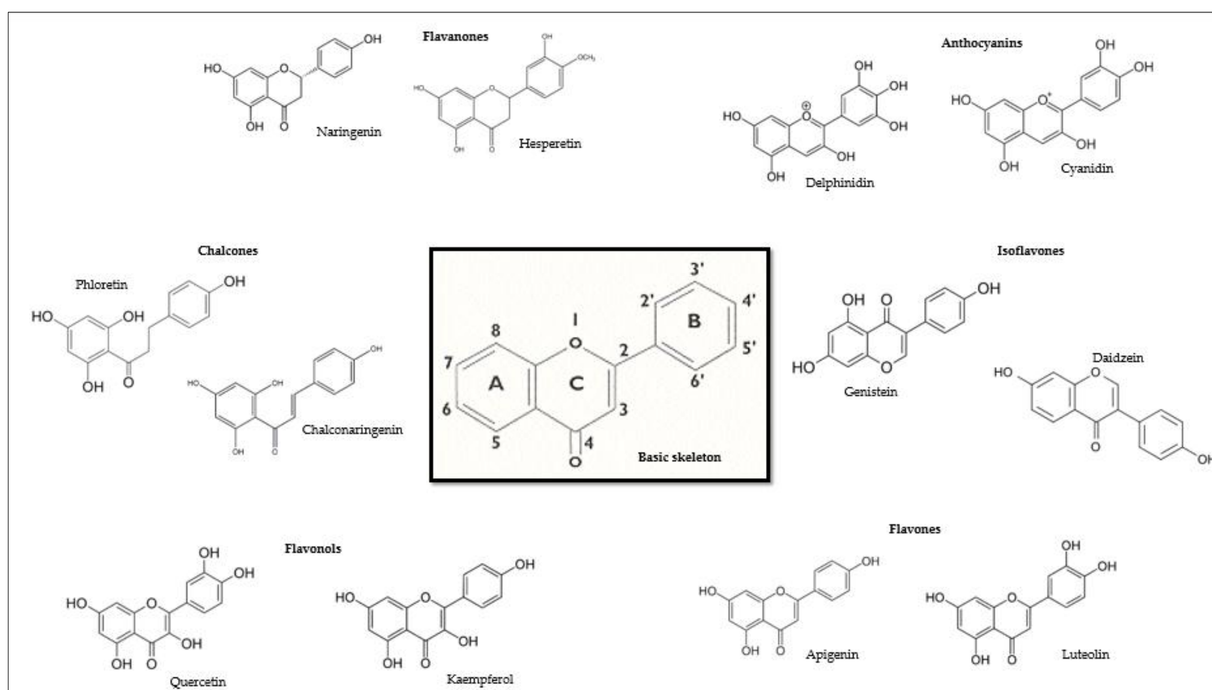

Figure 1. The basic skeleton structure of flavonoids and their classes.

The primary sources of flavonoids are fruits and vegetables

Typically, each type of flavonoids is found in specific sources. For example, Soybeans is widely reported as a source of isoflavones while grapes and red fruits are more associated

with a source of anthocyanins. Thus diets rich in fruits and vegetables are correlated with health-promoting effects; moreover, several reports have been focused in the quantification of flavonoids in these sources to relate their flavonoid concentration with their bioactivity [2].

Table 1. The flavonoids and their food sources and concentration.

Type of Flavonoid	Source	Reported flavonoids concentrations[1]	References
Flavones	Celery	5-10	[8]
	Parsley	10-50	[9]
	Hot peppers	0-5	[10]
Anthocyanidins	Berries	69.87	[11]
	Plums	215-237	[12]
	Red wine	1.8-2 g/L	[13]
	Grapes	2968.6	[14]
Flavonones	Citrus fruits	19.8-23.2	[15]
Isoflavones	Soybeans	55	[16]
Flavonols	Onions	6407-9571	[17]
	Leeks	3353	[18]
	Brussels sprouts	2040	[19]
	Kale	963	[20]
	Broccoli	7.45	[21]
	Green and black tea	20-50 mg/L	[22]
	Beans	5.143-5.74	[23]
	Apples	85-105	[24]
Flavanols	Cocoa	65-73	[25]

[1]The concentrations are reported in mg/100g dry weight. For sources such as red wine and green and black tea, flavonoid concentrations are expressed in g / L because they are liquid substances.

1.7 The metabolism of flavonoids in the human body.

Flavonoids are ingested, mostly, in the form of glycosides, with a proportion of the aglycone form released in the small intestine. If we compare the flavonoids with the macro and micronutrients that we obtain through the diet, a large part of the ingested flavonoids is not absorbed in the proximal intestine, and they reach the colon where the hydrolysis and the microbiome-mediated fermentation exert a direct action about these compounds [7]. Interestingly, when the flavonoids that we obtain from the diet interact with the microbiota present in the colon, they do so bi-directionally. On the one hand, polyphenolic compounds can modulate the composition of the microbiota, thus favoring the growth of

certain bacteria compared to others, we can say that this is a prebiotic effect, mainly prefers the growth of bacteria like lactobacilli and some bifidobacteria [8]. It is worth mentioning that colon bacteria transform flavonoids, giving rise to metabolites that are generally smaller, this makes them more bioavailable, and in most cases, they are attributed better biological effects compared to the flavonoids itself, before being transformed.

When the flavonoids are in the lumen of the small intestine, lactase phlorizin hydrolase (LPH) hydrolyzes flavonoids in the form of glycosides in aglycones [9]. It is a transmembrane protein that has high substrate specificity for flavonoids O-b-D glucosides. The flavonoids in the form of aglycones can enter the epithelial cells, by the passive diffusion method, this as a result of great lipophilicity. On the other hand, flavonoids can be transported directly to the epithelium using transporters such as the sodium-dependent glucose transporter [8]. Also, they can be absorbed once the glycosides are hydrolyzed by the cytosolic b-glucosidases (Figure 2) [9]. Interestingly, the glycosides could be hydrolyzed in the intestine light before being absorbed. However, anthocyanins are an exception, since we can find them present in plasma and urine in the form of glycosides [10]. The ATP-binding proteins bound to the membrane have a participation in the transcellular epithelial passage of many flavonoids [11]. This group of proteins are involved in the transport of bioactive compounds through the basolateral membrane into the bloodstream of the portal vein, this facilitates its absorption, or it can also happen that they return to the intestine, this reduces its bioavailability (Figure 2).

Once the flavonoids are absorbed, these compounds undergo a phase I metabolism in the liver, by the monooxygenases present in cytochrome P450 and 2C9 [7]. Most of the flavonoids suffer this type of oxidation. However, there are other types of reactions that flavonoids could experience, such as phase II reactions, for example, methylation, sulphitation, and glucuronidation. They could also occur in phase I substrates in the liver and small intestine, where phase II enzymes are involved, such as catechol-O-methyltransferases (COMTs), some sulfotranspheres, and 5U'-diphosphate glucuronosyltransferases (UGTs) [8]. Sulfated metabolites, such as glucuronide sulfate and methylated metabolites, are metabolites with more polarity and can be eliminated via

urine or bile. Generally, most of the conjugated metabolites present in plasma and urine are glucuronides. On the other hand, aglycones are usually in deficient concentrations or absent in plasma after ingestion. UGTs can catalyze and mediate the transfer of glucuronic acid from UDP-glucuronic acid to flavonoids. This glucuronidation is site-specific and will depend on the flavonoid [9]. On the other hand, sulfotransferases place a residue of sulfates on the flavonoids [10]. It has reported that sulfotransferases 1A1-4 and 1E1 are involved in the metabolism of flavonoids [7]. Interestingly, catechol -O-methyltransferases participate in methylation to the catechins, epicatechins, and epigallocatechins [8]. This methylation decreases the hydrophilicity of the compounds, and are vital to achieving an adequate elimination in the organism [11]. Flavonoids tend to be eliminated in the urine; however, they can also be excreted by the bile duct [10].

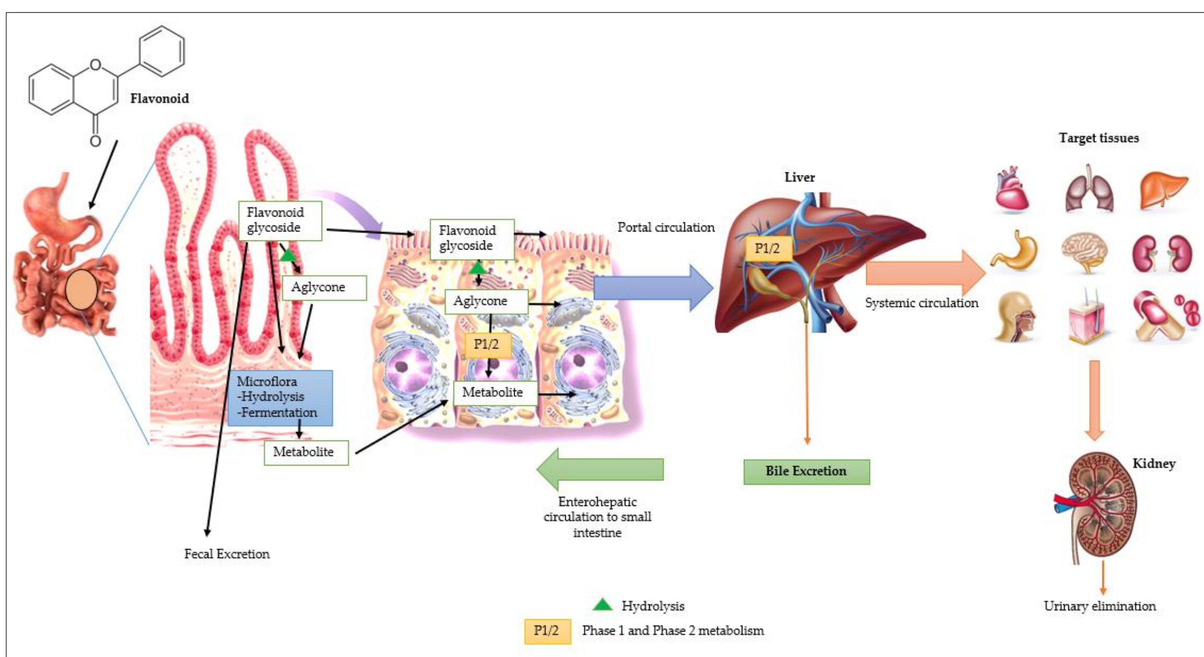

Figure 2. Overview of flavonoid absorption and post-absorptive metabolism.

1.8 Antioxidant activity of flavonoids.

The antioxidant activity has been considered a critical bioactive characteristic due to be the primary mechanism to prevent oxidative damage in cells by the removal of free radicals. In normal physiological conditions, the production of free radicals and other

reactive species is maintained in an equilibrium state by this antioxidant defense system. Free radicals occur in the form of reactive oxygen species and nitrogen (ROS and RNS), which result from a process of oxidation and reduction in the cell. An imbalance in those free radicals produce a damaging condition called oxidative stress [12]. It has been widely reported that oxidative stress plays an essential role in the development of chronic and degenerative diseases such as cancer, arthritis, autoimmune diseases, and cardiovascular diseases.

The main mechanism of action in the antioxidant activity of flavonoids is their ability to neutralize free radicals by their chemical structure. Reports described that the antioxidant capacity of flavonoids might be affected by the nature of the substituents present in their structure, as well as their position [13]. Significant antioxidant activity is presented with an ortho-dihydroxy substitution in the B ring, imparting stability to the free radicals formed by the oxidation of the flavonoid; the presence of hydroxyl groups in both carbons 3 and 5 that help the formation of stable structures once the flavonoids have been oxidized [14]. Interestingly, in the case of flavones and flavanones, which do not have substitution by the hydroxyl group in carbon 3, they do not have the significant antioxidant capacity.

Some enzymes can reduce the production of ROS and RNS, in this way can prevent cell oxidative damage. The proteins most reported for their antioxidant capacity are glutathione reductase (GRx), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD is the first line of defense against ROS and RNS, this catalyzes the dismutation of the superoxide anion (O_2), to convert it into hydrogen peroxide (H_2O_2) by reduction action. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by CAT or GPx. The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG) [15]. Interestingly the selenium is supplemented by dietary intake, and it has been related with the increase of the antioxidant activity of the GPx and SOD, which are the most relevant enzymes of the cell antioxidant defense system [16]. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing GSH. Another mechanism of antioxidant activity of flavonoids is the inhibition of oxidases, such as lipoxygenase (LO) and cyclooxygenase (CO), among others [17]. The presence of flavonoids in celery such

as quercetin, luteolin, kaempferol and apigenin, significantly reduce the activity of SOD and CAT [18]. Some flavonoids such as myricetin, epicatechin gallate, and epigallocatechin gallate carry out inhibition of CAT activity, due to the formation of hydrogen bonds between catalase and flavonoids. In the presence of some flavonoids, catalase can be inactivated [19]. Several in-vitro studies have described that the effect of polyphenols on the modulation of phase I enzymes is related to their chemical structure and the type of reaction. Polyphenols such as quercetin, apigenin, myricetin, kaempferol, and epigallocatechin gallate (EGCG) can inhibit CYP1A1, which contrasts with the mild effect of other polyphenols such as naringenin, hesperetin, naringin, hesperidin or rutin. Similarly, it appears that flavanols are more effective than flavanols in inhibiting the antioxidant enzyme quinone reductase (QR) [20].

Flavonoids inhibit the enzymes responsible for superoxide production, such as xanthine oxidase and protein kinase C [21]. For example, quercetin have been reported as inhibitors of the enzyme xanthine oxidase, resulting in a decrease in oxidative injury [22]. Luteolin has been reported to inhibit cyclooxygenase, lipoxygenase, microsome-succinoxidase activity, and NADH oxidase activity [22]. Also, the inhibition of protein kinase C was suggested to be a mechanism of inhibition of NADPH oxidase by quercetin. Potent flavonoid inhibitors of protein kinase C (e.g., quercetin, fisetin, and luteolin) possess a coplanar flavone structure with free hydroxyl substituents at the 3′, 4′ and 7-positions.

1.8.1 Chemo preventive effect by Antioxidant activity

Nitric oxide (NO) is vital in maintaining the dilation of blood vessels, but its high concentrations may result in oxidative damage. The oxidation of L-arginine, catalyzed by NO synthases (NOS) produce the NO. NO is toxic when is transformed to peroxynitrite, formed by the reaction of NO with O2. The intracellular accumulation of reactive oxygen and nitrogen species also plays an important role in the initiation of cell death processes. The exposure to oxidative stress may ultimately result in cell death, as a consequence of severe damage caused to biomolecules by reactive oxygen and nitrogen species [12]. The cells respond to DNA damage by increasing the production of p53, a potent inducer

of apoptosis. Mutations in p53 produce a defective protein that is often detected in cancer cells. The signaling and execution of apoptosis triggered by DNA damage are specific for cell type and genotoxin depending on p53 status (p63 and p73), the death receptor response, MAP kinase activation and, most importantly, the DNA repair capacity [23]. Tamarixetin is a flavonoid derived from quercetin and, interestingly, its viability has been studied on human tumor cell lines, and it was found to be cytotoxic against leukemic cells and, in particular, K562 / ADR cells that overexpress P-glycoprotein. Tamarixetin inhibited proliferation of leukemic cells in a dose-dependent manner, it also induced apoptosis and blocked the progression of the leukemic cell cycle in the G2-M phase. The overexpression of P-glycoprotein due to tamarixetin was associated with the accumulation of cyclin B1, Bub1 and p21 (Cip1 / Waf-1), changes in the phosphorylation state of cyclin B1, Cdk1, Cdc25C and MPM-2, and the inhibition of tubulin polymerization. Also, cell death was found to be associated with the release of cytochrome c and the cleavage of caspases and poly (ADP-ribose) polymerase, and the N-acetyl-L-cysteine radical scavenger free [24].

The accumulation of reactive species has been described to precede changes in the mitochondrial membrane, nuclear condensation, and other typical apoptotic events. Indeed, some studies have reported that an increase in ROS induces cytochrome c release from mitochondria (in a voltage-dependent anion channel (VDAC)-dependent way) and caspases activation. Many studies have shown other evidence for the induction of apoptotic pathways by reactive species [25]. Some mediators of apoptosis (e.g., JNK, ERK, and PTEN) have been reported to lead to increased levels of ROS. Similarly, the inhibition of the mitochondrial respiratory chain at complex I, or an impairment of the electron transfer chain by mutations in mitochondrial DNA, prevent the accumulation of ROS and consequently protect cells against apoptosis. In addition, lipid peroxidation has also been reported to occur following an apoptotic signal [26].

Another mechanism by which these types of molecules can support many of their biological activities can be the formation of metal complexes such as DNA protection, interaction with the DNA of tumor cells and regulation of the level of metals in the blood. This type of reactions takes place thanks to the proximity of two hydroxyl groups or a

carbonyl and a hydroxyl, such as the interaction of the catechol groups (hydroxyl groups ortho at the 3 'and 4' positions of the B ring), the 3-hydroxyl groups and 4-carbonyl and the 5-hydroxyl and 4-carbonyl groups [27].

The chemo-preventive or protective effect of polyphenols in black and green tea against the induction of different types of cancer in animals has been related to their ability to modulate the oxidative state of the cell. Said regulation occurs through the decrease of the activity of phase I enzymes (CYP1A1, CYP1B1, cytochrome b5 reductase, QR) or by increasing the expression of phase II enzymes (GST, UDP-glucuronosyltransferase) [28]. Similarly, polyphenols present in the apple, such as quercetin, can protect LT97 cells from colon adenocarcinoma against possible oxidative damage by increasing the expression of phase II enzymes such as GST and UDP-glucuronosyltransferase. For its part, chlorogenic acid plays a chemoprotective role against 13-acetate-12-O-tetradecanoyl phorbol (TPA) in mouse epidermal cells JB6 by increasing the activity of phase II enzymes GST and NQO, as well as that resveratrol promotes the activation of NQO-1 in mouse hepatoma cells [6].

The antioxidant action of most polyphenols is mediated by the antioxidant response element (ARE) (5'- (G/A) TGACNNNGC (G/A) -3'), which is located in the promoter region of the genes of various antioxidant enzymes and metabolism of xenobiotics [29]. Thus, black tea polyphenols play a protective role against oxidative damage in mouse hepatic and pulmonary cells, since they induce the increase of levels of detoxifying enzymes of phase II GST and NQO1, and promote the binding of Nrf2 to ARE. On the other hand, quercetin protects HepG2 cells from oxidative damage, induces the increase of NQO-1 expression through the activation of the Nrf2 signaling pathway, as does chlorogenic acid, which in cells Epidermal mouse JB6 promotes the binding of Nrf2 to the ARE and increases the expression of GSTA1. Likewise, coffee increases the expression of phase II enzymes, such as GST and also-keto reductase (AKR) through the induction of Nrf2 as a chemo-preventive mechanism of hepatocarcinogenesis. Polyphenolic grape extract of red wine was described to stimulate inhibition of receptor PECAM-1 (platelet endothelial cell adhesion molecule-1) thereby inhibiting platelet activation [30].

Cell death can occur by two main mechanisms: necrosis and programmed cell death (PCD). Necrosis is usually referred to as an "accidental," or uncontrolled, form of cell death. During this process, there is a rapid swelling of the cell, leading to the loss of membrane integrity and following the release of the cells' contents, which is known to induce an inflammatory response [26]. Quercetin causes the arrest of the S phase during the progression of the cell cycle in cancer cells tested. In addition, it induced tumor regression in mice at a concentration 3 times lower than ellagic acid. Importantly, the administration of quercetin leads to a 5-fold increase in the duration of life in the tumor-bearing mice. Interestingly, quercetin interacts directly with DNA and could be one of the mechanisms to induce apoptosis in both cancer cell lines and tumor tissues by activating the intrinsic pathway [31]. Epigallocatechin-3-gallate (EGCG) can reduce the inflammatory response associated with local tissue lesions, such as hepatocellular necrosis in acute liver injury induced by carbon tetrachloride. The protective effect of EGCG is due to its ability to decrease lipid peroxidation, oxidative stress and the production of nitric oxide (NO) radicals by inhibiting the expression of inducible nitric oxide synthase (iNOS). EGCG also improves the overproduction of proinflammatory cytokines and mediators, reduces the activity of the activated nuclear factor kappa-light-chain-enhancer of B-cell (NF-kβ) and transcription factor AP-1 and the subsequent formation of peroxynitrite with NO and reactive oxygen species [22]. Reports show that quercetin can inhibit and modulate the production and expression of the tumor necrosis factor (TNF-α) gene by human peripheral blood mononuclear cells (PBMC) [32].

1.9 Effect on cancer and inflammatory diseases

The relationship between inflammation and cancer is not new; it is widely known that sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma and DNA-damage-promoting agents, potentiates and/or promotes neoplastic risk [33]. The inflammatory cells promote the releasing of growth and survival factors and stimulate DNA damage. There is evidence that there are two distinct tumor-mediated mechanisms: a failure in the upregulation of the anti-inflammatory cytokines or deregulation of the host response resulting in desensitization of receptors due to high

chemokine and cytokine concentrations that then blunt systemic responses [33]. Quercetin and catechins are the most studied flavonoids in the promotion of anti-inflammatory effect by in-vitro and in-vivo models [34]. It has been suggested that flavonoids exert anti-inflammatory activity by the inhibition of pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase and inducible NO synthase, inhibition of NF-kB and activating protein-1 (AP-1) and activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C and nuclear factor-erythroid 2-related factor 2 [35]. Reports showed that anti-inflammatory effect and antiproliferative effect of flavonoids, such as apigenin, was related with the regulation of expression of genes involved in the apoptosis of human pancreatic cancer cells (BxPC-3 and PANC-1). Apoptosis was confirmed through flow cytometry assay, and it was concluded that apigenin affected the expression of genes involved in cell cycle, apoptosis, signal transduction, adhesion, angiogenesis, and invasion and metastasis in BxPC-3 cells [34]. Figure 3 shows the mechanism through which the possible interaction occurs in the proliferation process.

The flavonoids have been studied for their antiproliferative effects, without this effect being mediated by anti-inflammatory action. Reports showed that flavonoid-rich extract from Phaseolus vulgaris showed high cell inhibitions with specific cytotoxicity towards PC3 (prostate cancer cells), HepG2 (liver cancer cells) and Caco2 (colon cancer cells) with 77.7%, 90.1%, and 81.2% cell inhibition, respectively. The bioactivity of the extract was explained due to the presence of 116 mg/g extract of glycosylated flavonols such as myricetin-3-O-glucoside, quercetin- 4-O-galactoside and kaempferol-3-O-glucoside [35]. The evidence suggested that flavonoids in their glycoside form are more bioactive forms than aglycones. Moreover, glycoside forms have a significant antiproliferative effect than aglycones, and it is explained by their antioxidant activity.

Flavonoids can induce the synthesis of metabolic enzymes, both phase I and phase II, so they are considered, in general, bifunctional inducers.

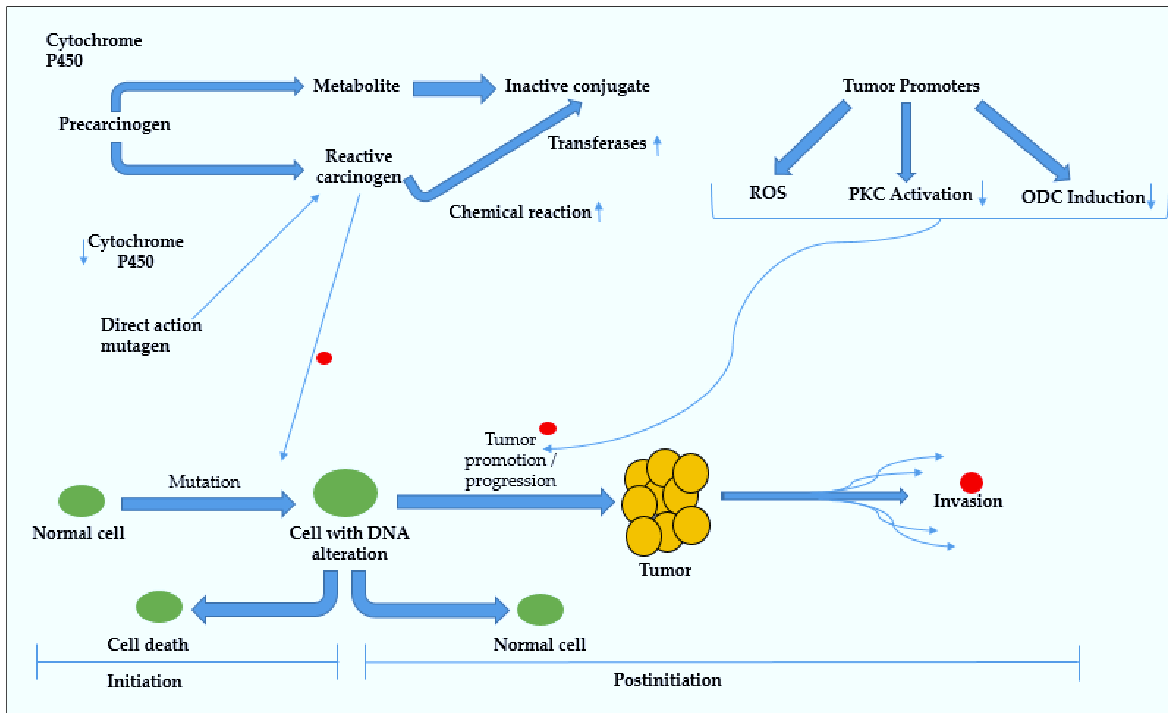

Figure 3. Possible interaction sites of the flavonoids with the carcinogenic process (marked with red circles). Short arrows indicate the enhancing effect (up arrow) or inhibitor (down arrow) activity. ROS: Oxygen Reactive Species; ODC: Ornithine Decarboxylase; PKC: Protein C kinase.

On the other hand, they can interfere with enzymatic activity directly. The most widely studied system has been cytochrome P450 and, among its isoenzymes, 1A is the most information, since the action on the other isoenzymes is less evident. On the phase II enzymes also some flavonoids have inhibitory activity [33]. The cytotoxic effect of quercetin has been associated with the inhibition of lactate release, with the decrease in the content of adenosine 5'-triphosphate (ATP), with the stimulation of the synthesis of transforming growth factor beta1-a recognized agent antiproliferative- and/or with the blocking of potassium channels [36]. Quercetin and other flavonoids can prevent the development of thermotolerance in cancer cells and, therefore, improve the efficacy of clinical hyperthermia as an anticancer therapy. Also, some flavonoids can act synergistically with other antitumor drugs [25].

1.10 Effects on Metabolic Syndrome

Lipotoxicity is a metabolic anomaly presented during the prevalence of obesity, and it refers to the induction of apoptosis of β- cells, myocardium and skeletal muscle leading to chronic diseases such as type 2 diabetes, cardiomyopathy, and insulin resistance respectively [37]. The relation of previously mentioned effects is known as metabolic syndrome. Development of coronary heart disease due to high cholesterol levels is one of the significant causes of death globally [38]. Diabetes, frequently caused by metabolic syndrome, is a public health problem in Mexico and other countries around the world. Moreover, Diabetes has considerable medical, social, and economic consequences and it ranks among the leading ten causes of death all over the country [39]. Hypertension and disorders in blood vessels are fundamental factors for the metabolic syndrome. Endothelial dysfunction and insulin resistance, common characteristics present in people with metabolic syndrome, are closely related. NO is involved in capillary vasodilation caused by insulin. The insulin signaling pathways that cause the release of NO is a vasodilator pathway with activation of NO synthase through Akt and a vasoconstrictor pathway that involves the release of endothelin-1 through mitogen-activated protein kinase (MAPK). Perivascular adipose tissue and the role of adipokines have vasoactive properties [40]. Hypertension is a highly prevalent disorder, occurring in approximately 50 million people in the United States and about one billion people worldwide.

Hyperlipidemia is a group of metabolic disorders characterized by elevated levels of cholesterol and triglycerides in plasma. The prevalence of hyperlipidemia has increased worldwide due to increased consumption of diets rich in saturated and trans-fatty acids [41].

Flavonoids such as quercetin, kaempferol, and catechin have been associated with the decrease of total cholesterol (TC) levels, the inhibition of LDL lipid peroxidation and the increase of HDL in humans [42]. Interestingly, it has been also reported that the decrease in TC is not related to the inhibition of cholesterol absorption through inhibited cholesterol micellization [37]. The mechanism of action by which flavonoids decrease TC and Increase HDL is by modulating cholesterol homeostasis via the transcription factors sterol regulatory element–binding proteins (SREBP) and liver x receptor (LXR). Lipid

homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors named sterol regulatory element–binding proteins (SREBPs). SREBPs directly activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol and fatty acids; many reports show that the expression of some of these genes is affected by the consumption of flavonoids. On the other hand, the LXR can modulate the expression of transporters ATP-binding cassette subfamily G members (ABCG5/G8). These reversed cholesterol transporters are glycoproteins synthesized in the endoplasmic reticulum (ER), and they are expressed in a tissue-specific manner in the liver, colon, and intestine [43]. Quercetin, one of the most common flavonols found in eatable plants, could modulate LXR activity by the change in the phosphorylation of AMPK. Thus it affects the expression of cholesterol transporters such as ABCG5/ABCG8. In consequence, they are responsible for limiting the intestinal absorption of cholesterol and promote biliary excretion of sterols [44].

Some studies suggested that there is a synergistic effect exerted by flavonoids and saponins to control lipogenesis, as well as reverse cholesterol transport [44]. The beneficial effect of flavonoids on the decrease of serum cholesterol levels in C57BL/6 mice was associated with a reduction of hepatic lipogenesis and cholesterol biosynthesis by negative regulation of SREBP1, FAS and HMGCR and ascending regulation of INSIG1/2 [44]. It has been shown that overexpression of INSIG1 traps the SCAP complex (SREBP cleavage activation protein) in the ER, promoting the degradation of SREBP and reducing the ability of SREBPs to activate the transcription of genes that encode enzymes involved in the biosynthesis of cholesterol and fatty acids [44]. A similar downward regulatory effect of lipogenic proteins has been demonstrated by consumption of wild-type diet enriched with cranberries [45] and coumarin, the latter related to the attenuation of hepatic steatosis [46]. Moreover, a flavonoid extract of Phaseolus vulgaris increased the expression of genes involved in the synthesis of bile acids, particularly CYP7A1, as well as in the reverse transport of cholesterol such as ABCG5/G8; also FSE stimulated the expression of ABCG5 / G8 in the intestine [44]. In the liver, ABCG5/G8 drives the elimination of hepatic cholesterol into the bile, whereas in the gut, it suppresses the absorption of biliary and dietary sterols. The active suppression of the accumulation of dietary cholesterol appears to require both intestinal and hepatic ABCG5/G8

overexpression. This effect has been shown to be related to protection against atherosclerosis [44].

There is recent evidence that shows that isoflavones, particularly genistein, can modulate LXR activity by controlling the phosphorylation state of this nuclear receptor [44]. Phosphorylation of AMPK, due to some flavonoids, might modify the action of LXR-α and LXR-β in. In another study, it was demonstrated that flavonoids such as Quercetin also stimulates the phosphorylation state of AMPK [44]. Therefore, evidence suggests that flavonoids up-regulate ABCG5/G8 and CYP7A1, and consequently promote of the excretion and catabolism of cholesterol, but was able to suppress lipogenic protein expression via LXR. On the other hand, LXR activation inhibits fatty acid oxidation by interfering with the binding of PPARα to its target sites [44].

1.11 Effect on Diabetes

According to the World Health Organization, 422 million adults worldwide had diabetes in 2014, compared to 108 million in 1980 [39]. It is a disease of high cost for the health sector because it causes other problems such as nephropathy, retinopathy, diabetic foot, neuropathy, among others. There are two types of glucose transporters, an energy-dependent transported and a sodium/glucose cotransporter (SGLT). SGLT family are expressed in the polarized epithelial cells which are located in the lumen of the small intestine and the proximal tubules of the kidney [47]. It has been shown that one of the most critical transporters of nutrients at the cellular level, is the transport of glucose through the plasma membrane by glucose transporters (GLUT), also called solute transporter family 2 (SLC2A) [48]. This family of transporters plays a vital role in the transport of monosaccharides, polyols, and other small carbon compounds through the membranes of eukaryotic cells [47]. In humans, fourteen GLUT proteins, called GLUT-1-12 and 14, are expressed as well as myoinositol transporter (HMIT) [48]. GLUTs function is to regulate the movement of glucose between the extracellular and intracellular compartments by maintaining homeostasis of glucose [47]. Insulin is a hypoglycemic hormone; it promotes the transport of glucose inside cells by activating the GLUT4 transporter, which is only found in the plasma membrane of adipocytes, in muscle fibers, in myocytes, and myocardiocytes. The insulin receptor is a complex transmembrane

glycoprotein consisting of 4 subunits: two alpha subunits of the extracellular side, two beta subunits that possess an extracellular domain, a transmembrane domain, and an intracellular domain. When activated by insulin, the intracellular part of one of the beta subunits acts as a specific tyrosine-protein kinase. The binding of insulin to the alpha subunits of the receptor causes a conformational change of the beta subunits, which induces autophosphorylation in 6 tyrosine residues. This phosphorylation induces covalent binding with other new specific proteins and is mainly phosphatidylinositol 3-kinase (PI3-kinase), which activates a membrane protein, the glucose transporter protein (GLU) that takes glucose from the extracellular medium and the transports to the interior of the cell [48].

The effects of some flavonoids have been reported in rats, for example, epigallocatechin gallate, given intraperitoneally to rats, determines a reduction of blood glucose and insulin level [49]. Similarly, genistein reduces blood glucose levels in diabetic rats, compared with the control, in glucose tolerance tests [50]. Similar data have been obtained with chronic treatments with genistein and daidzein in db/db mice and streptozotocin-induced rats [51]. Some flavonols, such as kaempferol, myricetin, rutin and its metabolite quercetin, show hypoglycemic activity. In particular, oral administration of rutin to diabetic rat's results in a plasma glucose levels reduction [49]. Another study reported that green tea improves glucose metabolism in healthy humans in oral glucose tolerance tests, this study was first tested in mice induced by streptozotocin and produces an antihyperglycemic effect without affecting the secretion of insulin [51]

Flavonoids can influence the synthesis and release of insulin from β cells. The mechanism underlying this biological effect may involve an increase in intracellular cAMP through increased adenylate cyclase activity and activation of protein kinase A (PKA), suggesting that genistein regulates insulinotropic action by activating the cAMP / PKA signaling cascade [50]. The α-Glucosidase is a membrane-bound enzyme located at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharides. Inhibitors of α-glucosidase may be used to control the blood sugar levels in type-2 diabetes [48]. The two anthocyanins cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside reduce glucose absorption and inhibit α-glucosidase

activity in vitro [51]. The latter action was also observed with luteolin, kaempferol, chrysin, and galangin. Luteolin-7-glucoside, luteolin, amentoflavone, and daidzein are the most potent inhibitors of α-glucosidase [48].

Insulin decreases the concentration of glucose in the blood. After eating, the concentration of glucose in the blood increases, which causes the β cells of the pancreas to secrete insulin, this reduces the secretion of glucagon, homeostasis occurs in the organism. Both hormones exert a direct effect on glycemia, although, in opposite ways, where there are low concentrations of glucose in the bloodstream, this induces the secretion of α cells, on the other hand, the cells of the pancreas β cells secrete insulin when they exist high concentrations of blood glucose [48]. When a person has diabetes, once glucagon is released is not due to a high concentration of glucose, the secretion of this hormone is deficient when there are high levels of glucose. Interestingly, intestine has an impact on the secretion of the hormones glucagon and insulin, because several peptides originate, such as gastrin, the gastric inhibitory polypeptide (GIP) and the glucagon-like peptide 1 (GLP-1). The release of the two main incretin hormones, GIP and GLP-1, decrease their concentration when there is a Type 2 Diabetes Mellitus condition [52]. The main effect of flavonoids in diabetes has to do with its action on several specific routes, such as the regulation of the route of the reduction of programmed cell death, among other molecular objectives [49]. These compounds also improve the growth and proliferation of pancreatic β-cells and also increase insulin secretion. On the other hand, flavonoids also participate in the modulation of glucose metabolism in liver cells, thereby improving hyperglycemia. Also, insulin resistance, inflammation and oxidative stress decrease in adipocytes and skeletal myofibers. In adipocytes, there are two parallel signaling pathways through which insulin translocation of the GLUT-4 transporter occurs, PI3K/AKT and CAP/Cb1/TC10 pathways [50]. Once the insulin receptor (IR) is activated, this leads to phosphorylation of the insulin receptor substrate (IRS), on the other hand, this causes the phosphoinositide 3-kinase (PI3K) to activate. Interestingly, PI3k, phosphorylates lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 3,4,4,5-triphosphate (PIP3). PIP3 then activates phosphoinositide-dependent protein kinase 1 (PDK) [52]. Once PDK1-mediated phosphorylation of protein kinase B (Akt) occurs, it allows the phosphorylation of the Rab GTPase activating protein

AS160, and this leads to the translocation of GLUT-4 from intracellular storage vesicles to plasma membrane and improves the glucose uptake. Once protein kinase B is activated, an inhibition of glycogen synthase kinase-3 (GSK-3) occurs, which subsequently phosphorylates and deactivates glycogen synthase (GS) [48]. Interestingly, AKT can phosphorylate O1 (FOXO1) of the forkhead box, which in turn, deactivates the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and thereby eliminates the process of gluconeogenesis in the liver [52]. The AMP-activated protein kinase (AMPK) plays an essential role as a regulator in the metabolism of cells, which also decreases the production of glucose from the liver through its role as mediator and modulator of PEPCK and G6Pase [49]. Interestingly, epicatechin and epigallocatechin reduce the blocking of insulin signaling that is induced by high concentrations of glucose by avoiding the decrease in IR, IRS-1, and IRS-2 levels; they also participate in inhibition of the PI3K / AKT and AMPK pathways. Moreover, it increases the phosphorylation reactions of Ser636 / 639 of IRS-1 in the HepG2 cell line. Also, the anthocyanins derived from the purple sweet potato have been shown to restore markedly the deterioration of the insulin signaling pathway IRS1 / PI3K / Akt in the livers of mice treated with high-fat diets [51].

There are many reports about the benefits of Opuntia ficus indica in lowering glucose, triglycerides and cholesterol levels in plasma. It is due to the 30% of soluble fiber that contains, which decreases the absorption of glucose and fat at the intestinal level and also prevents the concentration of glucose at mealtime. Also, it was reported that oral administration of Isorhamnetin-diglucoside to rats with diabetes induced by streptozotocin significantly reduced serum levels of glucose, hydroxymethylfurfural and serum levels reactive with thiobarbituric acid and liver and kidney mitochondria [50]. Other study reported that resveratrol supplementation for the treatment of T2DM produced clinically significant changes in fasting plasma glucose and insulin levels, systolic blood pressure, and diastolic blood pressure. Subgroup analysis showed a significant effect of resveratrol supplements at high doses (≥ 100 mg/d) in reducing fasting plasma glucose levels [49].

Table 2. Mechanisms and sources of flavonoids with antidiabetic potential.

Flavonoid	Plants/Dietary source	The specific mechanism of action	Model	Reference
Diosmin	*Scrophularia nodosa* L., and citrus fruits	Stimulate the insulin production from the existing B-cells of the pancreas.	STZ-nicotinamide-induced diabetic rats.	[51]
Fisetin	Strawberries, onion, and persimmon	Improve glucose homeostasis, decrease gluconeogenesis and increase glycolysis.	STZ-diabetic rats	[52]
Morin	*Prunus dulcis* (Mill.) D.A. Webb, *Chlorophora tinctoria* (L.) Gaud., *Psidium guajava* L., fruits, and wine	Inhibit PTP1B, which behaves as an activator and sensitizer of the insulin receptor stimulating the metabolic pathways. Also, preventing the destruction of B-cells from the islets of Langerhans.	HepG2 cell line and STZ induced diabetic rats	[53]
Hesperidin	Orange citrus aurantium	Down-regulate the generation of free radical, release of cytokines (TNF-alfa and IL-1B). Anti-angiogenic and anti-inflammatory effects.	Neuropathy rats and STZ-induced diabetic rats.	[54]

1.12 Cardiovascular diseases and vascular effects

Along with cancer, cardiovascular disease is one of the areas of medicine in which flavonoids have raised higher expectations [15]. The biological activities described for these compounds, both isolated and present in beverages, vegetables or fruit, make them compounds of interest in the cardiovascular field due to their effectiveness against the

oxidative damage of low-density lipoproteins and protector of vascular endothelium. They can also play an essential role in the regulation of platelet aggregation and vascular smooth muscle contractility [42]. Epicatechin has been shown, in animals and humans, to increase the production of new mitochondria in heart and muscle, a process called "mitochondrial biogenesis," while at the same time stimulating the regeneration of muscle tissue. Recent evidence indicates that epicatechin is acting as a mimic of a newly discovered human hormone released by normal muscles after exercise that may be responsible for the biogenesis of mitochondria and the stronger muscle fibers that generally occur after physical activity. This study also reported that epicatechin stimulates the production of NO in endothelial cells through the phosphorylation of eNOS, which can activate mitochondrial biogenesis [53].

Some polyphenols, extracts, and juices related to wine, or this one, have a vasodilatory effect depending on the endothelium in vitro. Although the mechanism that causes this behavior is far from clear, it has been suggested that stimulating the synthesis of nitric oxide or reducing nitric oxide degradation, can be one of the mechanisms of action [43]. Isoflavones and flavones appear to be similarly active, whereas the presence of a carbonyl group in position 4 and a C2-C3 double bond seem to be indispensable requisites for the vasodilator effect [53]. Moreover, the activity of specific kinases is related to the contractile function of muscle cells and flavonoids can modify the functioning of these enzymes [40]. Both the structural requirements for inhibition and the potency of this inhibition [53]. Flavonols and flavones are the most active inhibitors; on the other hand, chalcones behave as weaker inhibitors and flavanones are generally inactive [54]. However, there are no reports until now about the effects of flavonoids in the various PKC isoenzymes present in the vascular tissue. The kinase, of the light chain of myosin, is another essential enzyme in the development of contraction in smooth muscle. Kaempferol, a flavonol similar to quercetin, has been reported as a specific inhibitor of the activity of this purified protein from the bovine aorta [54].

At least part of the vasodilatory effect of flavonoids can be attributed to the blocking of voltage-activated calcium channels. Inhibition of the response to intracellular calcium release observed with some flavonoids may be due to decreased sensitivity to this ion as

a result of inhibition of PKC rather than emptying of intracellular deposits [15]. The increase in the cellular values of cyclic nucleotides participates in the relaxation of the vascular smooth muscle. Some flavonoids such as quercetin and epicatechin are capable of inhibiting the activity of 3',5'-guanosine monophosphate (GMP) and 3',5'-adenosine monophosphate (AMP), enzymes responsible for the degradation of these nucleotides [53]. They increase their concentration in this way, which can contribute to the relaxing action. Moreover, there is a relationship between the structure of the flavonoid and its effect on the different isoforms of this type of enzymes. For example, tea catechins can inhibit the activity of the phosphodiesterases GMP and AMP. The catechins can also block the entry of calcium from the extracellular space, without modulation of the functionality of the potassium channels being affected [54].

1.13 Conclusions

Flavonoids have been related to important health benefits and have also been shown to act through different mechanisms of action to improve the body's homeostasis. Among the main effects are those attributed to the prevention and treatment of chronic diseases. In cardiac diseases, there are flavonoids such as quercetin, epicatechin, rutin, and luteolin, which increase the production of new mitochondria in the heart. Also, they have antiatherosclerotic effects and platelet function. In diabetes, luteolin, kaempferol, chrysin, and galangin, have direct action in the synthesis and release of insulin and have a role at the level of glucose transporters, decreasing their concentration. Flavonoids also have antioxidant effects such as free radical scavenging and neutralization of some reactive oxygen and nitrogen species. In the case of cancer and inflammation-related diseases, these compounds exert anti-inflammatory activity by inhibiting pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase, and NO synthase, also some flavonoids such as apigenin are involved in regulation of genes that have to do with apoptosis. The type and, therefore, the function of the flavonoids depends on their plant source. Consequently, it is crucial to know what benefit has been attributed to what type of flavonoid. In some cases, the reported benefits of flavonoids have been robust enough for the specific mechanism at the cellular and molecular level has been reported by those who exert their effect. Consequently, its use has been proposed for the treatment of some

diseases such as diabetes, hypertension, and metabolic syndrome. Therefore, the use of flavonoids in the design of nutraceuticals and functional foods has been growing in recent years. Day after day more properties are attributed to the flavonoids. However, it is necessary to continue studies to ensure that these natural compounds are well used by the population.

CAPÍTULO II:

PRIMERA INTERVENCIÓN: El CONSUMO DIARIO DE UN CHOCOLATE RICO EN FLAVONOIDES DISMINUYE LA GENOTOXICIDAD CELULAR Y MEJORA PARÁMETROS BIOQUÍMICOS DEL METABOLISMO DE LÍPIDOS Y GLUCOSA.

2.1. Introduction.

Obesity is a rising global health problem that affects 600 millions of adults worldwide [55]. The prevalence of obesity is directly related to the development of cardiovascular diseases and diabetes through a condition known as metabolic syndrome [56,57]. Atherosclerotic Cardiovascular Disease (ACVD) is the leading cause of death around the world [56]. High levels of LDL and triglycerides, and low levels of HDL are associated with the development of ACVD [59]. Metabolic Syndrome is a condition that leads to other mortal conditions such as hypertension, dyslipidemia, ACVD, and diet is one of the major lifestyle factors that can significantly influence the incidence and progression of chronic diseases such as cardiovascular disease, diabetes, and cancer. Humans have consumed cocoa since at least 460 AD [56]. An increasing number of reports have shown that the consumption of cocoa and dark chocolate exerts several beneficial effects on cardiovascular health and endothelial dysfunction [59]; observations are consistent with reports that dark chocolate lowers blood pressure [60] and improves endothelium-dependent vasodilator responses [61]. Cocoa, especially dark chocolate, contains high levels of flavanols such as epicatechin, catechin, and procyanidins. Flavanols in cocoa are present as either the monomers (−) epicatechin and (+) catechin or oligomers of epicatechin and/or catechin, called proanthocyanidins or condensed tannins. Many beneficial effects have been attributed to flavonoids. For example, some reports have shown that epicatechins improved vascular function, reduced BP, improved insulin sensitivity, and reduced platelet activity [62]. Flavonoid content in chocolate varies among brands due to differences in the production process. The initial levels of flavonoids in the cocoa bean could change during fermentation, drying, roasting, alkalinization, and storage conditions, depending on the protocols of each company; even if the percentage of cocoa is the same, the final content of flavonoids might be different. Moreover, there is no specific regulation governing the labeling of dark chocolate or of its polyphenol or flavonoid content [63].

In metabolic syndrome, patients present a chronic systemic inflammation condition which has been widely related to cellular stress [56]. It is also known that cellular stress is related to the propensity to generate genetic mutation and cancer [64]. In recent years, increasing

attention has been given to compounds and conditions that induce genetic damage by various mechanisms. Specifically, the mutagenic events and genotoxic agents might play an important role in the cause and/or progression of human diseases other than cancer [65]. Physical activity and nutrition are important modifiers of systemic oxidative stress. The most reported metabolic disease that has been associated with DNA damage is diabetes. DNA damage is associated with diabetes; the extent of damage is greater in diabetics compared to non-diabetics [66] Interestingly, it has been reported that systemic DNA damage has a significant correlation with elevated parameters of metabolic syndrome [67]. Recently, oral epithelium cells have been used for the evaluation of exposure to various genotoxic agents, associated with its recognized sensitivity for the assessment of DNA damage. The micronucleus test is a multi-target genotoxic endpoint. Therefore, it can provide additional insight about epigenetic effects in different cell types caused by an agent or a condition. Thus, the determination of abnormalities in buccal epithelial cell nuclei has been used as a noninvasive assay for monitoring genotoxicity, and hence, DNA damage in humans.

In the present work, we evaluate the genoprotective effect of consuming a flavonoids-rich chocolate, and the improvement in the biochemical parameters related to cardiovascular risk and metabolic syndrome in young Mexican adults.

2.2 Results

2.2.1 Study Population

Table 1 describes participant characteristics in terms of sex, education level, physical activity, and cardiometabolic co-morbidities (diabetes and hypertension). Physical activity was classified as inactive, moderately active, and active, and it was assessed according to the International Physical Activity Questionnaire of World Health Organization [17]. The majority of the studied population was undergraduate students, with normal blood pressure but high triglyceride and glucose levels (Table 1). Participants presented an average body mass index (BMI) of 32.1, with an incidence of approximately 68% (Table 1). According to the World Health Organization, for adults over 20 years of age, a BMI

ranging from 30.0 to 34.9 falls within the Obesity Class I range [18]. The characteristics of each intervention group are described in Table 3. Since a randomized study was conducted, characteristics are similar in both groups.

Table 3. Characteristics of study population at recruitment.

Characteristics		*n*	(%)	FRC (*n*)	MC (*n*)	Observations
Participants		84	(100)	42	42	
Sex	Men	47	(55.90)	23	24	
	Women	37	(44.10)	19	18	
Education level (completed or in progress)	Underweight (Below 18.5)	0	(0.00)	0	0	According to the information provided in the surveys conducted.
	Normal weight (18.5–24.9)	9	(10.71)	4	5	
	Pre-obesity (25.0–29.9)	10	(11.90)	5	5	
	Obesity class I (30.0–34.9)	57	(67.85)	29	28	
	Obesity class II (35.0–39.9)	8	(9.52)	4	4	
	Obesity class III (Above 40)	0	(0.00)			
Risk Factors	Hypertension	18	(21.41)	9	9	Defined as blood pressure ≥140 mm Hg and/or ≥90 mm Hg for systolic and diastolic pressures respectively.
	Diabetes	12	(14.28)	6	6	Defined as taking antidiabetic medications and/or having fasting plasma glucose ≥120 mg/dL (≥7 mmol/L)
	Dyslipidemia	52	(61.90)	26	26	Defined as having at least one of the following anomalies: total cholesterol ≥190 mg/dL (≥4.9 mmol/L), TAG ≥150 mg/dL (≥1.7 mmol/L), LDL-cholesterol ≥115 mg/dL (≥3.0 mmol/L), HDL-cholesterol <40 mg/dL for men and <46 mg/dL for women.

FRC = commercial dark chocolate with 70% cocoa, MC = commercial milk chocolate.

2.2.2 Determination of Total Phenolic, Flavonoids Content and Antioxidant Capacity

Total phenolics content was quantified in commercial dark chocolate and milk chocolate (Table 4). Total phenolic content was 63.7 ± 0.2 and 56.3 ± 1.5 μmol of gallic acid equivalents per 1 g of dark chocolate and milk chocolate respectively. Dark chocolate and milk chocolate had 34.8 ± 0.5 and 10.4 ± 0.8 μmol of (-)-catechin equivalents per 1 g respectively. The percentage of flavonoids/phenolics (%) was significantly higher in dark chocolate than in milk chocolate. The antioxidant capacity (Table 4) was significantly greater in dark chocolate than in milk chocolate ($p < 0.05$).

Table 4. Phenolic and Flavonoid content in used dark mil chocolates for the study.

Treatment	Phenolic Content (μmol Gallic Aid Equivalents/g)	Flavonoid Content (μmol Catechin Equivalents/g)	Antioxidant Capacity (μmol Trolox Equivalents/g)	Flavonoids/Phenolics (%)
FRC	63.70 ± 0.20	34.80 ± 0.50	3.52 ± 0.60	54.63
MC	56.30 ± 1.50	10.40 ± 0.80	0.98 ± 0.02	18.47

FRC = commercial dark chocolate with 70% cocoa, MC = commercial milk chocolate.

2.2.3 Changes Caused by Daily consumption of Chocolate

The dietary variables did not change as a consequence of the consumption of flavonoid-rich chocolate. In contrast, some anthropometric and biochemical variables varied after the dietary intervention with dark chocolate (Table 4). Variables were evaluated before and after the treatment of either milk or dark chocolate daily consumption for 6 months.

2.2.4 Frequency of Nuclear Abnormalities in Buccal Epithelial Cells

At the beginning of the study, 14.4% of the buccal epithelial cells had abnormalities of the nuclei. Likewise, participants of both experimental groups showed nucleus abnormalities of broken egg, micronucleus, and binucleus at the beginning of the study (Figure 1). Interestingly, abnormalities of the nuclei in the buccal epithelial cells decreased significantly (less than 2%) after 6 month of daily consumption of 2 g of dark chocolate (Figure 1A). Milk chocolate intake (2 g per day for 6 months) did not affect the percentage of abnormalities observed in buccal cells.

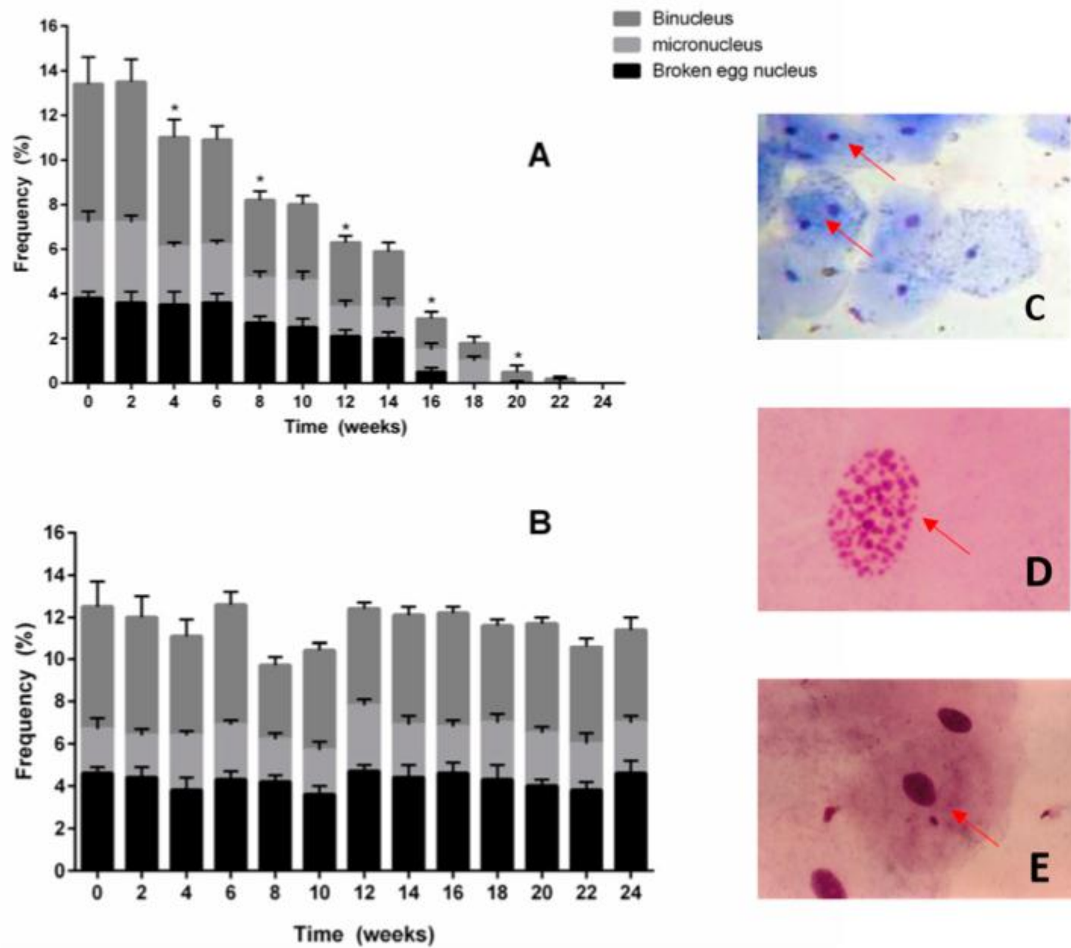

Figure 4. Frequency in nuclear abnormalities in buccal epithelial cells during consumption of dark chocolate with 70% cocoa (A) and milk chocolate (B). * Frequency was statistically compared with the frequency obtained in the previous week ($p < 0.05$). Observed nuclear abnormalities were (C) binucleus (D) micronucleus, and (E) broken egg.

The Correlation study through Pearson's linear regression analysis among the frequency of nuclear abnormalities in buccal epithelial cells and biochemical and anthropometrical variables was determined. The correlation study was performed with the biochemical and anthropometrical levels of the 84 participants at the beginning of the study. Figure 2 shows the parameters that had a significant correlation ($r \geq 0.5$, $p < 0.001$) with nuclear abnormalities in buccal epithelial cells. Waist circumference ($r = 837$, $p < 0.001$) and

Fasting glucose plasma (r = 785, p < 0.001) were the parameters with highest correlations with the frequency of nuclear abnormalities in buccal epithelial cells.

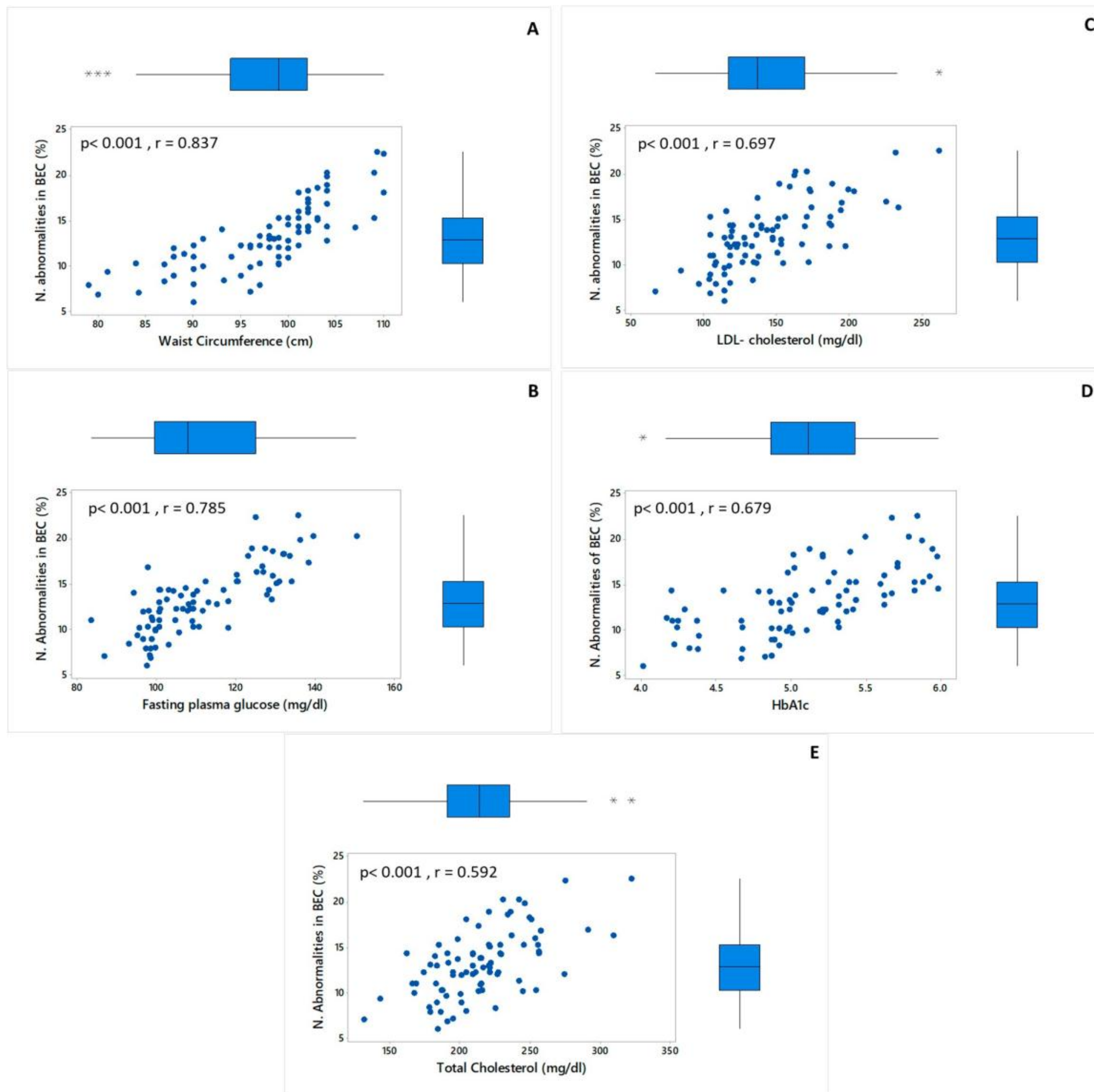

Figure 5. Correlation study through Pearson's linear regression analysis in the beginning of the study at the 84 participants. (A) Correlation between nuclear abnormalities in buccal epithelial cells (N. Abnormalities in BEC) and waist circumference ($p < 0.001$, $r = 837$). (B) Correlation between nuclear abnormalities in buccal epithelial cells (N. Abnormalities in BEC) and fasting plasma glucose ($p < 0.001$, $r = -0.785$). (C) Correlation between nuclear abnormalities in buccal epithelial cells (N. Abnormalities in BEC) and LDL-cholesterol levels ($p < 0.001$, $r = 0.697$). (D) Correlation between nuclear abnormalities in buccal epithelial cells (N. Abnormalities in BEC) and HbA1c ($p < 0.001$, $r = -0.679$). (E)

Correlation between nuclear abnormalities in bucall epithelial cells (N. Abnormalities in BEC) and total-cholesterol levels ($p < 0.001$, $r = 0.592$). Atypical points were marked with *.

2.3 Discussion

2.3.1 Determination of Total Phenolic and Flavonoids Content

Results showed that 54.6% of phenolic content in the dark chocolate was from the flavonoids group; this percentage of flavonoids was significantly higher compared with the 18% of flavonoids contained in the milk chocolate. The relation flavonoids/polyphenols showed that the dark chocolate with 70% cocoa was a significantly better source of flavonoids than milk chocolate. Taking into consideration that 2 g of dark chocolate were provided to each participant in the study, approximately 69.6 μmol of (-)-catechin equivalents of flavonoids were provided to each participant daily in the treatment with commercial dark chocolate with 70% cocoa (FRC), and 20.8 μmol of (−)-catechin equivalents of flavonoids were provided to each participant in the milk chocolate treatment (MC).

2.3.2 Characteristics of Study Population

A total of 84 participants were recruited for the present study. The majority of the participants were undergraduate students (94%), of which 38% were current smokers, 65% reported having a habit of moderate physical activity, and 67% presented a BMI in a range of 30–34.9 (falling into the Obesity class I range). Half of the participants ($n = 42$) were randomly included in the treatment group where commercial dark chocolate with 70% cocoa was provided, and the other half were treated with milk chocolate. The principal reported risk factor by participants was dyslipidemia, as 61.90% of participants presented at least one of the following anomalies: total cholesterol ≥190 mg/dL (≥4.9 mmol/L), TAG ≥150 mg/dL (≥1.7 mmol/L), LDL-cholesterol ≥115 mg/dL (≥3.0 mmol/L), HDL-cholesterol <40 mg/dL for men and <46 mg/dL for women.

2.3.3 Determination of Total Phenolic and Flavonoids Content

According to the results, the content of phenolics was slightly lower in milk chocolate compared to dark chocolate; however, it has been reported that sugar content could affect the measurement of phenolic content by Folin-Ciocalteu method. Interestingly, the flavonoid content was 3 fold higher in the dark chocolate compared with the milk chocolate used for the present study. Since the percentage of flavonoids/phenols (%) was significantly higher in dark chocolate than milk chocolate, the commercial dark chocolate used as a treatment was a better source of flavonoids compared with the commercial milk chocolate used in the present study.

There was no significant difference in the intake of fruits and vegetables reported by the participants (Table 5). However, a significant amount of flavonoids was provided on a daily basis by dark chocolate intake (Table 4), comparing the content of flavonoids of dark and milk chocolates (Table 4). Flavonoids are the most bioactive molecules reported among polyphenolic compounds. The flavonoids/phenols ratio shows the proportion of total polyphenolic compounds that belong to flavonoids. According to results, 54% of total phenolic compounds of dark chocolate are flavonoids, while only 18% of total phenolic compounds are flavonoids in milk chocolate. It has been reported that the most abundant flavonoid found in cocoa is the flavonol epichatechin, followed by chatechin. Also, the authors reported that some dimers and polymers of those flavonols were found in most of analyzed chocolates [66].

Table 5. Dietary, anthropometric and biochemical variables determined prior and after the dietary intervention with either flavonoid-rich chocolate or milk chocolate for 6 months.

Variables		Intervention Group	Beginning of the Study Mean ± SD	End of the Study Mean ± SD
Age		FRC MC	23.8 ± 3.4 23.6 ± 3.5	24.6 ± 3.1 23.8 ± 2.6
Number of Participants		FRC MC	42 42	42 42
Dietary variables	Fruit and vegetable intake (g/day)	FRC MC	523.7 ± 371.4 529.1 ± 329.6	548.2 ± 387.9 531.2 ± 356.21
	Total energy intake (kJ/day)	FRC MC	2214 ± 323 2208 ± 350	2298 ± 236 2310 ± 120
	Total carbohydrate (%E)	FRC MC	45.3 ± 8.2 46.7 ± 6.3	43.8 ± 8.9 45.8 ± 7.2
	Added sugar	FRC MC	5.3 ± 3.2 5.8 ± 4.1	6.1 ± 2.7 5.8 ± 3.8
	Total Fat (%E)	FRC MC	36.9 ± 8.1 34.8 ± 6.2	37.1 ± 7.6 36.5 ± 5.1
	Saturated Fat (%E)	FRC MC	20.8 ± 6.1 21.2 ± 3.1	16.9 ± 6.9 22.3 ± 5.9
	Unsaturated Fat (%E)	FRC MC	14.6 ± 4.1 14.5 ± 3.9	14.8 ± 4.6 15.9 ± 3.1
Anthropometric variables	BMI (Kg/m^2)	FRC MC	32.1 ± 3.8 31.4 ± 3.2	30.1 ± 2.2 32.4 ± 2.5
	Waist Circumference (cm)	FRC MC	98.7 ± 3.5 96.9 ± 4.1	**90.4 ± 4.5** * 94.9 ± 3.9
Biochemical variables	Total Cholesterol (mg/dL)	FRC MC	221.3 ± 16.7 224.3 ± 18.9	**201.2 ± 19.5** * 227.4 ± 12.4
	LDL-Cholesterol (mg/dL)	FRC MC	149.82 ± 18.4 147.23 ± 21.1	**116.2 ± 21.1** * 138.9 ± 19.1
	HDL-Cholesterol (mg/dL)	FRC MC	46.3 ± 12.5 45.4 ± 12.1	43.2 ± 10.9 44.2 ± 13.5
	Triglycerides (mg/dL)	FRC MC	228.25 ± 17.9 223.5 ± 21.1	**153.26 ± 18.95** * 224.1 ± 23.1
	HOMA-IR	FRC MC	2.3 ± 1.8 2.5 ± 1.6	**1.93 ± 1.1** * 2.4 ± 1.5
	Fasting plasma glucose (mg/dL)	FRC MC	114.23 ± 13.56 112.31 ± 16.71	**91.23 ± 9.25** * 111.67 ± 10.9
	HbA1c (%)	FRC MC	5.8 ± 1.0 4.7 ± 1.0	4.6 ± 1.1 4.5 ± 0.9
	Systolic blood pressure (mmHg)	FRC MC	139.2 ± 10.5 136.3 ± 21.5	**127.8 ± 11.2** * 133.9 ± 12.7
	Diastolic blood pressure (mmHg)	FRC MC	87.24 ± 11.8 87.28 ± 9.18	**84 ± 9.12** * 87.31 ± 9.44

FRC = commercial dark chocolate with 70% cocoa, MC = commercial milk chocolate. * *p* values for testing the differences among variables across two groups (before and after chocolate consumption) by using X^2 test. $p < 0.05$.

The antioxidant capacity was 3-fold greater in dark chocolate than in milk chocolate (Table 4). The antioxidant capacity has been previously related to the content of phenolic and flavonoid compounds. Moreover, the antioxidant capacity of cocoa might be higher than red wine and black tea [69,70].

2.3.4 Changes in Measured Parameters by Daily Dark Chocolate Consumption

Dietary variables did not significant change by the consumption of flavonoid-rich chocolate. However, certain anthropometric and biochemical variables varied after consumption of the flavonoid-rich chocolate (Table 4). The waist circumference of participants was significant lower ($p < 0.05$) after the study, a change that could not be attributed to changes in diet, since Total energy intake, and the proportion of carbohydrates and lipids were similar before and after the study. Interestingly, total blood cholesterol, triglycerides, and LDL-cholesterol significantly decreased after six months of daily intake of dark chocolate with high content of flavonoids ($p < 0.05$). It has been previously reported that dark chocolate intake could improve LDL levels in blood [65]. Moreover, it was also reported that the consumption of flavonoids could significantly increase lipid oxidation [68,69]; thus, flavonoids intake might improve lipid biochemical parameters by the modulation of reverse cholesterol transport in gut and liver [70]. With regards to HOMA-IR and fasting plasma glucose, both parameters were significantly decreased after 6 months of daily intake of the flavonoid-rich chocolate. HOMA-IR is a homeostatic model assessment (HOMA) to determine insulin resistance (IR) in β-cells. HOMA-IR is calculated with the proportion of plasma glucose and insulin levels. According to the results of the present study, and in accordance with previous reports [73], daily flavonoid-rich chocolate intake was significantly associated with a lower HOMA-IR ($p < 0.05$). Flavonoids such as genistein and epicatechin have been previously associated with a lowering of blood glucose concentration [74-78]. Finally, and no less important, blood pressure was significantly improved by the consumption of flavonoid-rich chocolate compared with milk chocolate (Table 5). Cocoa flavonoids have been previously reported for their effect in the improvement of cardiovascular parameters, such as blood pressure and platelet aggregation [79].

2.3.5 Frequency of Nuclear Abnormalities in Buccal Epithelial Cells

The abnormalities of the nuclei in the buccal epithelial cells were 14.4% at the beginning of the study. The participants of both experimental groups showed abnormalities of broken egg nucleus, micronucleus, and binucleus at the beginning of the study (Figure 4). Interestingly, waist circumference ($p < 0.001$, $r = 837$) and fasting plasma glucose ($p < 0.001$, $r = -0.785$) showed most significant correlation with the frequency of nuclear abnormalities in buccal epithelial cells at the beginning of the study (Figure 5). The results of the present study suggested that the risk factors of metabolic syndrome could be associated with the development of genomic instability (Figure 5). Other authors have shown an association of diabetic patients in the highest percentile of waist circumference, fasting plasma glucose, HbA1c, and cardiovascular risk with buccal-epithelia-cells increased genomic instability [80]. The exact mechanism that contributes to this genomic instability in obesity and metabolic syndrome is not clear, although the primordial link between them is oxidative stress, caused by chronic and systemic inflammation [55]. The increase in DNA damage might be occurring because of the increase in the imbalance between the production of oxidants and antioxidant defenses [81]. Moreover, epidemiologic studies have associated DNA damage with obesity and diabetes [76,82,83]. However, more in-depth studies should be conducted to determine the relationship between genomic instability and conditions of metabolic syndrome.

It was expected that nuclear abnormalities in buccal epithelial cells of participants would be found, because most of them presented risk factors that have been related with genotoxicity, such as the habit of smoking, obese state, and high glucose levels in plasma [80]. One of the most important findings of this study is that the daily consumption of 2 g of dark chocolate for 6 months could significantly decrease the genotoxicity and cellular damage of buccal epithelial cells. In particular, the frequency of nuclear abnormalities significantly decreased with the daily consumption of flavonoid-rich chocolate compared to milk chocolate intake. This might be the result of flavonoids increasing the antioxidant activity in the cells of the participants, and as a consequence, the DNA damage in buccal epithelial cells significantly decreased. The antioxidant capacity of flavonoids in dark chocolate (Table 4) could be related to its protective cellular effect, that decreases cellular

stress, and hence, DNA damage (Figure 4). This effect was a novel finding, because flavonoid consumption in dark chocolate has not been related with antigenotoxic effect before. The decreasing effect of genotoxicity might be related to the decreasing effect of oxidative stress in cells due to the antioxidant capacity and activity of flavonoids, and the modulation of expression of CYP450 [81]. Other genotoxic assays should be conducted to determine if the protective effect of flavonoids obtained in the present study is a systemic effect.

Interestingly, some studies have reported that Orlistat, an antiobesity drug, induced DNA damage in human cells, which suggested that regular consumption of orlistat needs careful circumspection [85]. In contrast, flavonoids from cocoa in dark chocolate are able to significantly decrease important risk factors such as waist circumference, total cholesterol, LDL-cholesterol, and triglycerides, without causing DNA damage, even having a celluloprotective effect that could reverse the DNA damage in buccal epithelial cells, which is apparently caused by the systemic inflammation of obesity, along with the fact that flavonoids have been long recognized for their anti-inflammatory effect [86].

2.4 Materials and Methods

2.4.1 Study Design and Participants

A randomized, placebo-controlled, double-blind study was performed in the Autonomous University of Baja California. The study was approved by the Ethics Committee of faculty of Medicine and Psychology of Autonomous University of Baja California (1120150617), and written consent was obtained from all subjects prior to enrollment in the study.

In brief, a random sample was recruited between November 2015 and January 2018. The inclusion criteria for the enrollment in the study were (1) Mexican Nationality with parents of Mexican Nationality–Hispanic Ethnicity; (2) Being from 20 to 35 years old; and (3) Having at least 3 of 5 risk factors: Glucose greater than 100 mg/dL, triglycerides levels greater than 160 mg/dL, LDL greater than 130 mg/dL, HDL lower than 45 mg/dL, and body mass index (BMI) greater than 29. Exclusion criteria for participation in the study were: taking antihypertensive, hypocholesterolemic, or weight-loss medications, or taking

any chocolate or cocoa extract regularly. The elimination criteria were to start taking antihypertensive, hypocholesterolemic, weight-loss medications, or any chocolate or cocoa extract during the experiment, or simply to decide to leave the study. Trained research staff provided the participants with detailed instructions for the study, assisted them in completing questions on dietary information, and then checked the completeness and accuracy of the responses. The dietary intervention was carried out during 6 months with a weekly follow-up of the participants. During the weekly interviews, a dietary survey was carried out; questions of the study were answered, and it was verified that the participants did not fall into the selimination criteria. The study began with 92 participants after data cleaning, particularly for poorly completed dietary data. During the study, 8 people were removed because of elimination criteria; the rest finished the study (n = 84).

2.4.2 Chocolate Consumption (Independent Variable)

The commercial dark chocolate of 70% cocoa content or milk chocolate was provided to participants by the research staff weekly, packaged in daily portions; the daily dose for each patient was 2 g of chocolate. The participants were blinded because the original packaging of the chocolates was removed; the chocolates were divided into 2 g portions, and packed again inside a white envelope. A weekly semi-quantitative survey was completed by the participants, including questions on habitual daily consumption of chocolate during the previous week. The participants reported their frequency of consumption, ranging from 1 to 7 days of consumption. They also selected the serving size based on how many packaged portions were ingested every day.

2.4.3 Determination of Total Phenolic Content

Total phenolic content determination was made using the Folin-Ciocalteu method [87,88], Briefly, extraction was performed with 80% methanol in water (v/v), then total polyphenols content was determined by using Beckman Coulter DU 520 Spectrophotometer (Beckman, Carlsbad, CA, USA) at 750 nm. A standard curve was developed using Gallic acid (Sigma-Aldrich, Lansing, MI, USA).

2.4.4 Determination of Flavonoids Content

Flavonoids were evaluated in 80% methanol in water (v/v) extracts with a colorimetric method, according to previous reports [86], using a Coulter DU 520 Spectrophotometer at 510 nm. A standard curve was developed with (−)-catechin (Sigma-Aldich).

2.4.5. Antioxidant Capacity

The capacity of dark and milk chocolates to scavenge the free radical cation of diammonium salt (ABTS+) was conducted as reported in [67]. Briefly, Trolox standard curves were used to measure the capacity to diminish free radicals of diammonium salt (ABTS) radical solution. Absorbance was measured using a Coulter DU 520 Spectrophotometer at 734 nm.

2.4.6 Epithelial Genotoxicity, Biochemical and Anthropometric Parameters Measure (Dependent Variables)

After 4 weeks of daily consumption of flavonoid-rich chocolate, a blood sample was obtained for each participant in order to measure biochemical parameters. Standard laboratory assays were used to measure Biochemical parameters with IDEXX Catalyst Dx® equipment (IDEXX, Westbrook, ME, USA). Anthropometric parameters were also measured every 4 weeks with a digital weighing scale and a measuring tape.

Genotoxicity was determined in each participant by a micronuclei assay. Briefly, exfoliated cells were collected by a non-invasive sampling method from oral mucosa. Buccal cavity cells are obtained by scraping the cheeks with a tongue depressor. The samples were transferred dropwise to pre-cleaned slides. Then, the slides were air-dried and fixed with 80% methanol. After that, slides were stained with hematoxylin for 3–5 min, and then with eosin for 5–8 min. Genotoxic damage was determined by

the observation of slides under the microscope; cellular malformations were observed and counted.

2.5 Statistical Analyses

Data were analyzed by using MiniTab and GraphPad Prism (version 6, GraphPad Software Inc., La Jolla, CA, USA) software. Results are reported as mean ± SEM. Statistical differences for mean data obtained from present study were analyzed by two-way ANOVA. Correlation study through Pearson's linear regression analysis was made using MiniTab 18.

5. Conclusions

In conclusion, flavonoids of cocoa had protective effects against DNA damage. This suggested that the reduction in genotoxic stress effect was related with the antioxidant activity of flavonoids and the modulation of CYP450. The modulation of CYP450 and the reduction in the expression of some interleukins have been related with the anti-inflammatory effect of flavonoids [88]. The antioxidant capacity of flavonoids contained in dark chocolate could be related to the decrease of cellular stress, and hence, to the DNA protection in the nucleus of cell. However, further in vivo studies are still needed to determine their mechanism of action of antigenotoxic effect. Biochemical parameters (total cholesterol, triglycerides, and LDL-cholesterol level in blood) and anthropometrical parameters (waist circumference) were also improved after six months of dark chocolate with a 70% of cocoa intake. Interestingly daily flavonoid-rich chocolate intake also improves fasting plasma glucose levels and insulin resistance parameter (HOMA-IR). These effects were attributed to the proportion of flavonoids in the chocolate which was 3-fold greater than in the milk chocolate. Together, these results suggested a potential beneficial effect as a consequence of the daily dark chocolate consumption in the lipid and glucose metabolism.

CAPÍTULO III:

SEGUNDA INTERVENCIÓN: EFECTO DE (-) - EPICATECINA Y QUERCETINA EN LA MEJORA DE LOS PARÁMETROS BIOQUÍMICOS Y EN LA DISMINUCIÓN DE GENOTOXICIDAD CELULAR DISMINUTIVA EN HUMANOS, CON UNA POSIBLE MEDIACIÓN POR SU EFECTO SINÉRGICO Y PROTECTOR IN VITRO EN LA DISHOMEOSTASIS DE CA +2 INTRACELULAR.

3.1 Introduction

Metabolic syndrome (MS) is a condition whose incidence around the world has been increasing in recent years [89]. It is characterized because patients present at least three of the following risk factors: excessive visceral fat storage, dyslipidemia, hypertension and hyperglycemia [90]. Diet is one of the major lifestyle factors that could significantly affect, positively or negatively, the progression of MS. During MS, patients present a chronic systemic inflammation, process which has been widely related with cellular stress and genetic mutations, frequently triggering deadly chronic diseases, such as: type 2 diabetes mellitus (T2DM), cardiovascular diseases (CV) and cancer [91,92,93]. Diabetes is the metabolic disease most commonly associated with genetic damage. Actually, systemic DNA damage has been correlated with elevated parameters of metabolic syndrome [94,95]. Evaluation of DNA integrity in oral epithelium cells by the micronucleus test, is a non-invasive technique that analyzes the genotoxic effect of a specific compound or condition; this test analyses the micronuclei that are formed because of DNA breakage or by disruption of the mitotic apparatus [96]. Also, intracellular calcium ([Ca2+] i) homeostasis is an important step in different signal transduction pathways. Controlled changes in [Ca2+]i are linked to several cellular functions; however, prolonged and overloading in the concentration of Ca2+, have been related to a number of chronic diseases, including obesity and dT2DM [96,97].

Flavonoids have been widely study because of their anti-inflammatory and antioxidant properties [98,99]. Some of their effects in lipid metabolism have also been reported before [97,98]. Although, most fruits and some legumes contain flavonoids, the reported levels in eatable parts are very low, varying from 4.5 mg/kg in kiwifruit to 610 mg/kg in black chocolate [99]. Even though the concentration, characterization and biological effect of flavonoids in fresh vegetables and fruits have been studied, little is known about the interactions, concentration and biological effect of flavonoids in bakery products, such as bread, which is an important component in the daily diet of European and American population.

In the present work, we evaluated the protective effect of two flavonoids: (-)-epicatechin and quercetin, on Mexican adult participants with at least three risk factors for MS. Bread

enriched was evaluated in terms of baking performance, shelf life and sensory properties. Enriched bread was consumed during 3 months, and we evaluated if there was an improvement in anthropometric and biochemical parameters. We also examine the genoprotective effect of consuming the enriched bread in buccal epithelium cells. Lastly, we investigated the protective effect of (-)-epicatechin, quercetin and the combination of both (1:1) by monitoring [Ca2+] i in Caco-2 cells stimulated with H2O2 as a pro-oxidative agent.

3.2 Materials and Methods

3.2.1 Bread making

The bread was made as reported before; the pup loaf straight-dough bread micro-baking method 10–10.03 was utilized [105]. Briefly, the formulations included composite flours enriched with 0.05% or 0% of a mixture 1:1 of (−)-epicatechin and quercetin. They were called bread with flavonoid mixture (BF) and control bread (CN), respectively. All ingredients were incorporated in the initial mixing step and doughs were mixed with the predetermined amount of 25°C distilled water using a 100–200 g dough mixer (National Manufacturing Co., Lincoln, Nebraska). Optimum water absorption and mixing times were determined by observing dough properties of gluten development (film formation, gloss and dough stickiness). Resulting doughs were weighed and then cut into two identical pieces before fermentation. Fermentation and baking conditions were made as previously reported [102]. Bake absorption, mixing time, proof height, loaf height, oven spring, loaf weight, loaf volume and loaf apparent density were determined. Proof height and loaf height were determined with a proof height meter (National Manufacturing Co., Lincoln, Nebraska). The difference between these values was recorded as oven spring. Loaf volume was determined by rapeseed displacement (National Manufacturing Co., Lincoln, Nebraska) according to method 10-05.01 of the AACC International [92]. Upon 30 min cooling at room temperature, BF and CN breads were cut into 15 mm thick slices, packaged in sealed polyethylene bags and stored at room temperature for 24 h for sensory analysis.

3.2.2 Sensory Analysis and color of bread crumb

Consumer panels (pilot consumer panels) made up of 40 consumers, evaluated the sensory features and overall acceptability of control (CN) and enriched breads (BF) after 24 h of baking. Bread evaluation was performed in food laboratory (UABC-Campus Tijuana) according with the guidelines described before [92]. Each consumer was simultaneously given 2 coded samples along with a ballot, and was asked to rate color, texture, flavor, odor, and overall quality on a 5-point hedonic scale, where 1 was “dislike very much” and 5 was “like very much”. Color of bread crumb samples (CN and BF) was measured on three different slices (1.5 cm) with a colorimeter (CR 300, Minolta, Japan) as reported before [101]. The color parameters measured were L* (luminosity), b* (yellowness) and a* (greenness).

3.2.3 Concentration of (-)-epicatechin and quercetin in bread samples

Concentration of flavonoids in the breads were evaluated in 80% methanol in water (v/v) extracts with a colorimetric method using a Beckman Coulter DU 520 Spectrophotometer (Brea, CA, USA) at 510 nm, according to previous reports [91]. A standard curve was developed with catechin (Sigma-Aldich, MI, USA).

3.2.4 Bread Shelf life

Relative humidity (RH), water activity (Aw) and microbiological assays were performed to study the evolution of both breads (CN and BF) during storage at 40°C, 25°C and 4°C, every day during 15 days.

3.2.5 Relative humidity (RH) and water activity (Aw)

Relative humidity (RH) of bread samples (CN and BF) was determined by gravimetric method. Briefly, bread samples were weighed before the procedure. Then, they were oven dried at 100 °C and accurately weighed at regular time intervals until constant weight

was reached. RH was expressed as grams of water/grams of total weight (g/100 g). The water activity (Aw) was measured with Pawkit Portable Water Activity Meter Kit (Vernon Hills, IL, USA).

3.2.5 Microbiological study

The microbiological evaluation of CN and BF was performed according to NOM-111-SSA1-1994 [100]. Briefly, 10 grams of every sample were manually homogenized in 90 ml of phosphate buffer (SRF) (1x10-1 solution). Then 1 ml of previous mentioned solution was transferred to 9 ml of SRF to obtain 1x10-2 solution. The procedure was repeated until 4 dilutions were obtained (1x10-1, 1x10-2, 1x10-3, and 1x10-4). Finally, every solution of each sample was cultivated in dextrose agar and incubated at 37°C. Colony-forming unit (CFU) and number of fungi and yeast (N°HL) were counted in the microscope (Zeiss Primo Star microscope (Zeiss Mexico, NL, Mexico) after 24 hours of incubation.

3.2.7 Study Design and biochemical and anthropometric parameters measure (dependent variables) in participants.

A randomized, placebo-controlled, double-blind study was undertaken in Rosarito, Baja California. The Ethics Committee of Hospital General de Baja California approved the study (CONBIOETICA2CEI01120150617), and written consent was obtained from all subjects prior to enrollment in the study. Briefly, a random sample was recruited between November 2017 and May 2018. The inclusion criteria for the enrollment in the study were: 1) Being Mexican of Mexican parents, 2) Being adults from 30 to 50 years old, and 3) To have at least 3 of the 5 risk factors for MS: Triglycerides greater than 160 mg/dl, Glucose greater than 100 mg/dl, HDL lower than 45 mg/dl, LDL greater than 130 mg/dl and Body mass index (BMI) greater than 29. Exclusion and elimination criteria for participation in the study were the following: to be taking hypocholesterolemic, weight-loss or antihypertensive medications. Another elimination criterion was to decide to leave the study. Trained research staff provided to participants detailed instructions of the study, including bread storage recommendations (store at 4°C for one week at most) and

assisted them in completing questions on dietary information. Table 6 describes the characteristics of participants. The dietary intervention consisted of daily consuming a 30 g slide of enriched bread (BF) or control bread (CN) during 3 months with a weekly follow-up of the participants. During the weekly interviews, a dietary survey was carried out and it was checked that the participants did not fall into elimination criteria. Every 4 weeks of daily consumption of BF or CN, a blood sample was obtained from each participant in order to measure biochemical parameters using IDEXX Catalyst Dx® equipment (Madrid, Spain). Anthropometric parameters were also measure every 4 weeks with a digital weighing scale and a measuring tape. The study began with 156 participants after data cleaning (particularly for poorly completed dietary data) and all participants finished the study (n=156).

Table 6. Characteristics of study population at recruitment.

Characteristics	n	(%)	Observations
Participants	156	(100)	
Sex			
Men	72	(46.1)	
Women	84	(53.8)	
Education Level (completed or in progress)			According to the information provided in the surveys conducted.
Primary School	2	(1.28)	
High School	15	(9.61)	
University	139	(89.1)	
Current smokers	58	(37.1)	
Physical activity			According to the information provided in the surveys conducted.
Inactive	47	(30.1)	
Moderate active	81	(51.9)	
Active	28	(17.9)	
Obesity (BMI range)			The classification was made according to the world health organization (WHO)
Underweight (Below 18.5)	0	(0.00)	
Normal weight (18.5–24.9)	21	(13.4)	
Pre-obesity (25.0–29.9)			
Obesity class I (30.0–34.9)	52	(33.3)	
Obesity class II (35.0–39.9)	64	(41.0)	
Obesity class III (Above 40)			
	19	(12.1)	
	0	(0.00)	

Risk Factors			
Hypertension	27	(17.3)	Defined as systolic blood pressure ≥140mmHg and/or diastolic blood pressure ≥90mmHg.
Diabetes	32	(20.5)	Having fasting plasma glucose ≥120 mg/dl (≥7 mmol/l).
Dyslipidemia	77	(49.3)	Defined as having at least one of the following anomalies: total cholesterol ≥190 mg/dl (≥4.9 mmol/l), Triglycerides ≥150 mg/dl (≥1.7 mmol/l), LDL-cholesterol ≥115 mg/dl (≥3.0 mmol/l), HDL-cholesterol <40 mg/dl for men and <46 mg/dl for women.

3.2.8 Genotoxicity in buccal epithelium cells.

Genotoxicity in buccal epithelium cells was measured in each participant by micronuclei assay as reported before [91]. Briefly, buccal cavity cells are obtained by scraping the cheeks with a tongue depressor. The samples were transferred dropwise to pre-cleaned slides. Next, the slides were air-dried and fixed with 80% methanol and were stained with hematoxylin for 3-5 minutes and then with eosin for 5-8 minutes. Genotoxic damage was determined by the observation of the slides in a Zeiss Primo Star microscope (Zeiss Mexico, NL, Mexico); the cellular malformations were observed and counted.

3.2.9 Protective effect of (-)-epicatechin and quercetin in Caco-2 cells.

The cell line of human colorectal adenocarcinoma epithelial cell line (Caco-2) was purchased from the American Type Culture Collection (ATCC ® HTB-37 ™), was cultured with 10% fetal bovine serum (Sigma-Aldrich, MO, USA) and supplemented with minimum essential media (Sigma-Aldrich, MO, USA).

The cells were kept in a humid atmosphere with 5% CO2/air, and the culture media was changed every 48 or 72 hours. When the confluence was observed between 70-80%, the cultured cells were recultivated on glass coverslips. The Caco-2 cells were infected with an adenoviral vector with the kit AdEasy-Adenoviral Vector System (Agilent Technologies, CA, USA), by adding a 1:500 virus dilution. Two days later, cells were ready for HyPer imaging. The coverslips were mounted in an open recording chamber, and the culture medium was replaced with HEPES-KRH Buffer (in mM: 140 NaCl, 4.7 KCl, 20 Hepes, 1.25 MgSO4 and 1.25 CaCl2, pH 7.4), supplemented with 5 mM glucose.

Cells were inoculated with FURA-2 probe, a fluorescent dye that binds to the intracellular $Ca2+$ and allows us to monitor it. The images were obtained in the inverted microscope (Nikon Ti Eclipse microscope, Nikon Products, Melville, NY, USA); technical adjustments were made using the micromanager software. Two wavelengths were used (420 and 490 nm). To obtain a radiometric measurement, the exposure time was the same for each excitation channel and constant throughout the experiment.

The fluorescence of any individual cell was quantified by drawing the same ROI (region of interest) in images derived from both channels. Both excitation wavelengths were acquired every 20 s and were expressed as a ratio (490 nm / 420 nm). For each record, an initial lapse of 20 minutes was made to guarantee a reliable baseline, called the baseline value. Then, a concentration of 5-10 μM of the flavonoid of interest (quercetin and (-)-epicatechin) was added, and we let the record run for 15-20 minutes. Subsequently, two consecutive pulses of H2O2 were applied.

The first was 50 μM; enough to evoke a moderate and transient increase in the HyPer relationship. The second was a saturation pulse of 500 μM H2O2, useful to determine the maximum response of HyPer. Finally, it was added an ionophore (ionomycin) as a positive control (calcium increase). Data were obtained from 26 experiments of each treatment in Caco-2 cells monitored for 80 minutes each.

3.3 Statistical analyses

Data were analyzed by using MiniTab 18 and GraphPad Prism (GraphPad Software Inc., CA, USA) softwares. Results are reported as mean ± SEM. Statistical differences for mean data obtained from present study were analyzed by two-way ANOVA. Correlation was made using MiniTab (MiniTab18 General Linear Model).

3.4 Results

3.4.1 Effect of adding (-)-epicatechin and quercetin (1:1) on baking performance and bread features.

The weight, height, volume, color and sensory evaluation of BF and CN were performed (Table 7). The addition of 0.05% of a mixture of (-)-epicatechin and quercetin in 1:1 proportion to whole wheat flour did not significantly affect baking properties. However, there was a significant difference in color; the bread containing the mixture of flavonoids (BF) had lower L* (luminosity), b* (yellowness) and a* (greenness) values ($p<0.005$) (Table 7, Figure 6).

Table 7. Effect of the addition of (-)-epicatechin and quercetin on baking properties and physical characteristics of the bread.

Attribute	Control bread (CN)	Bread with 0.05% mixture of (-)-epicatechin and quercetin in 1:1 proportion.
Baking properties		
Dough water absorption (%)	70.12 ± 3.1	71.01 ± 3.5
Dough mixing time (min)	4.56 ± 0.3	4.72 ± 0.4
Proof height (cm)	7.9 ± 0.3	7.9 ± 0.4
Bread height (cm)	8.3 ± 0.4	8.2 ± 0.3
Oven spring (cm)[1]	0.4 ± 0.1	0.3 ± 0.1
Bread weight (g)	152.1 ± 0.9	151.6 ± 1.2
Bread volume (cm^3)	663.2 ± 27.3	658. 3 ± 15.2
Apparently density (g/cm^3)	0.229 ± 0.1	0.230 ± 0.1
Crumb color		
*a**	4.38 ± 0.42	**3.14 ± 0.36†**
*b**	20.31 ± 0.53	**15.58 ± 0.42†**
*L**	67.21 ± 2.1	**55.01 ± 2.00†**
Sensory Evaluation[2]		
Color	3.21 ± 1.01	4.61 ± 0.91
Texture	3.5 ± 1.12	3.2 ± 1.01
Flavor	4.56 ± 1.31	4.38 ± 1.25
Odor	3.81 ± 1.15	3.85 ± 1.07
Overall acceptability	4.32 ± 1.01	4.56 ± 1.21

[1] Oven spring = bread height – proof height, [2] Measured on the scale of 1 to 5, where 1 = dislike very much and 5 = like very much. *a*, b** and *L** are the components of color according to the CIE International Commission on Illumination. † Parameters with statistical significance between CN and BF ($p<0.005$), mean comparison was performed with Student's T-test.

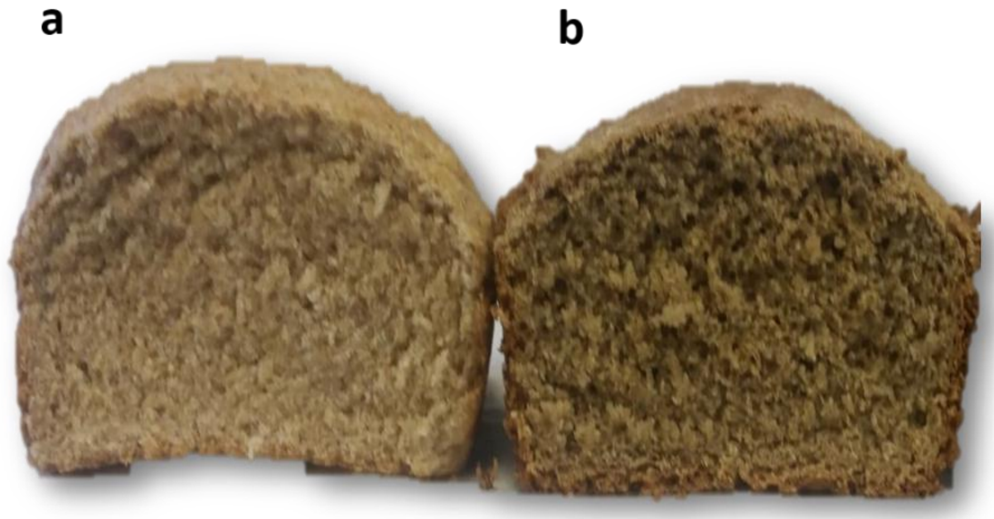

Figure 6. Appearance of (**a**) Control whole wheat bread (CN); (**b**) bread enriched with 0.05% of a mixture of (-)-epicatechin and quercetin in a proportion of 1:1 (BF).

3.4.2 Flavonoid content and shelf life of breads.

Flavonoid content, RH and Aw were measured in both bread samples (CN and BF) during 15 days (Figure 6). RH and Aw had no statistical differences between CN and BF. Nevertheless, flavonoid content was better retained ($p<0.005$) at lower temperatures. At storage temperature of 40°C, flavonoid content started significantly decreasing at the fourth day of storage, retaining 70% of total flavonoids approximately; and, on the seventh day of storage, bread BF had lost 100% of the added flavonoids. At storage temperature of 25°C, flavonoid content started significant declining at the eighth day ($p<0.005$), and the totality of flavonoid content was lost approximately at the fifteenth day. Meanwhile, at 4°C of storage, flavonoid content started significant decreasing at the eleventh day ($p<0.005$), and at the fifteenth day BF had retained approximately 36% of the initial flavonoid content.

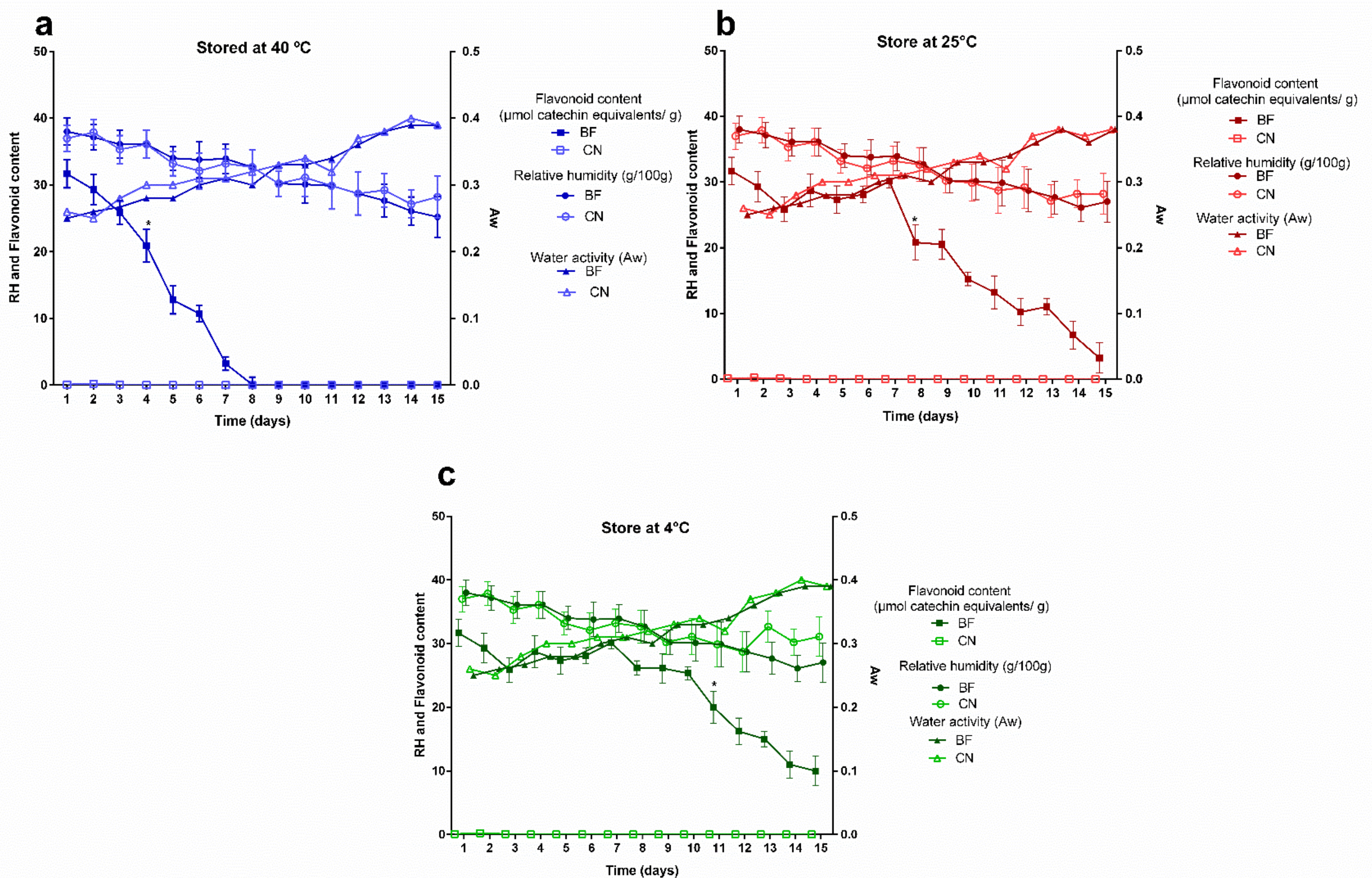

Figure 7. Flavonoid content, relative humidity (RH) and water activity (Aw) were measured during 15 days of storage. (a) Parameters of bread samples stored at 40°C are presented in blue, (b) those stored at 25°C are presented red and (c) at 4°C in green. Samples of control breads (CN) are the figures only outlined in the corresponding color, while samples of enriched breads with 0.05% of a mixture of (-)-epicatechin and quercetin (1:1) (BF) are represented with colored figures. *Time when flavonoid content started to decrease significantly ($p<0.005$).

3.4.3 Microbiological study.

Table 3 shows the results of the microbiological study in bread samples (CN and BF) stored at 4°C, 25°C and 40°C. There were no significant differences between CN and BF in the counted colony-forming unit (CFU) and number of fungi and yeast (N°HL). However, there were significant differences due to storage temperature (4°C, 25°C and 40°C). Figure 7 shows representative images of samples that were evaluated for the microbiological test at different temperatures.

Table 8. Microbiological effect of storage temperature on bread samples.[1]

4°C								
Dilution	DAY 1		DAY 6		DAY 12		DAY 15	
	CFU	N°HL	CFU	H/L	CFU	N°HL	CFU	N°HL
$1x10^{-1}$	ND	ND	ND	ND	15 ± 3	5.5 ± 1.0	416 ± 3	61.8 ± 1.0
$1x10^{-2}$	ND	ND	ND	ND	5 ± 1.2	ND	58 ± 1.2	21.3 ± 2.1
$1x10^{-3}$	ND	ND	ND	ND	2	ND	8.7 ± 3.1	ND
$1x10^{-4}$	ND	ND	ND	ND	ND	ND	1 ± 1.2	ND

25°C								
Dilution	DAY 1		DAY 6		DAY 12		DAY 15	
	CFU	N°HL	N°HL	N°HL	CFU	N°HL	CFU	N°HL
$1x10^{-1}$	ND	10 ± 2.6	10 ± 2.6	67 ± 6.2	182 ± 21.0	316 ± 29.1	819 ± 52.1	1276± 123.9
$1x10^{-2}$	ND	3 ± 0.21	3 ± 0.21	13 ± 3.4	100 ± 17.2	87.9 ± 12	310 ± 35.1	173 ± 11.9
$1x10^{-3}$	ND	ND	ND	1.2 ± 0.8	24 ± 2.9	16.8 ± 13	68.5 ± 4.3	27.98 ±7.5
$1x10^{-4}$	ND	ND	ND	ND	6.3 ± 0.9	1.3 ± 0.9	24.5 ± 3.7	12.6 ± 1.5

40 °C								
Dilution	DAY 1		DAY 6		DAY 12		DAY 15	
	UFC	N°H/L	UFC	N°H/L	UFC	N°H/L	UFC	N°H/L
$1x10^{-1}$	ND	ND	100 ± 31.1	56.2 ± 3.2	781 ± 22.1	418.7 ± 21.3	781 ± 22.1	4821.9 ± 94.7
$1x10^{-2}$	ND	ND	41 ± 9.7	20.2 ± 4.2	125 ± 19.2	110.1 ± 9.7	125 ± 19.2	512.5 ± 29.5
$1x10^{-3}$	ND	ND	18 ± 13.5	8.1 ± 0.3	67.3 ± 6.5	50.2 ± 10.2	67.3 ± 6.5	123.8 ± 10.9
$1x10^{-4}$	ND	ND	6.4 ± 8.7	1 ± 0.06	31.1 ± 3.2	6.8 ± 16.9	31.1 ± 3.2	79.8 ± 21.9

[1] There were no significant differences between bread enriched with a mixture of (-)-epicatechin and quercetin (1:1) and the control bread ($p<0.005$), experiments were performed in triplicate and it is showed the mean ± std. ND= No detectable.

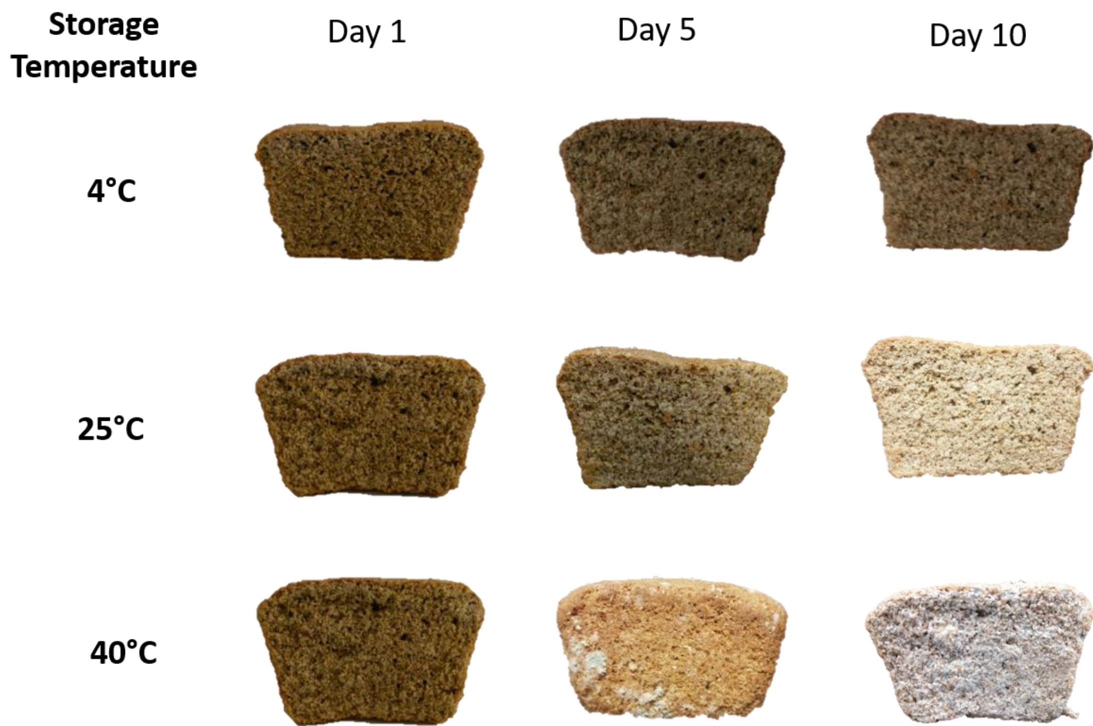

Figure 8. Effect of 3 storage temperatures (4°C, 25°C and 40°C) in breads enriched with 0.05% of a mixture of (-)-epicatechin and quercetin in 1:1 proportion. No differences in bread appearance were found compared with control.

3.4.4 Changes in measured parameters in the participants who consumed the enriched bread (BF).

Dietary, anthropometric and biochemical variables measured in the present studied are reported in Table 9. Dietary and anthropometric variables did not significant change by the consumption of BF in the participants. However, in several biochemical variables, such as: total cholesterol, LDL-cholesterol, total triglycerides and fasting plasma glucose ($P<0.005$), there was a significant improvement after 3 months of daily consumption of the enriched bread (BF), observed by lower values in these biochemical parameters in comparison with their values at the beginning of the study.

Table 9. Dietary, anthropometric and biochemical variables before and after 3 months of consumption of control bread or bread enriched with (-)-epicatechin and quercetin.

Variables	Intervention group	Beginning of the study Mean ± SD	End of the Study Mean ± SD
Age	BF	35.2 ± 3.4	35.6 ± 3.1
	CN	33.6 ± 3.5	34.1 ± 2.6
Participants	BF	78	78
	CN	78	78
Dietary variables			
Fruit and vegetable intake (g/d)	BF	473.9 ± 178.2	478.2 ± 187.2
	CN	475.8 ± 126.5	477.2 ± 153.2
Total energy intake (kJ/d)	BF	2210 ± 331	2220 ± 316
	CN	2208 ± 351	2210 ± 321
Total carbohydrate (%E)	BF	48.9 ± 7.2	47.8 ± 8.9
	CN	46.8 ± 9.3	51.1 ± 4.2
Added sugar	BF	8.3 ± 3.2	7.9 ± 3.5
	CN	8.2 ± 4.1	8.8 ± 3.5
Total Fat (%E)	BF	32.3 ± 6.1	35.3 ± 6.7
	CN	34.6 ± 6.3	36.2 ± 7.3
Saturated fat (%E)	BF	18.8 ± 6.1	16.9 ± 5.9
	CN	19.2 ± 5.1	20.3 ± 3.4
Unsaturated fat (%E)	BF	17.6 ± 5.8	22.8 ± 5.6
	CN	18.5 ± 2.9	20.1 ± 3.9
Anthropometric variables			
BMI (Kg/m2)	BF	32.9 ± 2.8	31.1 ± 2.2
	CN	31.4 ± 3.2	31.2 ± 2.5
Waist Circumference (cm)	BF	101.7 ± 3.5	99.4 ± 3.2
	CN	104.2 ± 4.1	103.8 ± 3.1
Biochemical variables			
Total Cholesterol (mg/dl)	BF	223.3 ± 16.7	**206.2 ± 15.7***
	CN	224.1 ± 19.3	220.4 ± 15.4
LDL-Cholesterol (mg/dl)	BF	149.72 ± 14.4	**123.5 ± 21.1***
	CN	148.23 ± 18.1	144.9 ± 23.4
HDL-Cholesterol (mg/dl)	BF	40.5 ± 12.8	45.2 ± 8.9
	CN	40.4 ± 10.1	42.1 ± 10.2
Triglycerides (mg/dl)	BF	218.34 ± 21.9	**164.73 ± 19.25***
	CN	213.7 ± 23.6	214.91 ± 20.1
Fasting plasma glucose (mg/dl)	BF	118.03 ± 12.71	**101.23 ± 13.25***
	CN	119.31 ± 10.73	120.67 ± 10.9
HbA1c (%)	BF	5.1 ± 1.0	4.4 ± 1.1
	CN	5.7 ± 1.0	5.7 ± 0.9
Systolic blood pressure (mmHg)	BF	139.2 ± 12.3	127.8 ± 12.3
	CN	139.3 ± 21.5	138.9 ± 17.1
Diastolic blood pressure (mmHg)	BF	87.26 ± 12.8	84.31 ±10.14
	CN	87.13 ± 10.13	86.02 ± 9.49

BF= bread enriched with 0.5% of a mixture of (-)-epicatechin and quercetin (1:1), CN = control bread. *P values for testing the differences among variables across two groups (before and after dietary intervention) by using $\chi 2$ test ($P<0{\cdot}05$).

3.4.5 Frequency in nuclear abnormalities in buccal epithelial cells

Next, we evaluated if the consumption of the enriched bread (BF) diminish genotoxicity in buccal epithelium cells. The abnormalities of the nuclei in the buccal epithelial cells at the beginning of the study were 15.8 ± 3.2 %, without statistical differences between groups consuming BF (Figure 9a) or CN (Figure 9b). Participants showed abnormalities of broken egg nucleus, micronucleus and binucleus (Figure 9c). After the dietary intervention with BF, participants significant decreased abnormalities in buccal epithelial cells (8.3 ± 1.0%, $p<0.05$). Interestingly, fasting plasma glucose (mg/dl) and LDL-Cholesterol (mg/dl) showed significant correlation ($p<0.0001$) with nuclear abnormalities in buccal epithelial cells (Figure 9d and 9f). In Figure 5, is showed the comparison of biochemical parameters, waist circumference and nuclear abnormalities in buccal epithelial cells of participants that consumed control bread and participants that consumed bread enriched with 0.05% of a mixture of (-)-epicatechin and quercetin (1:1), at the beginning of the study and at the end of the study.

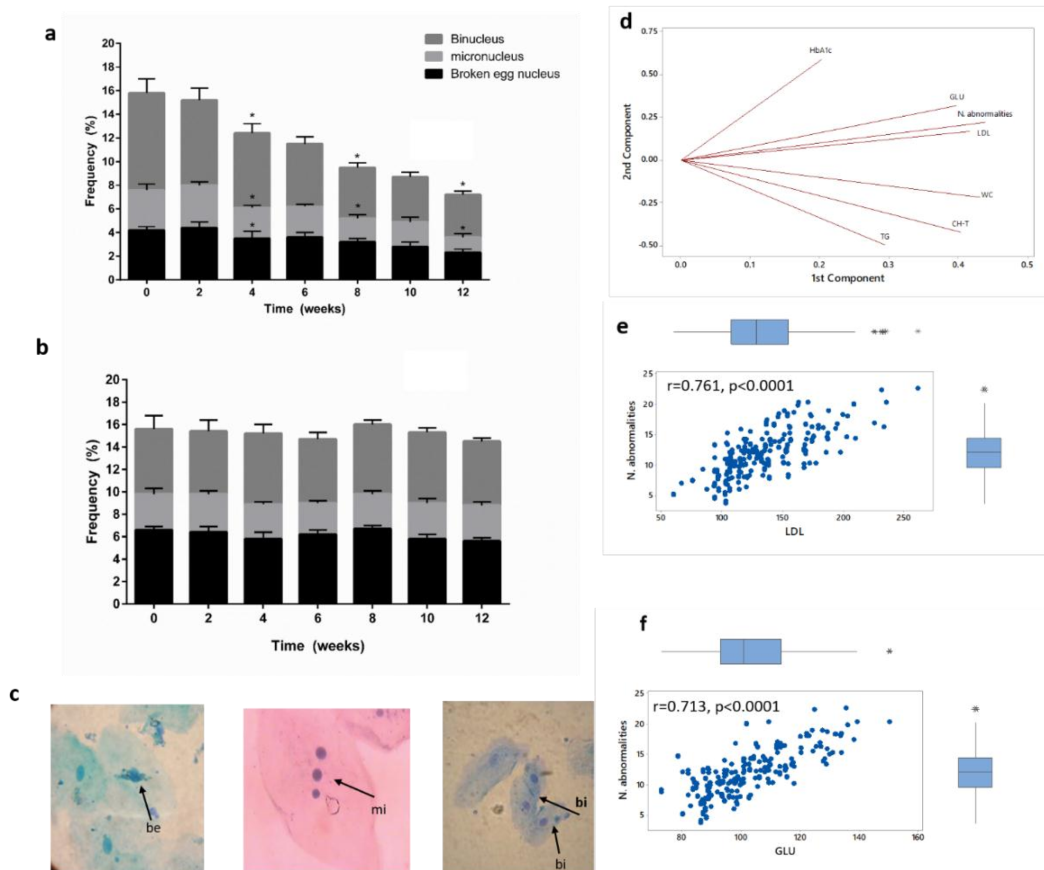

Figure 9. Frequency in nuclear abnormalities in buccal epithelial cells and its correlation with biochemical parameters. (a) The frequency of nuclear abnormalities in participants consuming bread enriched with 0.5% of (-)-epicatechin and quercetin (1:1). (b) The frequency of nuclear abnormalities in participants consuming control bread. (c) The most observed abnormalities were broken egg (be), micronucleus (mi) and binuclear cells (bi). (d) A component analysis was made and the variable most correlated with nuclear abnormalities were (e) LDL-Cholesterol (LDL) and (f) Fasting plasma glucose (GLU). * Significant decrease of abnormalities in buccal epithelial cells of participants compared to the previous week (P<0.005).

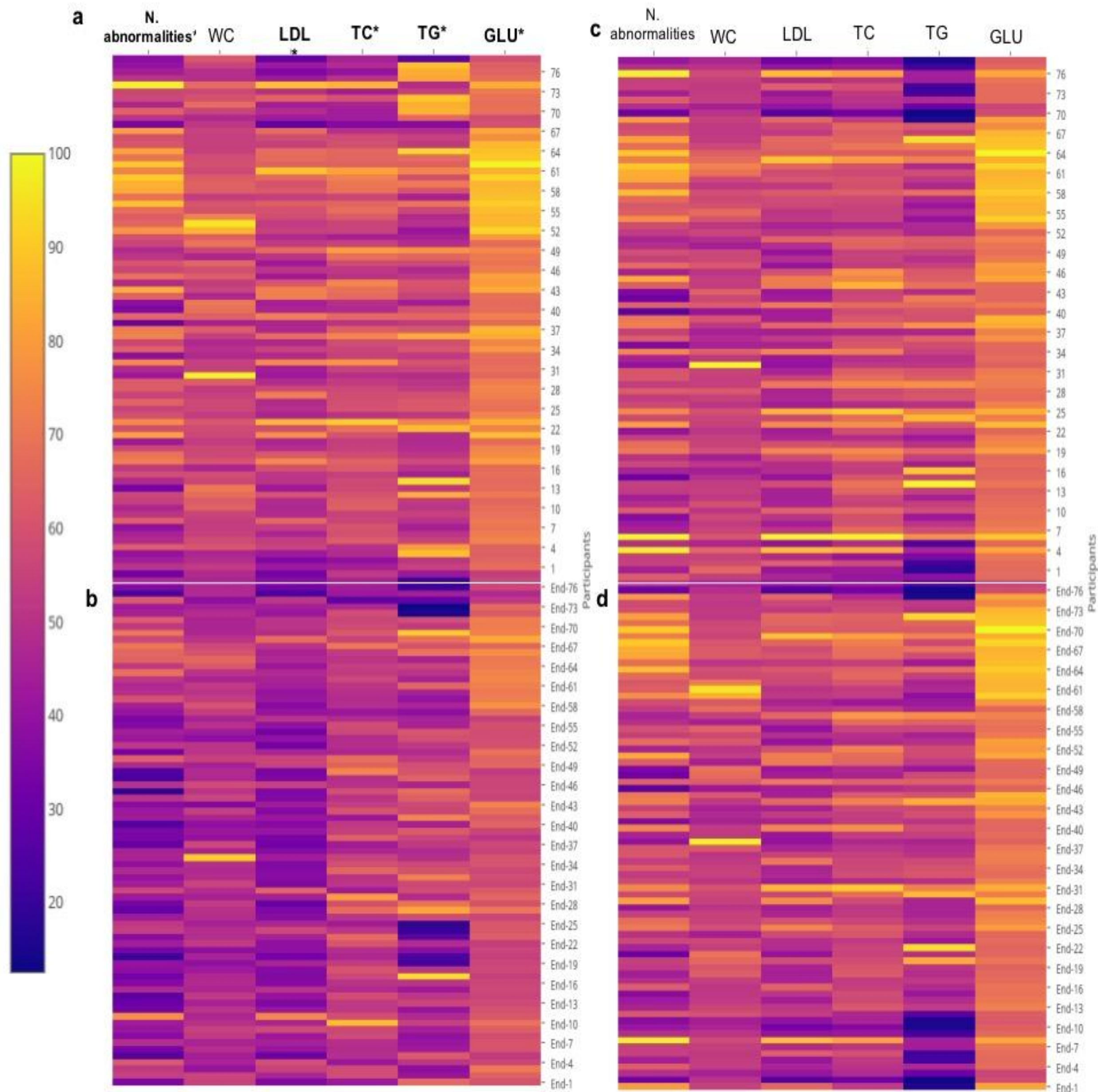

Figure 10. Comparison of relevant variables and nuclear abnormalities of buccal epithelial cells. It is Showed the parameters of the participants who consumed bread enriched with 0.5% of a mixture of -(-) epicatechin and quercetin (1:1), (a) at the beginning of the study and (b) after 3 months of daily

consumption. Also are presented (c) the initial and (d) final parameters of participants who consumed control bread during 3 months. The more yellow colors represent higher values, while the bluer colors represent smaller values

3.4.6 Protective effect of (-)-epicatechin and quercetin in Caco-2 cells.

Finally, to determine a possible cause of the decreased genotoxicity, we investigated their protective effect by monitoring [Ca2+]i in Caco-2 cells after a stimulus with quercetin (Que), (-)-epicatechin (Epi) or the mixture of both flavonoids in 1:1 proportion (Epi + Que); cells were then stimulated with two concentrations of H2O2 (50 and 500 µM) (Figure 6). The minimum concentration at which a protective effect was observed was 10µM, which represents 1/3 of the concentration of flavonoids in enriched bread (BF) (Figure 6). Addition of quercetin, (-)-epicatechin or the mixture of both flavonoids (Epi + Que) reduces significantly [Ca2+] i (Figure 11a). A subsequent stimulus with 500 µM of H2O2 generates [Ca2+] i overload, even in cells treated only with quercetin or (-)-epicatechin. However, cells pretreated with a combination of quercetin and (-)-epicatechin there was no significant [Ca2+] i overload compared to calcium baseline. Examples of the different treatments are represented in Figures 11b, 11c and 11e.

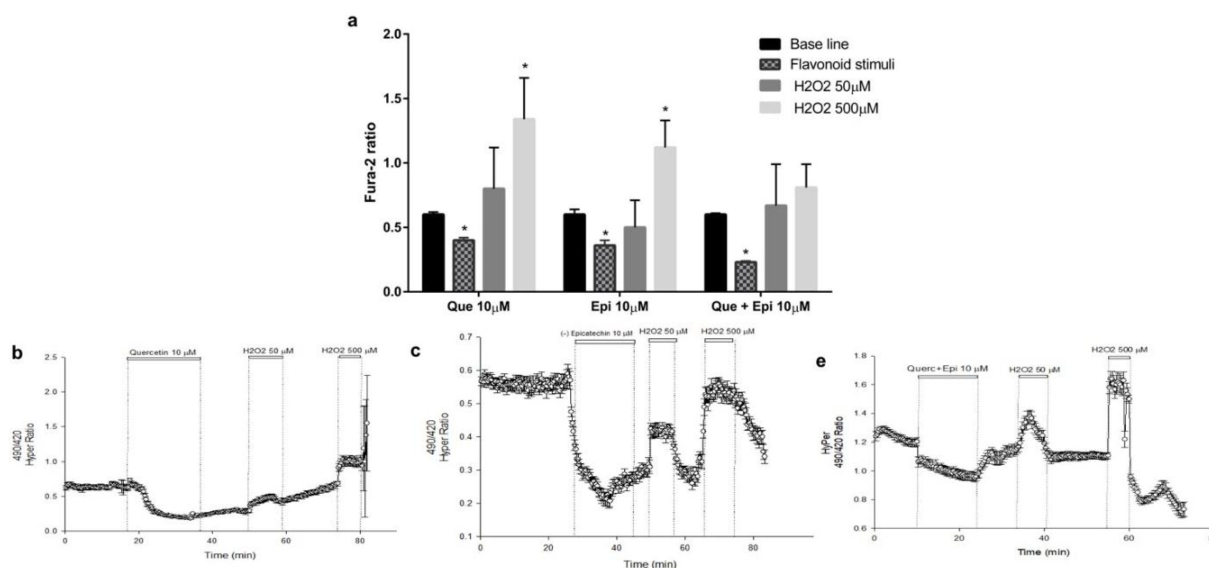

Figure 11. Intracellular calcium (Ca+2) behavior in Caco-2 cells after stimulus with 10 µM of quercetin (Que), (-)-epicatechin (Epi) or the mixture of both flavonoids in 1:1 proportion (Epi + Que), then stimulated

with H_2O_2 (50 and 500 μM). (a) Intracellular calcium determined by Fura-2 in Caco-2 cells without treatment (base line), after flavonoid stimuli (Que, Epi or Que + Epi), and stimulated with H_2O_2 (50 μM) and H_2O_2 (500 μM). (b) Representative graph of Ca^{2+} fluxes stimulated with Que (10 μM) followed by a stimulus of H_2O_2 (50 and 500 μM). (c) Representative graph of Ca^{2+} fluxes stimulated with Epi (10 μM) followed by a stimulus of H_2O_2 (50 and 500 μM). (e) Representative graph of Ca^{2+} fluxes stimulated with a mixture of Que + Epi (10 μM) followed by a stimulus of H_2O_2 (50 μM and 500 μM). *Significant differences $p<0.005$.

3.5 Discussion

3.5.1 Effect of added flavonoids on baking performance and bread features.

Cereal-based products are consumed as an important part of daily diet worldwide. Thus, is an important product of interest to be successfully used as a vehicle for the delivery of bioactive compounds that helps in the prevention of chronic diseases [101].

In the present work we made a whole wheat bread enriched with 0.05% of (-)-epicatechin and quercetin (1:1), and we evaluated bread features and bioactivity. Baking properties did not show significant differences between control bread (CN) and enriched bread (BF). However, as expected, sensory evaluation and color test showed that color significantly changed due to the addition of flavonoids ($p<0.005$). Bread containing flavonoids had lower L* (luminosity), b* (yellowness) and a* (greenness) values. It has been previously reported that the addition of polyphenols and flavonoids in bread significant decreases this values producing darker crumbs [102]. Participants that performed the sensory analysis did not notice significant differences between control bread and enriched bread in texture, flavor and odor. Thus, the overall acceptability of both breads (CN and BF) was similar.

The shelf-life of bread is limited by bread texture and microbial growth. Relative humidity (RH) and water activity (Aw) behavior were similar in both breads (CN and BF) during storage at different temperatures (40°C, 25°C and 4°C). Nevertheless, as shown in Figure 6, flavonoid content significantly decreased during storage, and the higher the storage temperature the greater the loss of flavonoid content over time ($p<0.005$). Similarly, it has been reported that the content of tocopherols and the antioxidant capacity during storage decreases in cereal grains, and that it could be an indicator of changes induced by

oxidation [102]. However, information on the stability of phytochemicals during storage in processed cereal-based food is limited. In fresh foods, the content of these compounds varies by the still active metabolism of the food, and these flavonoids are usually linked to the matrix of fresh food [103]. The decreasing concentration of flavonoid content observed in the present work could be related to oxidation of food products and/or microbial metabolism. Bacteria (CFU), fungi and yeast (N°HL) were observed after 9 days of storage at 4°C, 6 days at 25°C and 3 days at 40°C, also it was similar in both bread samples (CN and BF). The load of microorganism in breads (CN and BF) during the storage time could have been influenced by the lack of conservatives in formulation. Microbiological test was able to detect bacteria, fungi and yeast before being appreciated macroscopically in bread samples (Figure 7). Also, microbiological test did not showed differences in the load of microorganisms during storage between BF and CN. After these results we determined that the participants had to store the bread at 4ºC for a time not greater than 7 days.

3.5.2 Effect of flavonoids in its consumption in enriched bread and on *in-vitro* bioactivity.

Participants who consumed enriched bread (BF) for 3 months, significantly decreased the biochemical parameters of total cholesterol, LDL-Cholesterol, triglycerides and fasting plasma glucose ($p<0.005$), compared with participants that consumed control bread (CN). It has been reported that flavonoids could modulate lipid homeostasis through the regulation of LXR via AMPK phosphorylation [104]. Furthermore, data in the present work suggested that the improvement in biochemical parameters could also be related to genoprotective effect of flavonoids, since we found that in the group that consumed the enriched bread, the frequency of nuclear abnormalities in buccal epithelial cells (Figure 9) decreased significantly, from 15.8 ± 3.2 % to 8.3 ± 1.0% ($p<0.005$). The decreasing frequency of nuclear abnormalities was significant correlated to LDL-Cholesterol levels ($r=0.761$, $p<0.0001$) and fasting plasma glucose levels ($r=0.713$, $p<0.0001$). Previous studies have also showed the correlation between frequency of nuclear abnormalities and levels of LDL-Cholesterol and fasting plasma glucose [105]. This might be the result of

flavonoids increasing the antioxidant activity in the cells of the participants or regulating other important cellular process, like [Ca2+] i dyshomeostasis [106,112].

Data in the present study indicated that (-)-epicatechin, quercetin and the combination of both, were able to minimize the toxic effect induced by H_2O_2 at a concentration of 50μM, since there was no significant difference between [Ca2+] i at base line compared with the [Ca2+] i after stimulus with 50μM of H_2O_2. Interestingly, both flavonoids together (1:1 proportion) could modulate the stimulus caused by 500μM of H_2O_2, preventing [Ca2+] i increase; whereas each flavonoid alone, in the same concentration, could not modulate these effect ($p<0.005$). This suggests that it could be a synergistic effect of both flavonoids in grater challenges, which could not be modulated by each flavonoid by itself. Similarly, it has been previously reported the protective effect from non-physiological [Ca2+] increase due to natural products of olive pomace [106-108]. The increase of the intracellular calcium signal is closely linked with ROS production [108]. Authors suggested that calcium ions could accumulate in the nuclei of cells, leading to the formation of micronuclei and other damages in the DNA [109]. Also, increased levels of calcium in the nucleus could cause inhibition of the DNA repair function, which is also related with the formation of micronuclei, as well as the induction of mitotic alterations [109,110]. Thus, the consumption of the enriched bread (BF) could significantly modulate [Ca2+] i dyshomeostasis caused by chronic inflammation that characterizes participants with risk factors of metabolic syndrome increasing DNA damage, and therefore their nuclear abnormalities that we observed in buccal epithelial cells. Finally, 3 months were enough to see a significant effect on biochemical parameters; it was half the time that took to see a similar effect due to (-)-epicatechin alone in dark chocolate [91,111].

3.6 Conclusions

We evaluate the effect of adding (-)-epicatechin and quercetin (1:1) in bread at 0.05%. Baking performance and bread features were evaluated, including sensory analysis. Only crumb color parameters (L*, b*, and a*) were significant decreased ($p<0.005$) by flavonoids addition. Moreover, a study of changes in bread was made in 3 storage temperatures (4°C, 25°C and 40°C). There were no significant differences in control bread

and bread enriched with 0.05% with flavonoids. However, the concentration of flavonoids significantly started to decrease early during storage with a higher storage temperature. The decreasing concentration of flavonoid content observed could be related with oxidation of food products and/or microbial metabolism.

Regarding to the designed pilot trial, participants that consumed bread enriched with flavonoids (0.05%) significant decreased several biochemical parameters (total cholesterol, LDL-Cholesterol, triglycerides and fasting plasma glucose) related with metabolic syndrome, compared with participants that consumed control bread. In addition, there was a significant decrease ($p<0.05$) on the frequency of nuclear abnormalities of buccal epithelial cells of participant that consumed enriched bread compared with those who consumed control bread. Furthermore, only 3 months of daily consumption of BF were need to observed significant changes in biochemical parameters. Data in the present work suggest that beneficial effects of flavonoids could also be related to genoprotective effect of flavonoids due to regulation of non-physiological $[Ca2+]i$ increase, and that (-)-epicatechin and quercetin could be performing the effect synergistically.

CAPÍTULO IV:

FINAL CONCLUSIONS

CONCLUSIONS

- ❖ Flavonoids have been related to important health benefits and have also been shown to act through different mechanisms of action to improve the body's homeostasis.
- ❖ Among the main effects are those attributed to the prevention and treatment of chronic diseases. In cardiac diseases, there are flavonoids such as quercetin, epicatechin, rutin, and luteolin, which increase the production of new mitochondria in the heart.
- ❖ Also, they have antiatherosclerotic effects and platelet function. In diabetes, luteolin, kaempferol, chrysin, and galangin, have direct action in the synthesis and release of insulin and have a role at the level of glucose transporters, decreasing their concentration.
- ❖ Flavonoids also have antioxidant effects such as free radical scavenging and neutralization of some reactive oxygen and nitrogen species. In the case of cancer and inflammation-related diseases, these compounds exert anti-inflammatory activity by inhibiting pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase, and NO synthase, also some flavonoids such as apigenin are involved in regulation of genes that have to do with apoptosis.
- ❖ The use of flavonoids in the design of nutraceuticals and functional foods has been growing in recent years. Day after day more properties are attributed to the flavonoids.
- ❖ Flavonoids of cocoa had protective effects against DNA damage. This suggested that the reduction in genotoxic stress effect was related with the antioxidant activity of flavonoids and the modulation of CYP450. The modulation of CYP450 and the reduction in the expression of some interleukins have been related with the anti-inflammatory effect of flavonoids.
- ❖ The antioxidant capacity of flavonoids contained in dark chocolate could be related to the decrease of cellular stress, and hence, to the DNA protection in the nucleus of cell. However, further in vivo studies are still needed to determine their mechanism of action of antigenotoxic effect. Biochemical parameters (total cholesterol, triglycerides, and LDL-cholesterol level in blood) and anthropometrical parameters (waist circumference) were also improved after six months of dark chocolate with a 70% of cocoa intake. Interestingly daily flavonoid-rich chocolate intake also improves fasting plasma glucose levels and insulin resistance parameter (HOMA-IR). These effects

were attributed to the proportion of flavonoids in the chocolate which was 3-fold greater than in the milk chocolate. Together, these results suggested a potential beneficial effect as a consequence of the daily dark chocolate consumption in the lipid and glucose metabolism.

- We evaluate the effect of adding (-)-epicatechin and quercetin (1:1) in bread at 0.05%. Baking performance and bread features were evaluated, including sensory analysis. Only crumb color parameters (L*, b*, and a*) were significant decreased ($p<0.005$) by flavonoids addition. Moreover, a study of changes in bread was made in 3 storage temperatures (4°C, 25°C and 40°C). There were no significant differences in control bread and bread enriched with 0.05% with flavonoids.
- The concentration of flavonoids significantly started to decrease early during storage with a higher storage temperature. The decreasing concentration of flavonoid content observed could be related with oxidation of food products and/or microbial metabolism.
- Regarding to the designed pilot trial, participants that consumed bread enriched with flavonoids (0.05%) significant decreased several biochemical parameters (total cholesterol, LDL-Cholesterol, triglycerides and fasting plasma glucose) related with metabolic syndrome, compared with participants that consumed control bread. In addition, there was a significant decrease ($p<0.05$) on the frequency of nuclear abnormalities of buccal epithelial cells of participant that consumed enriched bread compared with those who consumed control bread.
- Only 3 months of daily consumption of BF were need to observed significant changes in biochemical parameters. Data in the present work suggest that beneficial effects of flavonoids could also be related to genoprotective effect of flavonoids due to regulation of non-physiological [Ca2+] i increase, and that (-)-epicatechin and quercetin could be performing the effect synergistically.

CAPÍTULO V:

FUTURE WORK

FUTURE WORK

- ❖ We intend to continue working on the improvement of functional foods and also in the search for new properties of flavonoids.
- ❖ A good start would be to evaluate the effect of ingredients or functional foods in the microbiota.
- ❖ Also, try other models of mammalian cells and a model of complete organism like Drosophila melanogaster, for example.
- ❖ We also intend to be able to evaluate how our functional ingredients behave, specifically the flavonoids, when loaded in different pharmaceutical forms, such as microparticles or nanoparticles.

CAPÍTULO VI:

REFERENCES

1. Panche AN, Diwan AD, Chandra SR (2016) Flavonoids: An overview. J Nutr Sci 5, doi:10.1017/jns.2016.41.
2. Kozłowska A, Szostak-Wegierek D (2014) Flavonoids- Food sources and health benefits. Rocz Panstw Zakl Hig 65(2),79-85.
3. Shashank K, Pandey AK (2013) Chemistry and biological activities of flavonoids. Sci World J 2013, 533–548.
4. Fernandes I, Pérez-Gregorio R, Soares S, Mateus N, de Freitas V (2017) Wine flavonoids in health and disease prevention. Molecules 14(22),2.
5. Galleano M, Oteiza PI, Fraga CG (2009) Cocoa, chocolate, and cardiovascular disease. J. Cardiovasc. Pharmacol 54, 483–490.
6. Grigalius I, Petrikaite V (2017) Relationship between antioxidant and anticancer activity of trihydroxyflavones. Molecules 7(22)2.
7. Cassidy A, Minihane AM (2017) The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. Am J Clin Nutr 105(1), 10–22.
8. Hollman P, Katan M (1997) Absorption, metabolism and health effects of dietary flavonoids in man. Biomed Pharmacother 51, 305–310.
9. Liu Y, Hu M (2002) Absorption and metabolism of flavonoids in the Caco-2 cell culture model and a perfused rat intestinal model. Drug Metab Dispos 30, 370–377.
10. Walle T (2004) Absorption and metabolism of flavonoids. Free Radic Biol Med 36, 829–837.
11. Hu M (2007) Commentary: Bioavailability of flavonoids and polyphenols: Call to arms. Mol Pharm 4, 803–806.
12. Silva JP, Coutinho OP (2010) Free radicals in the regulation of damage and cell death – basic mechanisms and prevention. Drug Discov Ther 4, 144–167.
13. Stankovic MS, Niciforovic N, Mihailovic V, Topuzovic M, Solujic S (2012) Antioxidant activity, total phenolic content and flavonoid concentrations of different plant parts of Teucrium polium L. subsp. polium. Acta Soc Bot. Pol 81, 117–122.

14. Di Majo D, Giammanco M, La Guardia M, Tripoli E, Giammanco S, Finotti E (2005) Flavanones in Citrus fruit: Structure-antioxidant activity relationships. Food Res. Int. 38, 1161–1166.
15. Álvarez E, Orallo F (2003) Actividad biológica de los flavonoides (II), acción cardiovascular y sanguínea. J. Farmacol. 22, 102–107.
16. Manganaris GA, Goulas V, Vicente AR, Terry LA (2014) Berry antioxidants: Small fruits providing large benefits. J Sci Food Agric 94, 825–833.
17. Lago JHG, Toledo-Arruda AC, Mernak M, Barrosa, K. H et al (2014) Structure-Activity association of flavonoids in lung diseases. Molecules 19, 3570–3595.
18. Cao J, Zhang X, Wang Q, Jia L et al (2012) Influence of flavonoid extracts from celery on oxidative stress induced by dichlorvos in rats. Hum Exp Toxicol 2012, 31, 617–625.
19. Kryc, J, Gebicka L (2013) Catalase is inhibited by flavonoids. Int. J. Biol. Macromol. 2013, 58, 148–153.
20. Ombra MN, D'Acierno A, Nazzaro, F, Riccardi R, et al (2016) Phenolic Composition and Antioxidant and Antiproliferative Activities of the Extracts of Twelve Common Bean (Phaseolus vulgaris L.) Endemic Ecotypes of Southern Italy before and after Cooking. Oxid Med Cell Longev 2016, PMC5220516.
21. Park HY, Choi HD, Eom H, Choi I (2013) Enzymatic modification enhances the protective activity of citrus flavonoids against alcohol-induced liver disease. Food Chem 139, 231–240.
22. Procházková D, Boušová I, Wilhelmová N (2011) Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82, 513–523.
23. Agati G, Azzarello E, Pollastri S, Tattini M (2012) Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci 196, 67–76.
24. Mansuri ML, Parihar P, Solanki I, Parihar MS (2014) Flavonoids in modulation of cell survival signalling pathways. Genes Nutr 9(3),400.
25. Wang JY (2011) DNA damage and apoptosis. Cell Death Differ. 2001, 8, 1047–8.

26. Nicolini F, Burmistrova O, Marrero MT, Torres F, et al (2014) Induction of G2/M phase arrest and apoptosis by the flavonoid tamarixetin on human leukemia cells. Mol Carcinog 53, 939–950.
27. Theurey P, Pizzo P (2018) The aging mitochondria. Genes 9 (9)1.
28. Márquez-Jurado S, Díaz-Colunga J, Das Neves RP, Martinez-Lorente A (2018) Mitochondrial levels determine variability in cell death by modulating apoptotic gene expression. Nat Commun. 389.
29. De Souza RF, De Giovani WF (2004) Antioxidant properties of complexes of flavonoids with metal ions. Redox Rep 9, 97–104.
30. Batra P, Sharma AK (2013) Anti-cancer potential of flavonoids: recent trends and future perspectives. 3 Biotech 3(6),439-459.
31. Busch C, Burkard M, Leischner C, Lauer UM et al (2015) Epigenetic activities of flavonoids in the prevention and treatment of cancer. Clin Epigenetics 7, 1–18.
32. Srivastava S, Somasagara RR, Hegde M, Nishana M (2016) Quercetin, a natural flavonoid interacts with DNA, arrests cell cycle and causes tumor regression by activating mitochondrial pathway of apoptosis. Sci Rep 6, 1–13.
33. Nair M, Mahajan S, Reynolds J, Aalinkeel R, Nair, H., Schwartz, S & Kandaswami, C. The Flavonoid Quercetin Inhibits Proinflammatory Cytokine (Tumor Necrosis Factor Alpha) Gene Expression in Normal Peripheral Blood Mononuclear Cells via Modulation of the NF-κβ System Clin Vaccine Immunol. 2006 13 (3),319-328.
34. Mosquera DMG (2018) Flavonoids from Boldoa purpurascens inhibit proinflammatory cytokines (TNF-α and IL-6) and the expression of COX-2. 3–7.
35. Johnson, J. L.; de Mejia, E. G. Flavonoid apigenin modified gene expression associated with inflammation and cancer and induced apoptosis in human pancreatic cancer cells through inhibition of GSK-3β/NF-κB signaling cascade. Mol. Nutr. Food Res. 2013, 57, 2112–27, doi:10.1002/mnfr.201300307.

36. Chávez-Santoscoy RA, Gutiérrez-Uribe JA, Serna-Saldívar SO (2013) Effect of Flavonoids and Saponins Extracted from Black Bean (Phaseolus vulgaris L.) Seed Coats as Cholesterol Micelle Disruptors. Plant Foods Hum Nutr 68, 416–23.
37. Roberts C, Andrea H, Barnard J (2014) Metabolic Syndrome and Insulin Resistance: Underlying Causes and Modification by Exercise Training. Compr Physiol 3, 1–58.
38. World Health Organization (2012) World Health Statistics Vol. 27.
39. Mendizábal Y, Llorens S, Nava E (2013) Hypertension in metabolic syndrome: Vascular pathophysiology. Int J Hypertens 2013,230868.
40. Berglund L, Brunzell JD, Goldberg AC, Goldberg IJ, et al (2014) Treatment options for hypertriglyceridemia: From risk reduction to pancreatitis. Best Pract Res Clin Endocrinol Metab 28, 423–437.
41. Kris-Etherton P, Hecker KD, Bonanome A, Coval SM, Binkoski AE (2002) Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med 113 Suppl, 71S–88S.
42. Calpe-Berdiel L, Rotllan N, Fiévet C, Roig R et al (2008) Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8. J Lipid Res 49, 1904–11.
43. Chavez-Santoscoy RA, Gutierrez-Uribe JA, Granados O, Torre-Villalvazo I et al (2014) Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. Br J Nutr 112, 886–899.
44. Vendrame S, Daugherty A, Kristo AS, Klimis-Zacas D (2014) Wild blueberry (Vaccinium angustifolium)-enriched diet improves dyslipidaemia and modulates the expression of genes related to lipid metabolism in obese Zucker rats. Br J Nutr 111, 194–200.
45. Um MY, Moon MK, Ahn J, Ha TY (2013) Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. Br J Nutr 109(9),1590-1597.

46. Nicolle E, Souard F, Faure P, Boumendjel A (2011) Flavonoids as Promising Lead Compounds in Type 2 Diabetes Mellitus: Molecules of Interest and Structure-Activity Relationship. Curr Med Chem 18, 2661–2672.
47. Chang L, Chiang SH, Saltiel AR (2005) Insulin Signaling and the Regulation of Glucose Transport. Mol Med 10, 65–71.
48. Pinent M, Blay M, Bladé MC, Salvadó MJ (2004) Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology 145, 4985–4990.
49. Ae Park S, Choi MS, Cho SY, Seo JS et al (2006) Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. Life Sci. 79, 1207–1213.
50. Hiroshi T, Ishizuka M, Terasawa M, Jin-Bin W et al (2004) Effect of green tea on blood glucose levels and serum proteomic patterns in diabetic (db/db) mice and on glucose metabolism in healthy humans. BMC Pharmacol. 2004, 4, 18.
51. Liu D, Zhen W, Yan Z, Carter JD et al (2006) Genistein acutely stimulates insulin secretion in pancreatic β-cells through a cAMP-dependent protein kinase pathway. Diabetes 55, 1043–1050.
52. Feliciano RP, Pritzel S, Heiss C, Rodriguez-Mateos A (2015) Flavonoid intake and cardiovascular disease risk. Curr Opin Food Sci 2, 92–99.
53. Almeida Rezende B, Pereira AC, Cortes SF, Soares Lemos V (2016) Vascular Effects of Flavonoids. Curr Med Chem 23, 87–102.
54. Ajay M, Gilani AUH, Mustafa MR (2003) Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. Life Sci 74, 603–612.
55. World Health Organization. Prevention of Cardiovascular Disease. 2011. Available online: http://www. who.int/cardiovascular_diseases/en/ (accessed on 28 August 2011).
56. Esser, N.; Legrand-Poels, S.; Piette, J.; Scheen, A.J.; Paquot, N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res. Clin. Pract. 2014, 105, 141–150.

57. Torres, N.; Torre-Villalvazo, I.; Tovar, A.R. Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. J. Nutr. Biochem. 2006, 17, 365–373.
58. O'Donnell, C.J.; Elosua, R. Cardiovascular Risk Factors. Insights from Framingham Heart Study. Rev. Española Cardiol. 2008, 61, 299–310.
59. McGillicuddy, F.C.; Roche, H.M. Nutritional status, genetic susceptibility, and insulin resistance–important precedents to atherosclerosis. Mol. Nutr. Food Res. 2018, 56, 1173–1184.
60. Torres, N.T.; Palacios-Gonzalez, B.; Noriega-Lopez, L.; Tovar-Palacio, A.R. Glycaemic index, insulinemic index and glycaemic load of soya drinks with low and high levels of carbohydrates. Rev. Investig. Clin. 2006, 58, 487–497.
61. Ginsberg, H.N. Insulin resistance and cardiovascular disease. J. Clin. Investig. 2000, 106, 453–458.
62. Surujdeo-Maharaj, S.; Sreenivasan, T.N.; Motilal, L.A.; Umaharan, P. Black pod and other phytophthora induced diseases of cacao: History, biology, and control. In Cacao Diseases; Bailey, B., Meinhardt, L., Eds.; Springer: Cham, Switzerland, 2016; pp. 213–266. ISBN 978-3-319-24789-2.
63. Grassi, D.; Socci, V.; Tempesta, D.; Ferri, C.; De Gennaro, L.; Desideri, G.; Ferrara, M. Flavanol-rich chocolate acutely improves arterial function and working memory performance counteracting the effects of sleep deprivation in healthy individuals. J. Hypertens. 2016, 34, 1298–1308.
64. Flammer, A.J.; Sudano, I.; Wolfrum, M.; Thomas, R.; Enseleit, F.; Périat, D.; Kaiser, P.; Hirt, A.; Hermann, M.; Serafini, M.; et al. Cardiovascular effects of flavanol-rich chocolate in patients with heart failure. Eur. Heart J. 2012, 33, 2172–2180.
65. Stahl, L.; Miller, K.B.; Apgar, J.; Sweigart, D.S.; Stuart, D.A.; McHale, N.; Ou, B.; Kondo, M.; Hurst, W.J. Preservation of cocoa antioxidant activity, total polyphenols, flavan-3-ols, and procyanidin content in foods prepared with cocoa powder. J. Food Sci. 2009, 74, C456–C461.
66. Langer, S.; Marshall, L.J.; Day, A.J.; Morgan, M.R.A. Flavanols and methylxanthines in commercially available dark chocolate: A study of the

correlation with nonfat cocoa solids. J. Agric. Food Chem. 2011, 59, 8435–8441.
67. Coussens, L.M.; Werb, Z. Inflammation and cancer. Nature 2002, 420, 860–867.
68. Gupta, P.K. Toxicological testing: Genesis. In Fundamentals of Toxicology: Essential Concepts and Applications; Erin Hill-Parks, Ed.; Elsevier Inc.: Bareilly, India, 2016; pp. 111–122, ISBN 978-0-12-805426-0.
69. Arif, M.; Islam, M.R.; Waise, T.M.Z.; Hassan, F.; Mondal, S.I.; Kabir, Y. DNA damage and plasma antioxidant indices in Bangladeshi type 2 diabetic patients. Diabetes Metab. 2010, 36, 51–57.
70. Erol, A. Systemic DNA damage response and metabolic syndrome as a premalignant state. Curr. Mol. Med. 2010, 10, 321–334. [CrossRef] [PubMed]
71. World Health Organization. Global Physical Activity Questionnaire (GPAQ) Analysis Guide. 2018. Available online: https://epi.grants.cancer.gov/paq/q078.html (accessed on 24 April 2018).
72. National Center for Chronic Disease Prevention and Health Promotion. Body mass index—BMI. 2018. Available online: https://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/index.html (accessed on 24 April 2018).
73. Lee, K.W.; Kim, Y.J.; Lee, H.J.; Lee, C.Y. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem. 2003, 51, 7292–7295.
74. Ioannone, F.; Sacchetti, G.; Serafini, M. Effect of dark chocolate extracts on phorbol 12-myristate 13-acetate-induced oxidative burst in leukocytes isolated by normo-weight and overweight/obese subjects. Front. Nutr. 2017, 4, 23.
75. Palacios-González, B.; Zarain-Herzberg, A.; Flores-Galicia, I.; Noriega, L.G.; Alemán-Escondrillas, G.; Zariñan, T.; Ulloa-Aguirre, A.; Torres, N.; Tovar, A.R. Genistein stimulates fatty acid oxidation in a leptin receptor-independent manner through the JAK2-mediated phosphorylation and activation of AMPK in skeletal muscle. Biochim. Biophys. Acta 2014, 1841, 132–140.

76. Chavez-Santoscoy, R.C.; Gutierrez-Uribe, J.A.; Granados, O.; Torre-Villalvazo, I.; Serna-Saldivar, S.O.; Torres, N.; Palacios-González, B.; Tovar, A.R. Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. Br. J. Nutr. 2014, 112, 886–899.
77. Function, H.D.L.; Millar, C.L.; Duclos, Q.; Blesso, C.N. Effects of dietary flavonoids on reverse cholesterol transport, HDL metabolism, and HDL function. Adv. Nutr. 2017, 8, 226–239.
78. Alkerwi, A.; Sauvageot, N.; Crichton, G.E.; Elias, M.F.; Stranges, S. Daily chocolate consumption is inversely associated with insulin resistance and liver enzymes in the observation of cardiovascular risk factors in luxembourg study. Br. J. Nutr. 2016, 115, 1661–1668.
79. Ramos, S.; Rodríguez-Ramiro, I.; Martín, M.A.; Goya, L.; Bravo, L. Dietary flavanols exert different effects on antioxidant defenses and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. Toxicol. In Vitro 2011, 25, 1771–1781.
80. Rull, G.; Mohd-Zain, Z.N.; Shiel, J.; Lundberg, M.H.; Collier, D.J.; Johnston, A.; Warner, T.D.; Corder, R. Effects of high flavanol dark chocolate on cardiovascular function and platelet aggregation. Vascul. Pharmacol. 2015, 71, 70–78.
81. Müllner, E.; Brath, H.; Nersesyan, A.; Nitz, M.; Petschnig, A.; Wallner, M.; Knasmüller, S.; Wagner, K.H. Nuclear anomalies in exfoliated buccal cells in healthy and diabetic individuals and the impact of a dietary intervention. Mutagenesis 2014, 29, 1–6.
82. Demirbag, R.; Yilmaz, R.; Gur, M.; Celik, H.; Guzel, S.; Selek, S.; Kocyigit, A. DNA damage in metabolic syndrome and its association with antioxidative and oxidative measurements. Int. J. Clin. Pract. 2006, 60, 1187–1193.
83. Jain, N.; Naseem, I.; Ahmad, J. Evaluation of DNA damage and metabolic syndrome parameters in diabetic rabbits supplemented with antioxidants. Fundam. Clin. Pharmacol. 2009, 23, 197–205.

84. Damasceno, D.C.; Sinzato, Y.K.; Bueno, A.; Dallaqua, B.; Lima, P.H.; Calderon, I.M.P.; Rudge, M.V.C.; Campos, K.E. Metabolic profile and genotoxicity in obese rats exposed to cigarette smoke. Obesity 2013, 21, 1596–1601.

85. Luca, V.S.; Miron, A.; Aprotosoaie, A.C. The antigenotoxic potential of dietary flavonoids. Phytochem. Rev. 2016, 15, 591–625.

86. Chakrabarti, M.; Ghosh, I.; Jana, A.; Ghosh, M.; Mukherjee, A. Genotoxicity of antiobesity drug orlistat and effect of caffeine intervention: An in vitro study. Drug Chem. Toxicol. 2017, 40, 339–343.

87. Serafini, M.; Peluso, I.; Raguzzini, A. Flavonoids as anti-inflammatory agents. Proc. Nutr. Soc. 2010, 69, 273–278.

88. Pimentel, F.A.; Nitzke, J.A.; Klipel, C.B.; De Jong, E.V. Chocolate and red wine–A comparison between flavonoids content. Food Chem. 2010, 120, 109–112.

89. Saklayen, M.G., The Global Epidemic of the Metabolic Syndrome. Curr Hypertens Rep. 2018 20, 2, 12.

90. Mohamed, S., Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease. Trends Food Sci. Technol. 2014, 35, 2, 114–128.

91. Esser, N., Legrand-Poels, S., Piette, J., Scheen, A.J., Paquot, N., Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes Res. Clin. Pract. 2014, 105, 2, 141–150.

92. Torres, N., Torre-Villalvazo, I., Tovar, A.R., Regulation of lipid metabolism by soy protein and its implication in diseases mediated by lipid disorders. J. Nutr. Biochem. 2006, 17, 6, 365–373.

93. Deng, T., Lyon, C.J., Bergin, S., Caligiuri, M.A., Hsueh, W.A., Obesity, inflammation and cancer. Annu Rev Pathol. 2016, 23, 11, 421-449.

94. Erol, A., Systemic DNA damage response and metabolic syndrome as a premalignant state. Curr. Mol. Med. 2010, 10, 3, 321–334.

95. Leyva-Soto, A., Chavez-Santoscoy, R.A., Lara-Jacobo, L.R., Chavez-Santoscoy, A.V., Gonzalez-Cobian, L.N., Daily consumption of chocolate rich

in flavonoids decreases cellular genotoxicity and improves biochemical parameters of lipid and glucose metabolism. Molecules 2018, 23, 9, 1–12.

96. Bolognesi, C., Bonassi, S., Knasmueller, S., Fenech, M., Bruzzone, M., et al., The human MicroNucleus project on exfoliated buccal cells. Mutation research 2011, 728(3), 88-97.
97. Averna, M., Casazza, A., Martines, A., Pedrazzi, M., Franchi, A., et al., Cell protection from Ca2+-overloading by bioactive molecules extracted from olive pomace. Natural Product Research. 2018, 6419, 1-7.
98. Arruda, A.P., Hotamisligil, G.S., Calcium Homeostasis and Organelle Function in the Pathogenesis of Obesity and Diabetes. Cell Metabolism, 2015, 22, 3, 381-397.
99. Chandra, S., De Mejia Gonzalez, E., Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of Ardisia compressa in comparison to mate (Ilex paraguariensis) and green (Camellia sinensis) teas. J. Agric. Food Chem. 2004, 52, 11, 3583–3589.
100. Secretaría de Salud. NORMA Oficial Mexicana NOM-111-SSA1-1994. Mexico, 1994. Available online: www.salud.gob.mx/unidades/cdi/nom/111ssa14.html (accessed on 22 03 2019).
101. Stahl, L., Miller, K.B., Apgar, J., Sweigart, D.S., Stuart, D.A., et al., Preservation of cocoa antioxidant activity, total polyphenols, flavan-3-ols, and procyanidin content in foods prepared with cocoa powder. J. Food Sci. 2009, 74, 6, C456-c461.
102. Belguith-Hadriche, O., Bouaziz, M., Jamoussi, K., El Feki, A., Sayadi, S., et al., Lipid-lowering and antioxidant effects of an ethyl acetate extract of fenugreek seeds in high-cholesterol-fed rats. J. Agric. Food Chem. 2010, 58, 4, 2116–2222.
103. Bladé, C., Arola, L., Salvadó, M.J., Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. Mol. Nutr. Food Res. 2010, 54, 1, 37–59.

104. Chavez-Santoscoy, R.C., Gutierrez-Uribe, J.A., Granados, O., Torre-Villalvazo, I., Serna-Saldivar, S., et al., Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. Br. J. Nutr. 2014, 112, 6, 886–899.
105. Kozłowska, A., Szostak-Wegierek, D., Flavonoids- Food sources and health benefits. Rocz. Rocz Panstw Zakl Hig. 2014, 65, 2, 79-85.
106. Chávez-Santoscoy, R.A., Lazo-Vélez, M.A., Serna-Sáldivar, S.O., Gutiérrez-Uribe, J.A., Delivery of flavonoids and saponins from black bean (Phaseolus vulgaris) seed coats incorporated into whole wheat bread. Int. J. Mol. Sci. 2016, 17, 2.
107. Lazo-Vélez, M.A., Chuck-Hernandez, C., Serna-Saldívar, S.O., Evaluation of the functionality of five different soybean proteins in yeast-leavened pan breads. J. Cereal Sci. 2015, 64, 63–69.
108. Dziki, D., Różyło, R., Gawlik-Dziki, U., Świeca, M., Current trends in the enhancement of antioxidant activity of wheat bread by the addition of plant materials rich in phenolic compounds. Trends in Food Science & Technology, 2014, 40, 1, 48–61.
109. Ktenioudaki, A., Alvarez-Jubete L., Gallagher, E., A review of the process-induced changes in the phytochemical content of cereal grains: The breadmaking process. Critical Reviews in Food Science and Nutrition 2015, 55, 5, 611-619.
110. Higashi, T., Mai, Y., Mazaki, Y., Miwa, S., Intracellular Ca2+ is an essential factor for cell damage induced by unsaturated carbonyl compounds. Journal of Bioscience and Bioengineering 2017, 124, 6, 680-684.
111. Vara-Ciruelos, D., Dandapani, M., Gray, A., Ejaife O., Egbani, A., et al., Genotoxic Damage Activates the AMPK-α1 Isoform in the Nucleus via Ca2+/CaMKK2 Signaling to Enhance Tumor Cell Survival. Molecular cancer research 2018, 16, 2, 345-357.

CAPÍTULO VII:

ANNEXES

Annex 1. Scientific publication made in the international journal Molecules: An Open Access Journal from MDPI.