BRNO UNIVERSITY OF TECHNOLOGY

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY

FAKULTA CHEMICKÁ

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

POLYPHENOLS IN NUTRITIONS AND THEIR EFFECT ON DNA

POLYFENOLY VE VÝŽIVĚ A JEJICH VLIV NA DNA

BACHELOR'S THESIS

BAKALÁŘSKÁ PRÁCE

AUTHOR   Juan Osorio
AUTOR PRÁCE

SUPERVISOR   doc. Mgr. Václav Brázda, Ph.D.
VEDOUCÍ PRÁCE

BRNO 2022

Assignment Bachelor's Thesis

Project no.:	FCH-BAK1731/2021	Academic year: 2021/22
Department:	Institute of Food Science and Biotechnology	
Student:	**Juan Osorio**	
Study programme:	Chemistry and Technology of Foodstuffs	
Study field:	Food Chemistry and Technology	
Head of thesis:	**doc. Mgr. Václav Brázda, Ph.D.**	

Title of Bachelor's Thesis:

Polyphenols in nutritions and their effect on DNA

Bachelor's Thesis:

– Cultivation of human cell lines.
– Testing the effect of selected substances in vivo.
– Testing the effect of selected substances in vitro (CD spectroscopy, ThT assay).
– Determination of global methylation before and after exposure to selected substances.
– Detection of epigenetic markers.

Deadline for Bachelor's Thesis delivery: 27.5.2022:

Bachelor's Thesis should be submitted to the institute's secretariat in a number of copies as set by the dean This specification is part of Bachelor's Thesis

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      Juan Osorio   doc. Mgr. Václav Brázda, Ph.D.   prof. RNDr. Ivana Márová, CSc.
        student   Head of thesis   Head of department

- - - - - - - - - - - - -

      prof. Ing. Michal Veselý, CSc.
            Dean

In Brno dated 1.2.2022

ABSTRAKT

Epidemiologické studie prokázaly vliv konzumace rostlinných potravin v prevenci široké škály nemocí. Přírodní antioxidanty přítomné v těchto potravinách, mezi nimiž jsou velmi důležité polyfenoly, mohou být zodpovědné za tuto činnost podporující zdraví. Cílem bakalářské práce je ukázat interakci určitých polyfenolů s genetickým materiálem prostřednictvím různých signálních mechanismů, zejména pokud jde o stabilizaci nekanonické struktury DNA G-kvadruplex a poukázat tak na nejselektivnější látku pro inhibici biochemických procesy. Dále práce obsahuje podrobné informace, které mohou pomoci pochopit, jak mohou polyfenolové sloučeniny interagovat s DNA prostřednictvím epigenetických mechanismů a G4 struktur, a které faktory mohou ovlivnit jejich účinnost. Různé experimenty, biologickým a experimentálním opakováním, byly použity k potvrzení interakce mezi sloučeninami a DNA.

ABSTRACT

Epidemiological studies have shown the effect of plant-derived food consumption in the prevention of a wide range of diseases. The natural antioxidants present in these foods, among which polyphenols are very relevant, may be responsible for this health-promoting activity. The aim of this bachelor thesis is to submit evidence in the interaction of certain polyphenols with the genetic material through different signaling mechanisms, especially regarding the stabilization of the non-canonical DNA structure G-quadruplex and therefore point out the most selective substance to inhibit biochemical processes. Furthermore, the thesis includes detailed information that might help understand how polyphenol compounds can be attached to the DNA through epigenetic mechanisms and G4 structures, and which factors may affect the efficiency of these. Different experiments, by biological and experimental repetition, were used to corroborate the interaction between the compounds and DNA.

KLÍČOVÁ SLOVA

Polyfenoly, antioxidanty, výživa, epigenetika, DNA, G-kvadruplex, G4-ligandy.

KEYWORDS

Polyphenols, antioxidants, nutrition, epigenetics, DNA, G-quadruplex, G4-ligands.

BIBLIOGRAPHIC CITATION

DECLARATION

I declare that the bachelor thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the bachelor thesis is the property of the Faculty of Chemistry of Brno University of Technology, and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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Juan Osorio

ACKNOWLEDGEMENT

CONTENT

1. INTRODUCTION

Nowadays, the human population is exposed to numerous xenobiotics due to external factors such as population growth, environmental pollution, technology, and food; considering the number of diseases that afflict the population, there is a specific group that poses a higher risk to life. They can cause chronic, degenerative, and mutagenic diseases; where in most cases, it is the oxidative stress, caused by the imbalance between the generation of oxidizing species and the ability of the cellular antioxidant system to act, the key factor in the development and progress of these diseases. To mitigate them, prevention is the best treatment strategy, reducing the risk of cardiovascular, degenerative diseases, or even cancer [1]. A balanced diet, rich in plant-based foods, offers micronutrients such as vitamins, minerals and phenolic compounds, which are important for specific biochemical processes that in turn regulate and inhibit these oxidative processes; consequently, reducing the risk of diseases associated with oxidative stress [2]. Phenolic or polyphenol compounds are the most extensive group of non-energetic substances present in foods of plant origin; these could modulate the activity of different enzymes, interfere in signaling mechanisms with participation of non-canonical DNA structure such as G-quadruplex, as well as in different cellular processes [2]. This may be partially due to their physicochemical characteristics, allowing them to take part in different cellular metabolic oxidation-reduction reactions [2]. Therefore, these substances are known as antioxidant agents, therefore justifying their health benefits. Antioxidant substances prevent the formation of oxides, which means, they have the ability to delay or prevent the oxidation of other molecules, which in their absence can produce free radicals, hence, triggering cellular damage.

The aim of this thesis is the study of polyphenols related to plant-derived food for daily consumption and how they affect or interact with the cellular genetic material; through the culture of cancer and non-cancer cells, evaluating their coordination at the molecular level in the presence of these substances, through controlled *in vivo* and *in vitro* experiments to finally review the cytotoxicity of the compared substances.

2. CONTEXT

Genetics is not the only factor that influences cell arrangement and, consequently, the proper functioning of the organism; there are other variables that together may or may not favor its operation; among them we find external factors such as the environment, society, coexistence, population growth and diet; factors that in one way or another are related to free will, and thereby can be modifiable [2].

Nutrition and diet are concepts widely known nowadays, as they coincide with great public interest due to their fundamental role in health and development, where good nutrition as a pillar in the lifestyle is directly related to a stronger immune system, safer pregnancies and deliveries, longevity; as well as in the prevention, treatment and reduction of risks in hereditary diseases [3].

The main source of energy is food, which after biological processes is transformed and from it the necessary nutrients are obtained to maintain or improve health [4]; nutrients such as fats, carbohydrates, proteins, vitamins, minerals and other vital micronutrients necessary for the day-to-day. As soon as this interest in healthy lifestyles began to grow, remarkable scientific breakthroughs have coincided in an attempt to understand the epigenetic biology behind diseases directly related to lifestyle habits, trying to decipher the biological mechanisms that underlie them, and which are closely related to oxidative stress [5]. Among them, epigenetics studies the hereditary changes caused by the activation and deactivation of genes without any change in the underlying DNA sequence of the organism [6]. Additionally, other important concepts attached to this field of study have been created, which lead to the study of nutritional variables such as in nutrigenetics, a scientific field that tries to understand those processes where the genetic sequence of an individual is influenced in response to their diet, associating genetic variants with differential responses to nutrients and with the risk of developing a disease [7]. Accordingly with these above-mentioned concepts, a more recent one has been coined, nutriepigenomics, studying the effects of nutrients and food on human health through epigenetic modifications [7].

During the study of these nutritional properties, it was shown that, if a disproportionate diet or food intake plus metabolic alterations occur during the critical moments of the development or during life itself, the resulting epigenetic alterations can lead to permanent changes in the expression of certain genes that affect the structure and/or functions of organs, predisposing the individual to a disease, which in some cases, can become hereditary; or changes that could enhance the development of life and lead to preventive activities against the progress of chronic diseases.

In this attempt to understand the biological mechanisms that underlie and explain the behaviors and consequences of a diet, as well as the external factors already mentioned above, epigenetic mechanisms are given rise; these encompass processes that alter gene expression with resulting effects on the phenotype without changes in DNA sequence, and include DNA methylation, covalent histone modifications, remodeling or changes that affect chromatin folding or chromatin's preservations and chromosome stability; and in general, all those phenomena that affect gene expression patterns without altering or mediating the DNA sequence, such as the activities of non-coding RNAs (ncRNAs), transposons, chaperones, among others [7].

From the previous mechanisms mentioned, the most studied ones are DNA methylation, covalent modifications in histones, and non-coding RNAs [7]. Epigenetic mechanisms regulate gene expression at the transcriptional and post-transcriptional level and therefore contribute to phenotypic manifestations [8].

These processes are responsible for most of the observable differences between monozygotic twins, or for explaining how cells or tissues carrying the same nucleotide sequence can generate different cell types and different responses under the same exposure to hormones and nutrients [7].

Hence, epigenetics is situated in the middle of the innate part, the genetics, and the acquired part, other external situations outside the organism that affect it directly or indirectly; and together with the mechanisms of action, keep or intercede in the development of life. For this reason, emphasis is always placed on the diet that is favorable for health and development, which includes the least amount of processed foods, such as fruits and vegetables.

In their natural state, these foods offer greater nutritional value, which generally provide these natural substances for a healthy lifestyle, including micronutrients such as vitamins, minerals and products of the secondary metabolism of plants, such as polyphenols.

3. THEORETICAL PART

This work focused on the effect of specific phenolic compounds through their antioxidant activity, present in commonly consumed plant-based foods, over the genetic material. The polyphenols targeted in this work belong to the phenolic acids subclass, specifically gallic acid; tannins, specifically ellagic acid, and curcuminoids, specifically curcumin.

3.1. Phenolic compounds

They are a heterogeneous set of molecules of natural origin, characterized by the presence of one or more phenolic rings in their molecular structure. They originate mainly in plants, as products of secondary metabolism. These metabolites are essential for the physiology and protection of plants, being participants in different stages of their life cycle [9]. Phenolic compounds are related to the sensory quality of plant-based foods, both fresh and processed and are easily spotted through the unique pigmentation given to fruits and vegetables, this, being responsible for the red, blue, violet and orange color in them and their products. In addition, the oxidation reaction of phenolic components towards the formation of quinones, catalyzed by polyphenol oxidase enzymes, produces enzymatic browning in foods, a phenomenon of vital importance used to spot the quality of fruits and vegetables during processing [10].

Phenolic compounds present a hydroxylated benzene ring as a common element in their molecular structures, which can include functional groups such as esters and glycosides, among others [9]. Although there is a great variety of phenolic compounds being known, around 8 000 so far, all phenolic compounds of plant origin derive from an intermediate substance in plant metabolism, phenylalanine, or a close precursor, shikimic acid; occurring by two main synthetic pathways: the shikimate pathway and the acetate pathway [11, 12]. Polyphenols are mainly conjugated forms with one or more sugar residues attached to hydroxyl groups, as well as directly attached to an aromatic carbon [11]. Additionally, it is common to find associations between these compounds and other substances, being the most relevant carboxylic acids, organic acids, amines, lipids, or other phenolic groups [12].

3.1.1. Biosynthesis

As mentioned above, the biosynthesis of polyphenols occurs through two primary routes - the shikimic acid route and the polyacetates route.

The shikimic acid pathway, depending on light, results in the synthesis of aromatic amino acids, cinnamic acids and their derivatives, such as phenylalanine, simple phenols, phenolic acids, coumarins, among others [2, 11]. The polyacetates route results in the synthesis of quinones and xanthones, through the condensation of acetyl coenzyme A (Acetyl-CoA) and later by reduction of the polyacetates; the formation of fatty acids occurs, and consequently, by cyclization, it produces aromatic compounds [2]. Precursors of both metabolic routes form another type of polyphenols, known as flavonoids.

3.1.2. Classification

Phenolic compounds can generally be classified into simple and polyphenolic compounds. Simple phenolic compounds are those that contain one phenol unit or its derivative; in other words, they are substituted phenol compounds, where the group denoted by "R", as shown in figure 1, can be an organic compound such as alkyl, alkenyl, aryl, or hydroxyl groups, alkoxy or amino; which in turn can be found in *ortho (o), meta (m)* or *para (p)* aromatic ring positions [13].
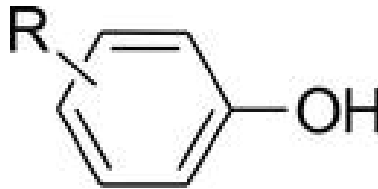

Figure 1. General structure of simple phenolic compound [13].

Simple phenols are subdivided into [13]:
- Simple phenolics,
- Phenolic acids,
  - Hydroxybenzoic acids,
  - Hydroxycinnamic acids,
  - Coumarins.

On the other hand, polyphenolic compounds are characterized by containing more than one phenolic unit, that is, highly polymerized compounds and these are subdivided into [13]:
- Flavonoids,
- Tannins.

Similarly, there are other phenolic compounds that are not governed by these previous classifications, they generally have a different molecular conformation that does not satisfy the previous ones, these are [13]:
- Stilbenes,
- Lignans,
- Curcuminoids.

3.1.2.1. Phenolic acids

They are a subclass of phenolic compounds, representing about 30 % of them and are produced through the shikimic acid pathway, as by-products of the monolignol route, by decomposition of cell wall polymers and, in some cases, produced by microbes [14]. They have a carboxylic acid group and are characterized by having a phenolic residue and a structure stabilized by resonance that allows the donation of the hydrogen atom as a mechanism of participation in the oxidation-reduction reactions, therefore having antioxidant properties through the mechanism of radical scavenging [14].

In addition, other mechanisms of participation in the elimination or extinction of radicals are known, through the donation of electrons or the extinction of singlet oxygen, giving the antioxidant nature to phenolic acids [14].

Phenolic acids are subdivided into three subgroups, hydroxybenzoic acids, hydroxycinnamic acids and coumarins [13, 14]. They are widely distributed in plant-based foods, concentrated mainly on seeds, leaves and fruit skins.

Gallic acid

Is the most widely studied hydroxybenzoic acid. It is characterized by its three hydroxy groups at positions 3, 4 and 5, as seen in the figure 2. It is an astringent, a cyclooxygenase2 inhibitor, an antioxidant, a neoplastic agent, a human xenobiotic metabolite, an apoptosis inducer and a geroprotector. It is found both as a free state and a constituent of tannins, named gallotannins. The gallic acid is present in nearly every part of the plant, from the roots to the bark, including the seeds, leaf and fruit. Although there is plenty of food sources of gallic acid, like strawberries, grapes, bananas, blueberries walnuts, cashews, hazelnuts, blackcurrants, guavas, mangos, pomegranates; is mainly abundant in tea leaves, tree bark and processed beverages like red wine and green tea [15].
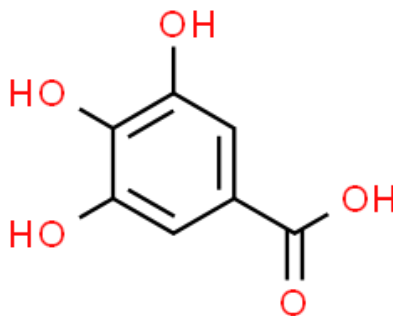

Figure 2. Gallic acid chemical structure [16].

3.1.2.2. Tannins

They are a subclass of highly polymerized phenolic compounds and are known to bind to and precipitate proteins, such as albumin, amino acids and heavy metals. They are subdivided into three types: hydrolyzable, condensed and complex tannins [13].

Hydrolyzable tannins can be gallotannins or ellagitannins. Gallotannins are polyols that are substituted with gallic acid units, where the galloyl units in gallotannins are linked by ester bonds. Commonly, the polyol core is a D-glucose that is substituted with gallic acid units [13].

Similar to gallotannins, ellagitannins are hydrolyzable 1,2,3,4,6-pentagalloylglucose. However, unlike gallotannins characterized by ester linkages, adjacent galloyl groups in ellagitannins are linked by C-C bonds [13].

In other words, hydrolyzable tannins are heterogeneous polymers and are formed by units of gallic acid and sugars, where their bond can be ester or C-C type. From its name comes its ability to hydrolyze easily.

On the other hand, condensed tannins are polymeric phenolic compounds that consist of catechin units. And last, complex tannins are gallotannins or ellagitannins bonded to a catechin unit [13].

Tannins are recognized by their bitter and astringent taste when consumed. They are more concentrated in the bark of trees, wood, stem and leaves of plants, as well as in red fruits, blueberries, pomegranates, guavas, apples, grapes, leaves of tea and in beverages such as wine, tea and coffee.

Ellagic acid

Chemically known as hexa-hydroxydiphenoic acid dilactone, it is a hydrolyzable tannin, derived from gallic acid, as shown in figure 3; it resulted from formal dimerization by oxidative aromatic coupling and with intramolecular lactonization of both carboxylic acid groups of the resulting biaryl [17]. It is an antioxidant, an inhibitor of enzymes participating in the DNA transcription mechanism and a geroprotective agent. It is found in different fruits and vegetables, especially in red fruits such as strawberries, raspberries, blueberries, blackberries and pomegranates, seeds such as walnuts and pecans, and in several types of honey [17].
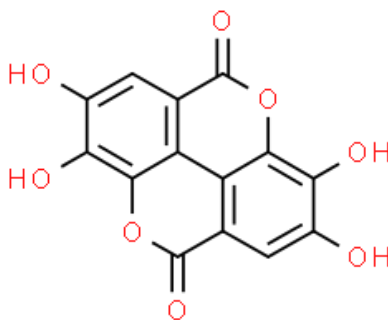

Figure 3. Ellagic acid chemical structure [16].

According to the physicochemical properties of ellagic acid, it has been found that it participates in epigenetic processes connected with DNA transcription and replication through binding mechanisms related to secondary structures, the G-quadruplex [18].

In vitro studies, as well as absorption and fluorescence spectroscopic studies, indicate that ellagic acid binds to G4 structures with a specific affinity, stabilizing the G4 structures of the KRAS oncogene, for example. Additional confirmation was given by showing an increase in the melting point of the quadruplex structure, a property that demonstrates the stability provided by the bond formed between the G4 structure and ellagic acid [19].

3.1.2.3. Curcuminoids

Curcuminoids are natural phenolic compounds from the dry rhizome of *Curcuma longa* plant. Curcuminoids include curcumin, demethoxycurcumin, and bisdemethoxycurcumin. While turmeric contains only, 2-9 % curcuminoids, 75 % of these active curcuminoids are curcumin [20].

Curcumin

Curcumin or diferuloylmethane is a natural occurring molecule in the root and rhizome of the plant. It exists in different tautomeric forms, the 1,3-diketo form and two equivalent enol forms, as shown in figure 4 and 5. The antioxidant activity of curcumin depends on its structural form, where the keto form exerts antioxidant action, while the enol form is prone to degradation. In polar and acidic medium, curcumin stays in the keto form, while in non-polar and basic medium it undergoes in the enol form, hence, degradation [21].
Among its therapeutic antioxidant property, it is anti-inflammatory and antibacterial, including the capacity of induce apoptosis in tumor cells. [21].
Due to its extensive properties, its analysis and research are wide. It is found exclusively in turmeric and is commonly used as a preservative and food colorant, as well as in the production of beer, butter, and meat [22]. Over the centuries, in the traditional Indian medicine has been used to treat different diseases, from cough, to rheumatism and liver disorders [23].
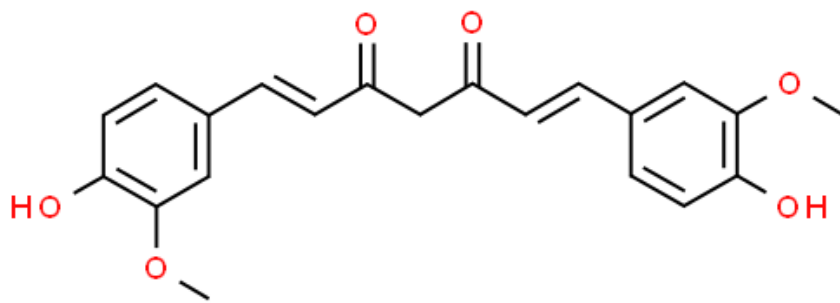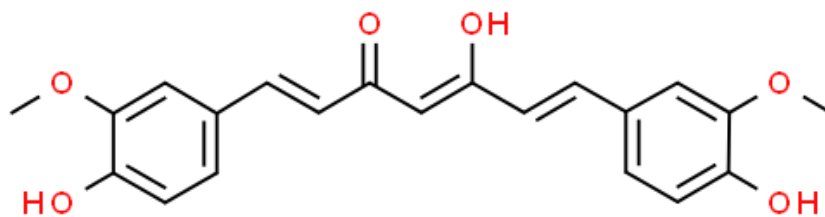

Figure 4. Keto form, solid curcumin [16].

Figure 5. Enol form, liquid curcumin [16].

According to the physicochemical properties of curcumin, it has been found that it participates in epigenetic processes connected with DNA transcription and replication through binding mechanisms related to secondary structures, the G-quadruplex [18].
In vitro studies, as well as absorption and fluorescence spectroscopic studies, indicate that curcumin binds to G4 structures with a specific affinity. It is known that in solution the keto and

enol form of curcumin coexists; however, the keto form showed less binding constant and energy towards duplex DNA than towards KRAS quadruplex [19]. In other words, curcumin binds to G-quadruplex more specifically than duplex DNA and therefore showed its participation in epigenetic modifications trough G-quadruplex [19].

3.2. Oxidative stress

Oxidative stress is a phenomenon caused by the imbalance between the production and accumulation of reactive oxygen species in cells and tissues, and the ability of a biological system to eliminate these reactive products [24].

3.2.1. Reactive species

A free radical is an atom or molecule with one or more unpaired electrons in the last orbital, which makes it highly unstable and reactive. It is capable of reacting with multiple adjacent biomolecules through oxidation-reduction reactions [25].

The effect of free radicals on the cell is dual; the beneficial effects occur at low concentrations when they participate in different physiological functions of the cell, such as defense against infectious agents and cell localization systems during mitosis, for example. On the other hand, the detrimental effect occurs in biological systems by producing oxidative stress, considered to be the most responsible for various human pathologies caused by a deficiency in the cellular antioxidant system, due to accumulation or increase in the concentration of free radicals [25].

Oxidative stress is a result of the metabolic reactions that use O_2 and represents an alteration in the pro-oxidant/antioxidant balance in living systems, with the ability to oxidize biomolecules (proteins, lipids, carbohydrates nucleic acids), inhibit their structure and impair their function. For this reason, the balance between the beneficial and harmful effects of free radicals must be maintained through redox regulation mechanisms, thus protecting living organisms from oxidative stress, maintaining control of the redox state through the cellular antioxidant system and through free radical scavengers [25].

Among the reactive species are the reduced forms of O_2 and hydrogen peroxide H_2O_2, the most frequent. These free radicals are products of cellular metabolism and exogenous sources. The main endogenous source of oxidant species and free radicals in the body is the generation of reactive species during mitochondrial respiration and lipid peroxidation chain reactions. On the other hand, exogenous stimuli such as ionizing radiation, environmental pollutants, intense physical activity, drug metabolism, cell actions of the immune system, hypercaloric diets or diets deficient in antioxidants, increase the generation of oxidant species [24].

3.2.2. Oxidative metabolism

3.2.2.1. The generation of reactive species during mitochondrial respiration

During energy production (ATP formation) for cellular processes in aerobic organisms, O_2 is required, this occurs during oxidative metabolism and mitochondrial electron transport, O_2 is reduced to water (H_2O) after accepting four electrons by the cytochrome complex-oxidase of the mitochondria, or the tetravalent pathway. There, O_2 is reduced in four stages, in each of which an

electron is transferred. However, mitochondrial electron transport is imperfect, and during the process, about 2 % of oxygen is incompletely reduced, and therefore generates one of the reactive oxygen species, superoxide anion O_2^-. The spontaneous and enzymatic dismutation of the superoxide anion O_2^- gives rise to the formation of hydrogen peroxide, and both can generate hydroxyl radicals ($\cdot\,OH$) through Fenton reactions catalyzed by transition metals [25].

3.2.2.2. Chain reactions of lipid peroxidation

Free radicals are capable of extracting a hydrogen atom from a saturated fatty acid, generating a lipid radical ($R\cdot$), which can react with O_2 and give rise to a peroxyl radical ($ROO\cdot$). This contributes to a series of chain reactions, generating new lipid radicals ($R\cdot$) and lipid hydroperoxides (ROOH), where the last mentioned can break down into alkoxyl ($RO\cdot$) and peroxyl radicals ($ROO\cdot$), contributing to oxidative damage. This decomposition is facilitated by exposure to ultraviolet light or by the presence of metal ions through the Fenton reaction mechanism [25].

The main reactive oxygen and nitrogen species or pro-oxidant substances are [24]:

- Hydroxyl radical ($\cdot\,OH$),
- Hydroperoxyl ($HO_2\cdot$),
- Hydrogen peroxide (H_2O_2),
- Superoxide anion (O_2^-),
- Singlet oxygen ($^1O_2$),
- Alkoxyl ($RO\cdot$),
- Peroxide ($ROO\cdot$),
- Nitric oxide (NO),
- Nitrogen dioxide (NO_2),
- Nitrate radical (NO_3),
- Semiquinone (Q),
- Ozone (O_3).

3.3. Oxidative stress and disease

The imbalance between oxidizing species and antioxidant species refers to oxidative stress, and as already mentioned, an imbalance in favor of oxidizing species is what triggers potential oxidative damage to biomolecules, reaching even the genetic material.

The oxidizing action of reactive species can severely affect cell structures such as membranes, lipids, proteins, lipoproteins, and DNA. Reactive species have been associated with numerous chronic and degenerative diseases, as well as accelerating the aging process [24].

The main effect of membrane lipid oxidation, through lipid peroxidation chain reactions, is the loss of membrane fluidity, affecting its properties and functionality. These chain reactions are capable of reacting and releasing proteins, as well as causing structural modifications in DNA [24].

Oxidative damage to proteins occurs through oxidation reactions of sulfhydryl groups, catalyzed by metals, which induce bonds between amino acid residues, reactions with aldehydes, cross-links between proteins, or fragmentation of peptides [24]. Protein lesions are important because they introduce modifications that can affect receptors, enzymes, transport proteins, and generate new antigens capable of triggering the immune response and inhibiting its functionality [25].

Consequently, protein damage could contribute to secondary damage of other biomolecules, such as DNA repair enzymes, or alter the functioning of DNA polymerases during the DNA replication process. Likewise, DNA is susceptible to oxidative damage. This occurs through modifications of nitrogenous bases, the formation of adducts between bases and sugars, unions between thymine and tyrosine, breaks in the DNA strand and cross-links with other molecules [25].

3.4. DNA secondary structures

Besides the canonical dextrorotatory double helix structure of DNA, there are other types of structures present in alternative conformations, which are based on specific sequence motifs and interactions with different proteins. These intermolecular and intramolecular secondary structures were characterized *in vitro* by biophysical techniques; however, their common presence in all organisms was soon determined, since they have been of great relevance due to their possible role within mechanisms of modulation or regulation of various biological functions. Among the non-B-form DNA (Classic double helix form) secondary structures analyzed, the most studied ones are listed and showed in figure 6. [26–28]:

- Z-DNA (a),
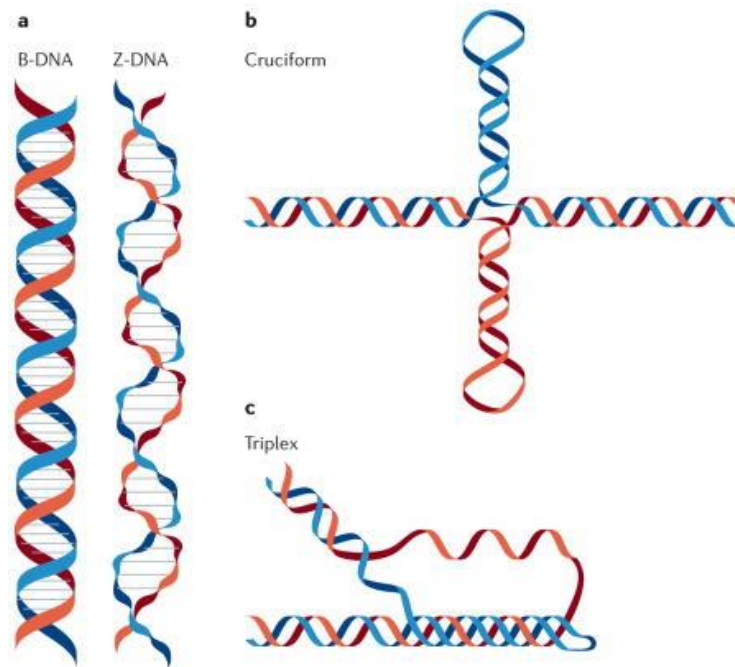- Cruciform structure (b),
- Triplex DNA (c),
- G-quadruplex DNA.

Figure 6. Other non-B-form DNA secondary structures [27].

G-quadruplex DNA

G4 or G-quadruplexes are noncanonical secondary structures formed in DNA sequences containing consecutive runs of guanines. Is a four-stranded DNA structure that contains stacked G-tetrad planes of four guanines connected by a network of Hoogsteen hydrogen bonding, as seen in section A of figure 7. [27, 29].

G-quadruplexes can be formed with one, two, or four G-rich DNA molecules, figure 7. B, and this G-strands in the structure can be parallel or antiparallel conformation, thus regarding the glycosidic bond configuration [27, 29].
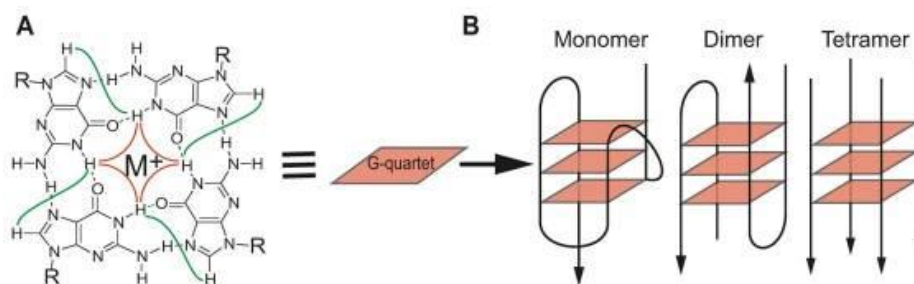
Figure 7. Schematic diagram of the interaction in a G-quartet (A), and intramolecular and intermolecular G-quadruplex DNA structures (B) [29].

Regarding the genome, many regions have the ability to form G4 structures; however, studies *in vivo* and *in vitro* indicate that unresolved G4 structures may participate during DNA replication by slowing or stalling the replication fork machinery. In addition, G4 structures are postulated to block transcription by inhibiting polymerase, facilitate transcription by retaining the single-stranded conformation of DNA, stimulate transcription by recruiting proteins that recruit or stimulate polymerase and are suggested to block transcription via the recruitment of G4 binding proteins, which directly or indirectly repress transcription [27, 29].

3.5.   Cellular antioxidant system

It is evident that free radicals are generated at the intracellular level through oxidative metabolism, and extracellularly, through exogenous stimuli. To counteract the harmful effects of free radicals, the body has its own defense mechanism, made up of enzymatic systems, as the first line of action, and non-enzymatic ones, such as molecules and chemical scavengers, which prevent oxidative damage. Antioxidants can be grouped according to their mechanism of action and their chemical nature [25]:

- **Enzymes:** They act on free radicals, degrading them into less harmful molecules through specific biochemical mechanisms, by the action of superoxidase dismutase (SOD), followed by catalase (CAT) and glutathione peroxidase (GPx). The activity of these enzymes must be in equilibrium to maintain intracellular redox balance [25].
- **Preventive antioxidants:** Molecules responsible for sequestering the initiators of the oxidative process, generally they are transition metals such as iron (Fe) and copper (Cu) in their reduced state [25].
- **Antioxidant scavengers of reactive oxygen species:** They inhibit the reaction chain and propagation in the formation of free radicals [25].
- **Nutritional antioxidants:** Diet is the greatest source of antioxidants and important microelements for the synthesis of antioxidant enzymes. Among the microelements, there are several transition metals such as iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), and others, such as selenium (Se), which participate mainly as components or cofactors in specific biochemical processes during the synthesis of enzymes. In addition, there are chemical components of plant origin, such as vitamins and phenolic compounds, which act

as scavengers of reactive oxygen species or as donors of hydrogen atoms to free radicals [25].

In general, aerobic metabolism, *in vivo*, and exogenous stimuli are factors that involve the production of reactive species capable of generating oxidative stress, reacting with biomolecules and participating in degenerative and pathophysiological processes [24]. However, the body has the ability through different mechanisms, enzymatic and non-enzymatic, to counteract the damaging action of free radicals. From the mechanisms of the cellular antioxidant system, nutritional antioxidants are the most striking, since they are based on diet and eating habits that a pro-oxidant/antioxidant balance, necessary for the correct functioning of the organism, can be achieved.

One of the recommendations that are gaining popularity in medical treatments today is to focus more on prevention than on the disease itself; this is because it is easier to modify life habits than to properly treat diseases whose progress is partially or totally unknown. Therefore, it has been proposed that the beginning of the prevention of damage to genetic material derived from oxidative stress lies in the consumption of natural chemical agents that reduce or inhibit the mutagenic or carcinogenic activity of compounds [24].

Evidence obtained from epidemiological studies suggests that the foods consumed contain compounds that may play an important role in reducing DNA damage. Although the mechanisms of action are partially or not yet known, their antioxidant properties are of greatest interest.

3.6.   Epigenetic modifications

Epigenetic modifications are heritable changes in gene expression patterns that are independent of changes in primary DNA sequence and affect the outcome of a locus or chromosome without altering the underlying DNA sequence itself [30]. In other words, they are reversible changes that act as a lock on chromatin that opens or closes, by relaxing or condensing it, to allow or prevent transcription factors from joining genes. This creates patterns of epigenetic modulation and regulation of expression in the individual, with the probability of becoming hereditary. The most studied epigenetic mechanisms by which genetic regulation is carried out, as shown in figure 8., are DNA methylation, histone modifications, and non-coding ribonucleic acids (ncRNAs) [31].
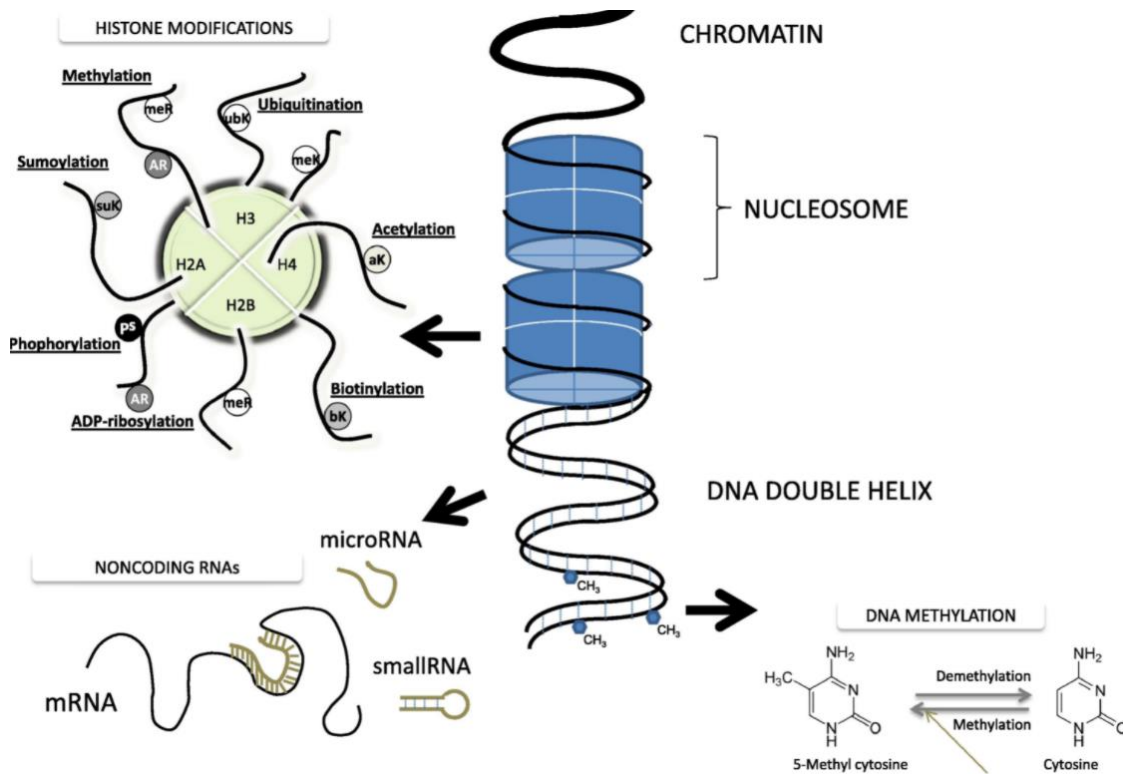
Figure 8. Major epigenetic mechanisms involved in gene expression regulation [31].

3.6.1. DNA methylation

DNA methyltransferases (DNMT *de novo* or *maintenance* enzymes) participate in this process, either by introducing the initial pattern of methyl groups on the DNA sequence, where it was not previously present; or, by copying the methylation pattern of a pre-existing DNA strand after cell replication. Adequate DNA methylation is suggested to be essential for cell differentiation and embryonic development, as well as for regulation of gene expression in different cell types. In the promoter region, where regulatory elements come together to help obtain the transcribed RNA, and with respect to DNA methylation, there are highly methylated genes that tend to be more packed, leading to a reduction in their expression; in contrast, genes with less mutilation tend to exhibit more flexible packaging, facilitating their expression [31–33].

In other words, methyltransferase enzymes add methyl groups at the 5' position of the cytosine of the CpG dinucleotide, a structure that is constantly repeated and forms CpG islands, regions of DNA where there is a high concentration of pairs of cytosine and guanine linked by phosphates, which make up about 40 % of gene promoters in mammals [24, 31, 34].

The addition of methyl groups to DNA does not allow the separation of both strands, therefore preventing the transcription of the DNA process and its carrier of genetic information, the mRNA. The mechanisms of DNA methylation are hypermethylation and hypomethylation [31].

3.6.1.1. Hypermethylation

The number of attached methyl groups increases in DNA, thereby suppressing gene expression (gene silencing), so there is no expression of proteins [31].

3.6.1.2. Hypomethylation

The number of attached methyl groups to DNA is reduced, allowing gene expression and resulting in an overexpression of proteins [31].
Consequently, both cases can produce cell damage due to lack or accumulation of a protein, which may be essential in some cell processes.

3.6.2. Histone modifications

Histones, which are the proteins responsible for packaging DNA and forming chromatin, are subject to a wide variety of post-translational modifications including, acetylation of lysines, methylation of arginine and lysine, phosphorylation of serine and threonine, or ubiquitination and sumoylation of lysine. These transformations occur mainly within the amino-terminal tails of histones that protrude from the surface of the nucleosome, as well as in the region of the globular nucleus [31, 35].
These histone modifications can affect chromosome function through two mechanisms, the first mechanism suggests alterations in the electrostatic charge of histones, structurally changing histones or binding to DNA. The second mechanism involves binding sites for protein recognition modules, such as Bromo-domains or Chromium-domains, which recognize, respectively, acetylated or methylated lysines. Finally, both mechanisms influence the regulation of gene expression through the rearrangement of chromatin and the formation of heterochromatin, chromosomal segments that appear more condensed [31].

3.6.3. Non-coding ribonucleic acid (ncRNAs)

ncRNAs are RNA molecules present in eukaryotic cells that do not code for any protein. These are classified into small (sncRNA, with less than 200 nucleotides) and long (lncRNA, from 200 to more than 100 kb). lncRNAs can act at the transcriptional level by binding to histones in unmethylated regions, remodeling chromatin, as is the case of lncRNA-MBD1, which binds to histone H19 forming a complex that prevents the DNA double helix from separating, so transcription cannot start; but it can also act at the post-transcriptional level, where lncRNAs can bind to regulatory proteins, allowing the activation or repression of transcription [30, 31].
On the other hand, within the group of sncRNAs, it is found the microRNAs (miRNAs), whose function is the silence mRNA and post-transcriptional regulation of protein synthesis, it plays an important role in the development of diseases (different types of cancer, cardiovascular and obesity) and has great potential as a biomarker and even as a therapeutic agent [36].
In other words, the epigenetic modifications act in coordination with each other to achieve the modulation of gene expression, creating a specific pattern of epigenetic marks on the genome (epigenome), which define the gene expression profile in each cell and tissue.

3.7. Oxidative stress and epigenetic modifications

Oxidative stress can modify the epigenome due to the effect that reactive oxygen species have on methylation, occurring through one of the two mechanisms of action or even the hypo and hypermethylation occurring at the same time. According to the literature, duality in methylation can occur due to various factors; however, hypomethylation specifically can be due to three processes [35–38]:

1. The elimination of reactive oxygen species where the synthesis of the endogenous antioxidant glutathione (GHS) is required, for which homocysteine is necessary [38]. Homocysteine requires S-adenosylmethionine (SAM) to be synthesized, which causes a depletion of this, and since SAM is the donor of methyl groups, DNMT cannot carry out DNA methylation [38].
2. The oxidative environment activates the translocation enzyme, which is involved in DNA demethylation processes by hydrolyzing 5-methylcytosine into 5-hydroxymethylcytosine, which is replaced by a demethylated cytosine through the base excision repair system [38].
3. Oxidative stress damages the guanine bases, forming 8-hydroxylguanine or 8-oxoguanine, also modifying the guanines of the CpG dinucleotide, so methylation does not take place [38].

On the other hand, regarding hypermethylation, it can occur due to the oxidative environment, which promotes the formation of DNMT-polycomb gene silencing complexes in addition to deacetylation processes [38].

Additionally, oxidative stress induces changes in histone acetylation and methylation, by acting on the enzymes responsible for chromatin maintenance, as well as affecting post-translational modifications of histones that regulate chromatin. However, the sensitivity of different histone groups varies, so the effect of oxidative stress varies based on its target [38].

Similarly, transcriptional regulation mediated by non-coding RNAs is also altered in several ways by oxidative stress, as it has been mentioned for methylation and histones modifications [38].

Accordingly with the literature, there is two new emerging properties of DNA that can help to understand why thermodynamically stable G-rich sequences would be enriched in regions that must be actively opened for mRNA synthesis [39].

First, due to GC-richness around alternative transcription start sites exists an enrichment of non-canonical G4 structures, i-motifs and Z-DNA [39].

Second, the G runs associated with potential G-quadruplex sequences render this sites highly sensitive to oxidative modification as a result of π-stacking of adjacent Gs lowering the ionization potential of the 5` most G in the run [39].

In other words, besides that G4 structures have shown their impact in the state of chromatin and thought to cause replication fork stalling [18, 27], these two features serve as a regulatory system during oxidative stress, hence, working as a G4 switch that can overcome the thermodynamic barrier to control gene expression and therefore, having an ambiguous functionality, when the structure itself positioned in the key genome areas, interrupting the replication fork progression,

and also, possibly acting as a recognition point for proteins involved in genome editing and DNA unwinding. [39].

To summarize, the physicochemical properties of the aforementioned dietary polyphenols, allows them to interact, intervene, induce, and regulate important biochemical process, especially the ones regarding the genetic material of the organism; through epigenetic modifications that can facilitate, maintain or regulate the formation of reactive oxygen species and therefore induce the oxidative stress. Although the mechanisms of action are partially or not yet well known, previous investigations provided that G-quadruplex formation is regulated and can respond to temporal signals associated with the cell cycle and development, as well as insights into how factors that act at G4 DNA structures may contribute to cancer and genetic disease.

4. EXPERIMENTAL PART

4.1. Materials

4.1.1. Phenolic compounds

The analyzed phenolic compounds were provided by:

Gallic acid (ACROS ORGANICS),

Ellagic acid (SIGMA-ALDRICH),

Curcumin (SIGMA-ALDRICH).

The solutions made of the chosen phenolic compounds were diluted in Milli-Q water at the concentration of interest for the purpose of each experiment and stored at 4 °C.

4.1.2. Cell culture

Used cell lines:

MCF-7,

H1299,

HDF164.

Epithelial cells MCF-7 are primary tumor cells line derived from adenocarcinoma breast and mammary gland tissue; they were isolated in 1970 from a 69-year-old white woman and the sample was provided by the Masaryk Institute of Oncology in Brno, Czech Republic.

Human non-small cell lung carcinoma cells H1299 were provided by the Masaryk Institute of Oncology in Brno, Czech Republic.

The human dermal fibroblasts (HDFs) were isolated from skin biopsies of a healthy adult donor (164), used as non-tumor cell line and provided by Evercyte, located in Vienna, Austria.

The isolation of the cells was approved by the respective local ethics commission and all donors gave informed consent.

Cell lines were grown in DMEM culture medium (Gibco) with stable glutamine (final concentration 4 mM), supplemented with 10 % Fetal Bovine Serum (FBS, SIGMA-ALDRICH) and 1x Penicillin-Streptomycin (Thermo Fisher scientific) in incubator with humidified atmosphere and 5 % CO_2, at 37 °C.

4.1.3. Oligonucleotides

MycPU52 (SIGMA)

Sequence: 5'-TTGGGGCGCTTATGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGGAGGAGACT
Length: 52 [nt].

hTel51 (SIGMA)

Sequence: 5'-GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG
Length: 51 [nt].

Random (SIGMA)

Sequence: 5'-ACCAGAGCTGATGGTATCCTAAGTTGACGACCCCGAGGGTGCCGCAAGGA
Length: 50 [nt].

4.1.4. Chemicals and solutions

75 % ethanol (Denatured Methanol),
AlamarBlue (Thermo Fisher Scientific),
Buffer Tris-HCl (pH 7,5) (SIGMA-ALDRICH),
Distilled water,
DMEM/F12 (1:1) High Glucose-Dulbecco's Modified Eagle Medium (Gibco),
DPBS-Dulbecco's Phosphate-Buffered Saline (SIGMA),
FBS-Fetal Bovine Serum (SIGMA-ALDRICH),
Glutamine Stable 100x 200 mM (biosera),
Milli-Q water,
Pen/Strep-Penicillin-Streptomycin solution 100x (biosera),
ThT-Thioflavin T, (SIGMA-ALDRICH),
Trypsin-EDTA 10x (biosera).

4.2. Instruments and Equipment

Analytical balance (Weight) XS105 (METTLER TOLEDO),
Autoclave Microjet Personal Microwave Autoclave (ENBIO),
CD spectrometer, J-815, (JASCO INTERNATIONAL CO.),
Cell culture flasks T25 and T75, (TPP),
Centrifuge 5804 refrigerated, (eppendorf),
CO_2 INCUBATOR, (CelCulture, ESCO),
Cordless electronic pipette filling device (accu-jet),
Counting chamber double net ruling with clamps (Bürker-Türker),
Deep freezing box in nitrogen refrigerated liquid LABS-40K (Taylor-Wharton),
Disposable sterile pipette of 5 ml, 10 ml and 25 ml (SPL),
Equipment for the production of Milli-Q Water (Synergy UV),
Fixable volume micropipettes from the volume of 0,1 to 1000 µl (eppendorf and VWR),
General material for daily use in the laboratory,
Graduated conical test tubes of 15 ml and 50 ml (TPP),
High-speed refrigerated centrifuge, minispin plus (eppendorf),
Inverted optical microscopy (VWR),
Laminar flow cabinet (ESCO),
Micro test plate 384-well (CORNING),
Micro test plate 96-well (TPP),
Micro test tubes 1,5 ml and 2 ml (eppendorf),
Multichannel variable volume micropipette from 20 to 200 µl (GILSON),
PC for Jasco 815 (Jasco International Co., Ltd., Tokyo, Japan),
PC with software Gen5 (BioTek)
Shaker MS3 (IKA),
Synergy H1 Hybrid Reader, (BioTek),

Thermomixer comfort 1,5 ml (eppendorf),
Vortex, Vortex 4 digital, (IKA).

4.3. Methods

4.3.1. ThT assay

Thioflavin T (ThT) is a benzothiazole dye used regularly to quantify the formation and inhibition of amyloid fibrils, which increases in fluorescence upon binding to it, in the presence of anti-amyloidogenic compounds or anti-aggregates [40–42].

Although the mechanism of interaction between ThT and amyloid fibrils remains poorly understood, the most widely accepted theory involves the intercalation of ThT molecules within grooves between solvent-exposed side chains of the amyloid fibril that run parallel to the fibril axis, as shown in the figure 9. [40–42].

As there is a stoichiometric and saturable interaction between ThT and amyloid fibrils, fluorescence from the amyloid–ThT complex provides accurate quantification of amyloid fibril formation as a function of amyloid fibril length and number. In this assay, ThT was simply added to samples containing fibril structures at a concentration in excess of the number of potential ThT–fibril binding sites, and steady-state ThT fluorescence emission was monitored at 490 nm after excitation at 425 nm [40–42].

In other words, is used as a fluorescent light-up probe for the formation of G-quadruplexes; when ThT is added to the DNA structure pre-formed in Tris-HCl and KCl buffer, where it becomes fluorescent in the presence of the G4 structure. Even though, ThT can possibly bind to single-stranded or double-stranded oligonucleotides, the signal is significantly lower compared to G4 structures [43].
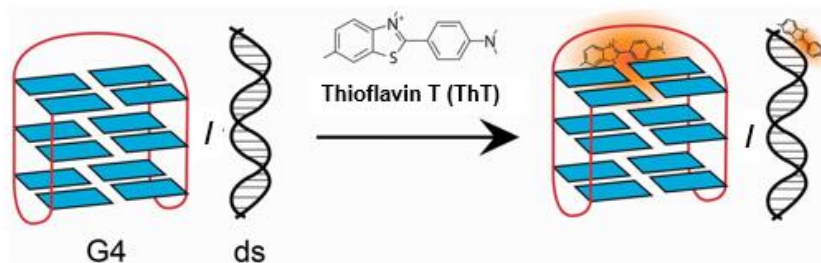
Figure 9. Principle of ThT test [43].

ThT was diluted in milli-Q water to a final concentration of 100 μM. After that, the dilution of ThT in milli-Q water were set at a final concentration of 1 μM and stored in a dark place at 4 °C.

The oligonucleotides were diluted in 100 mM Tris-HCl (pH 7,5) and 100 mM KCl buffer to a final concentration of 2 μM. The samples were heated to 95 °C for five minutes and then slowly cooled to room temperature.

The wells in the black 384-well microplate were filled with 10 μl of a prepared solutions of ThT, Milli-Q water and the phenolic compound of interest. Table 1. refers to the concentration and volume used for the direction of the experiment.

After distributing the solutions of different phenolic compounds in the black 384-well microplate, the same amount of oligonucleotide, 10 μl, at 2 μM concentration was added, which means a final ratio of 1:0,5 between the oligonucleotides and ThT, to reach a final volume of 20 μl per well.

After homogenizing the solution with a shaker for 15 minutes, the fluorescence emissions were measured at 490 nm after excitation at 425 nm.

All experiments were performed in triplicate, chemically and for three oligonucleotides in total, two containing G4 motif and one not containing it, as control, at room temperature.

Table 1. Concentration and volume used for competition test.

Concentration [μM]	ThT volume [μl]	Ligand's volume [μl]	Water volume [μl]
0,00	1,00	0,00	9,00
1,00	1,00	1,00	8,00
2,00	1,00	2,00	7,00
4,00	1,00	4,00	5,00
8,00	1,00	8,00	1,00

4.3.2. CD spectroscopy

Circular dichroism spectroscopy is a technique where the difference in the absorption of left and right circularly polarized light in optically active substances is measured. The technique is based in electromagnetic waves, which contains electric and magnetic field components that oscillate perpendicularly in the direction of a light beam's propagation. The directionality of these components defines the waves' polarization [44, 45].

In unpolarized light or white light, the electric and magnetic fields oscillate in many different directions; in linearly polarized light, the electromagnetic wave oscillates along a single plane and in circularly polarized light (CPL) two electromagnetic wave planes are at a 90° phase difference to one another and this plane rotates as the light beam propagates, as shown in figure 10 [44, 45].

The CD spectra of DNA provides information on the molecule's secondary structure that is dependent on the stacking orientation of the base pairs. While the base pairs themselves are not intrinsically chiral and alone would not exhibit a CD signal, they are covalently bound to the DNA backbone chain composed of sugar moieties [44, 45].

In other words, circular dichroism is an advanced biophysical technique for studying G4 structures and ligand binding since certain molecules interact differently with left and right circularly polarized light.
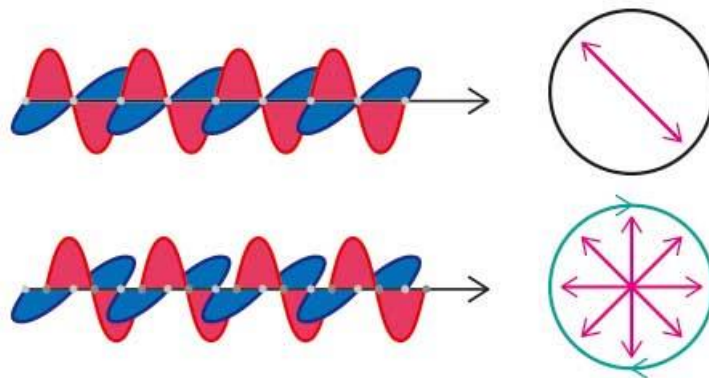
Figure 10. Linearly polarized light (upper), circularly polarized light (lower) [45].

The CD spectra were measured on Jasco J815 spectropolarimeter in 1 cm cuvettes at 20 °C. The oligonucleotides were diluted in buffer containing Tris-HCl (10 mM and pH 7,5) at a concentration of $1,176 \times 10^{-6}$ M for hTel51 and $1,154 \times 10^{-6}$ M for MycPU52, with a final volume of 220 μl. DNA samples were prepared by heating at 95 °C for five minutes and then slowly cooled to room temperature.

For each sample, a series of four scans in the wavelength range 210–330 nm, with a step of 1 nm and a scan speed of 100 nm/min, were taken, averaged, smoothed, and the background read. To achieve the desired effects, aliquots of all three ligands were added in steps at a ratio of 0,1; 1; 2; 3; 4 and 5 molar equivalents to DNA. Table 2. refers to the concentration and volume used for the direction of the experiment.

Table 2. Molar equivalence, concentration and volume of selected ligands and oligonucleotides.

Molar equivalence [e]	Concentration [μM]	Oligonucleotide's volume [μl]	Ligand's volume [μl]
0,00	0,00		0,00
0,10	0,12		+ 0,52
1,00	1,18		+ 4,68
2,00	2,36	220,00	+ 5,20
3,00	3,54		+ 5,20
4,00	4,72		+ 5,20
5,00	5,90		+ 5,20

4.3.3. AlamarBlue (AB) assay

It is a fluorometric assay also known as resazurin reduction assay, it is based on the conversion of the non-fluorescent blue dye resazurin, a phenoxazin-3-one dye, into pink/red fluorescent resorufin by mitochondrial enzymes and others such as diaphorases [46].

This dye acts as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor [46].

Viable cells with active metabolism are permeated by this dye and during their function continuously reduce resazurin to resorufin, as shown in figure 11., increasing the overall fluorescence and color of the cell culture medium [46].
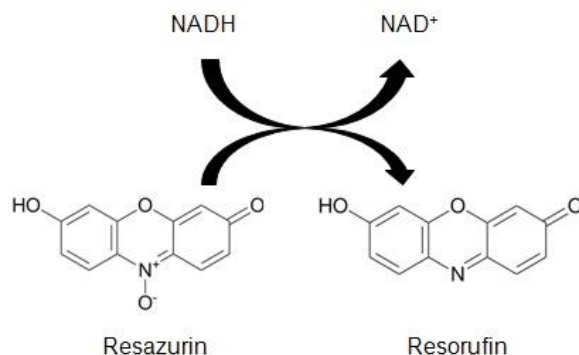

Figure 11. Structure of resazurin substrate and resorufin product resulting from reduction in viable cells [46].

Cell cytotoxicity assays were carried out separately for the different phenolic compounds (Gallic acid, Ellagic acid and Curcumin). Cells were plated in micro test plate 96-well in their complete medium at the optimal density, literature suggestion of 3,5 x 10^3 cell/cm^2.

After 24 h, medium was removed and replaced by only medium [(control) in the first column] and by medium supplemented with increasing concentrations of compounds diluted, up to 72 h treatment.

Cells were observed with optical inverted microscope during the time treatment to check on morphological changes, cell suffering or death. On the fourth day after seeding, cell cytotoxicity was determined by AlamarBlue (AB) assay.

Briefly, a 1:11 ratio solution of AlamarBlue and media was prepared according to the volume required (100 μl per well). After the addition, was handled in the absence of light due to light sensitivity and fluorescence was measured in emission at 595 nm after excitation at 555 nm using the Synergy H1 Hybrid Reader, being this the measurement at time zero.

After an additional incubation of 2 h at 37 °C, fluorescence was measured again under same conditions. All experiments were performed in triplicate, technically and biologically.

Relative IC$_{50}$ values were determined by nonlinear regression of variable slope (four parameters) model by GraphPad Prism version 9.3.1 for Windows, GraphPad software by Dotmatics, www.graphpad.com.

5. RESULTS AND DISCUSSION

5.1. *In vitro* G4-ligand interaction test

As it was mentioned during the theoretical part, the G4, secondary structures of DNA, rich in guanine and that can be formed from one, two or four strands; occur naturally near the ends of chromosomes, the telomeric regions, and in transcriptional regulatory regions of multiple genes [27, 29, 39].

The placement and formation of G-quadruplex is not random and has very unusual functional purposes, related to biochemical mechanisms of high importance in genetic regulation and functionality. its study lies in the possible interaction of specific phenolic compounds in the stabilization of the G4 structure that consequently promotes its functionality regarding the biochemical mechanisms related to epigenetic modifications and oxidative stress [27, 29, 39].

5.1.1. ThT competition test

The interaction between G4 and G4-ligands was first verified *in vitro* using a ThT competition assay. Thioflavin T acts as a fluorescent light-up probe, where an increase in the fluorescent signal at 490 nm is detected, means that ThT is binding to the G4 structure [43].

The oligonucleotides hTel51, MycPU52 and Random sequence were tested. The Random oligonucleotide does not contain a G4 sequence, hence, no binding occurred after the addition of ThT, and therefore the fluorescence signal measured at 490 nm should be the lowest of all three oligonucleotides.

The remaining two oligonucleotides contain a G4 sequence, therefore G4-ligands bind was expected.

The intensity in the fluorescence signal, shown in graph 1., was higher for MycPU52 than for hTel51 or the random sequence, meaning that MycPU52 was more prone to form G4 structures in this environment, this can be possible due to the guanine rich regions present in the oligonucleotides, implicating that is more abundance on G-rich regions in the MycPU52 oligonucleotide.

The three phenolic compounds (Gallic acid, Ellagic acid and Curcumin) were added to these oligonucleotides at different concentrations - 1, 2, 4 and 8 μM, where each of the additions were performed in triplicate.

The principle is that a G4-ligand replaces Thioflavin T into binding to G4, which results in a decrease in the fluorescence signal. The results, Graph 2. and Graph 3., show that with increasing concentration of ligands, the fluorescence signal decreased – if the ligand thus replaced ThT in binding to G4. The values in the graphs are expressed as percentages, with the signal of the oligonucleotides alone with ThT being 100 %. For ligands where the autofluorescence was an issue, were measured by itself and then subtracted from the measure. In addition, the standard deviations from the three replicates were marked on the histogram.

The result for the random oligonucleotide is available in the attachment 1.
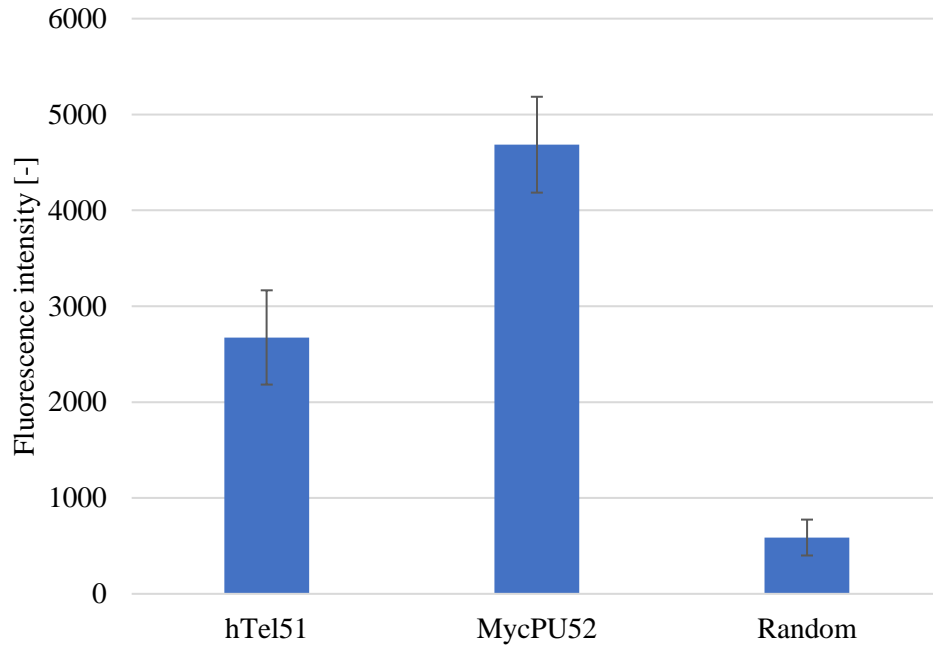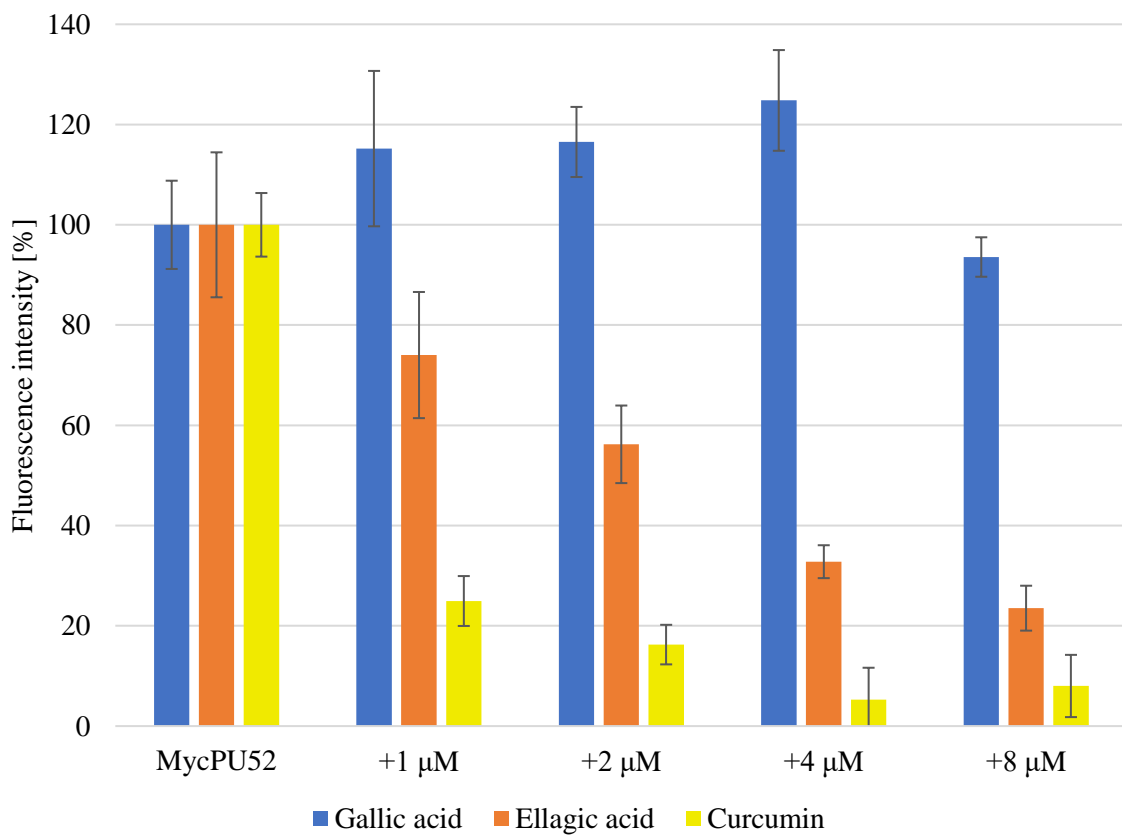
Graph 1. ThT competition assay for oligonucleotides.
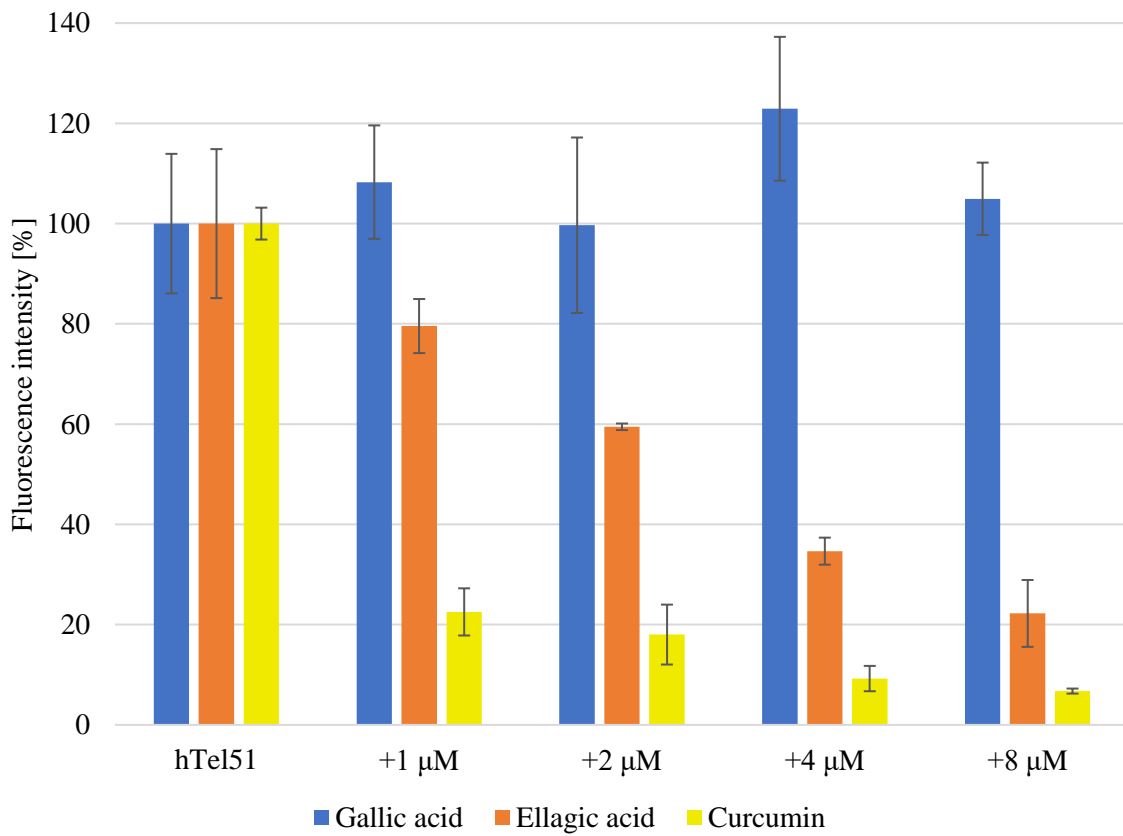
Graph 2. ThT competition assay for oligonucleotide hTel51 and G4-ligands gallic acid, ellagic acid and curcumin.

Regarding the ThT assay for the oligonucleotide hTel51 (Graph 2.), which contains the zones rich in guanine and therefore promotes the formation of G4 structures, a decrease in the fluorescence signal was observed during the addition of increasing concentrations in ellagic acid and curcumin, as expected.

However, regarding gallic acid, inconsistencies were observed in the measurement, indicating that this specific phenolic compound does not bind to G-motif in this system or is not strong enough and therefore did not participate as a stabilizer of G4 structures and therefore, through its physicochemical properties, acts differently regarding its antioxidant properties. Though, to have certitude about this, it is recommended further experiments that can corroborate the statement.

In the case of ellagic acid and curcumin, it showed that curcumin had greater stabilizing power of G-quadruplex structures, which, when adding 1 µM of curcumin, the intensity of fluorescence was reduced by 80 % approximately, compared to the 20 % reduced of ellagic acid.

Graph 3. ThT competition assay for oligonucleotide MycPU52 and G4-ligands gallic acid, ellagic acid and curcumin.

The situation was similar for the oligonucleotide MycPU52 (Graph 3.), which contained G4 motif and where the fluorescence signal decreased after the addition of the phenolic compounds ellagic acid and curcumin. However, the variable fluorescence signal for gallic acid persists.

For both oligonucleotides, curcumin showed the best binding properties when compared to ellagic acid, thus binding more stably to the G4 motif.

Based on the results, it can be inferred that the most efficient G4-ligand was curcumin due to the most significant decrease in the fluorescence signal detected for each phenolic compound.

5.1.2. CD spectroscopy

G-quadruplex structures plays an important role in gene regulation and some ligands have recently been identified to be very effective for stabilizing them; therefore, CD spectroscopy is widely used to monitor thermal unfolding of G-quadruplex structures [19].

In the UV region of the spectrum, a peak around 260 nm and a trough around 240 nm are the specific signatures of a parallel G-quadruplex formation, whereas a positive band at 295 nm and a negative band at 260 nm are indicative of an antiparallel conformation [19]. In addition, the linear increase or the shifting of the peak due to the increased concentration of the ligand, indicates a greater stabilization of the G4-quadruplex.
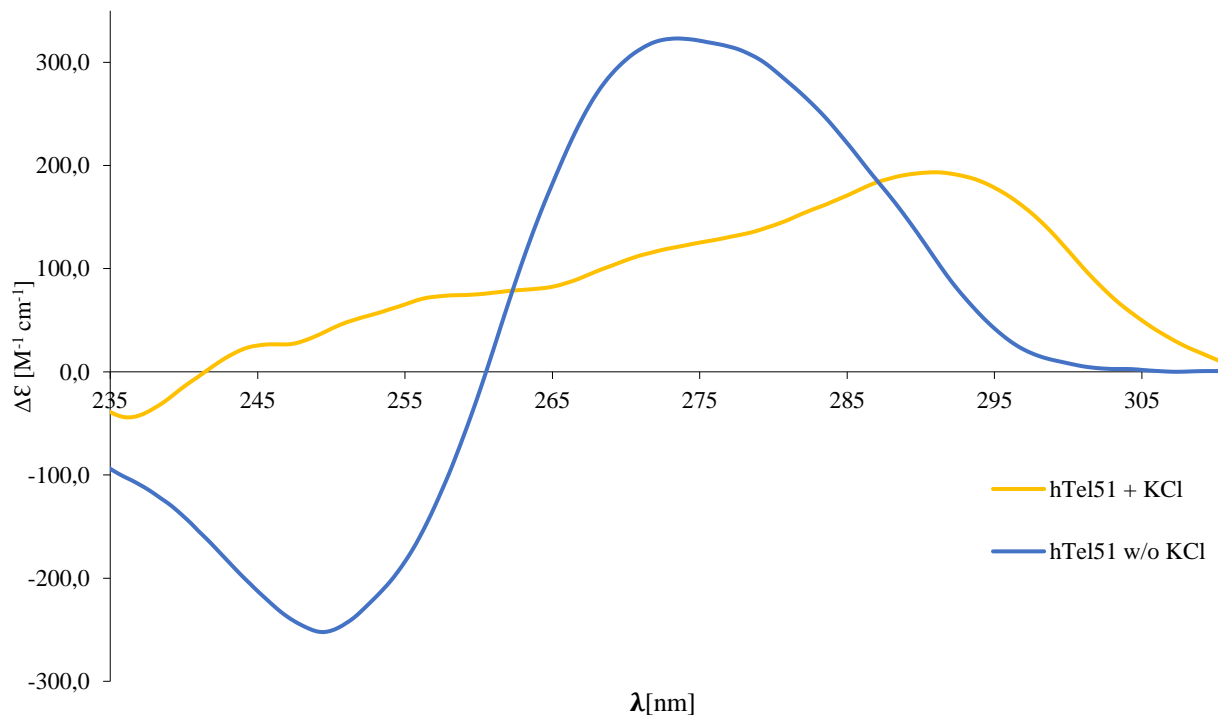
Graph 4. CD spectroscopy of G4 oligonucleotide hTel51 and its stabilization by KCl.

As it was showed in graph 4. and regarding the literature, the hTel51 oligonucleotide in addition with the stabilizer KCl, presented an antiparallel conformation for G-quadruplex due to the peak at 295 nm. On the other hand, as showed in the graph 5., the oligonucleotide MycPU52 was associated with a negative peak around 240 nm and a positive peak around 260 nm, therefore associated to the specific signatures of a parallel G-quadruplex formation.
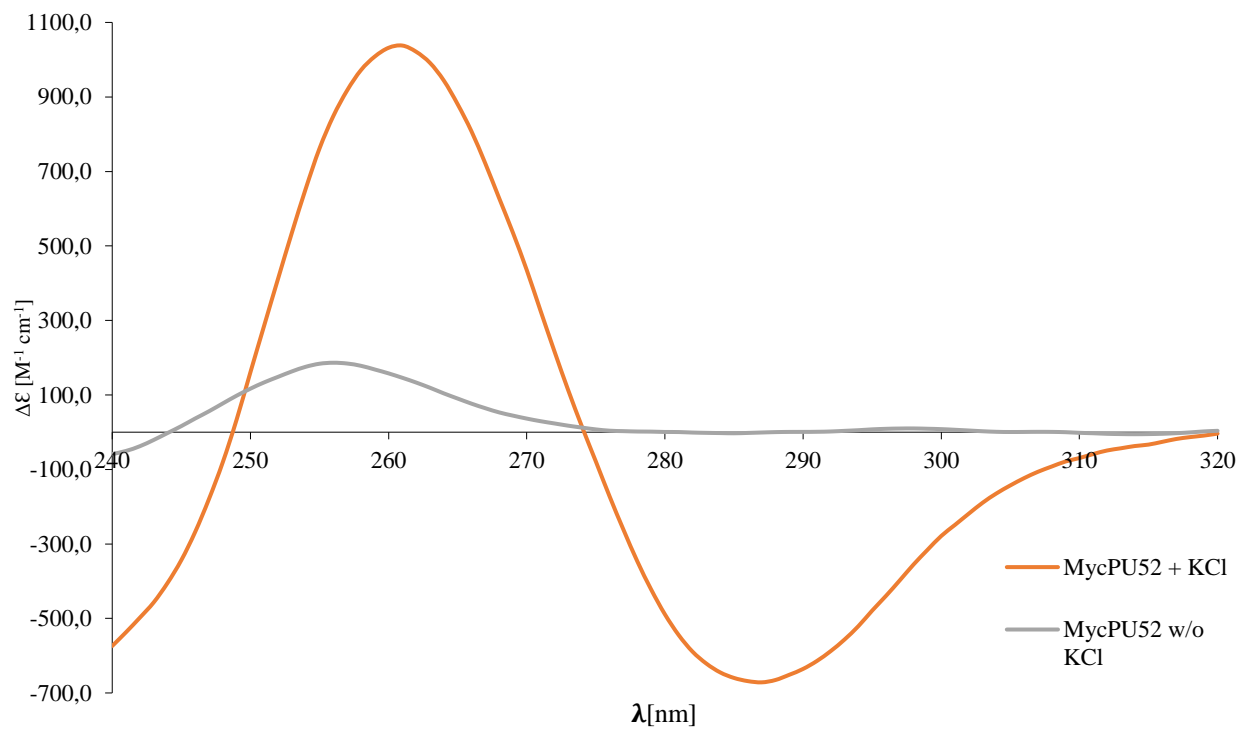
Graph 5. CD spectroscopy of G4 oligonucleotide MycPU52 and its stabilization by KCl.
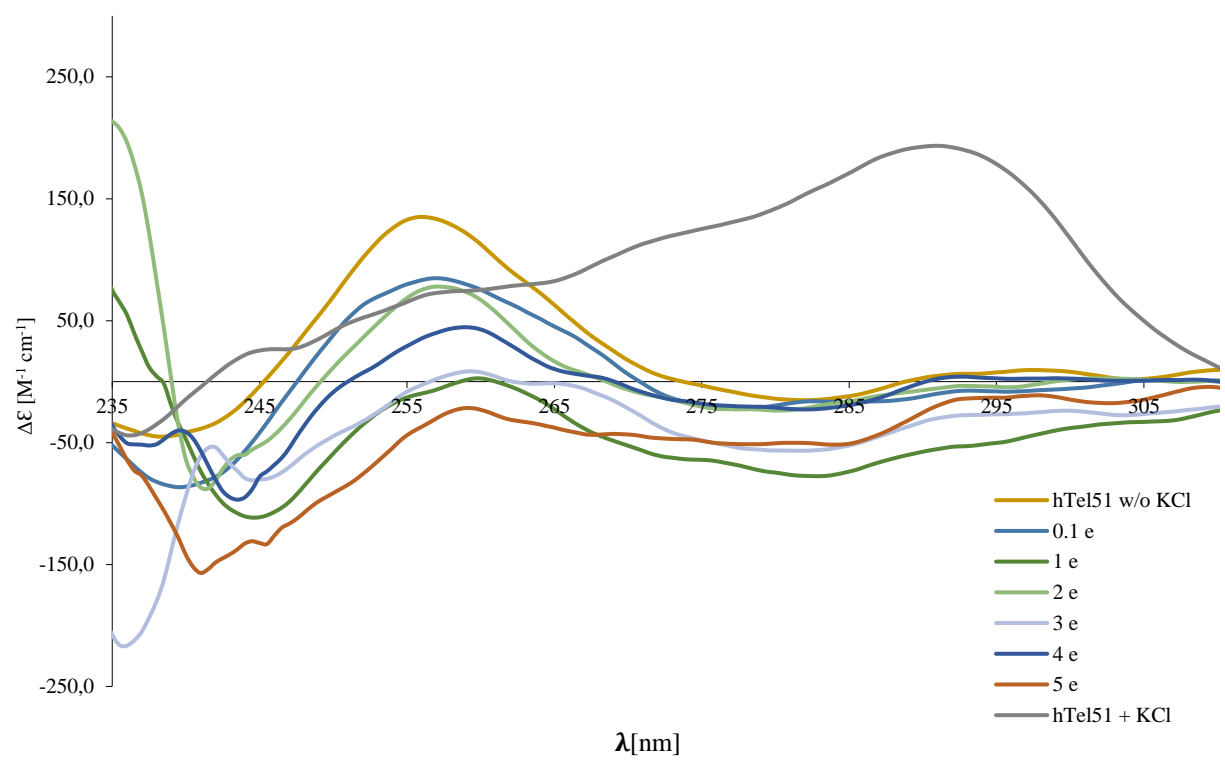
Graph 6. CD spectroscopy of G4 oligonucleotide hTel51 in addition of gallic acid at increasing concentration from 0.1 to 5 molar equivalents [e].
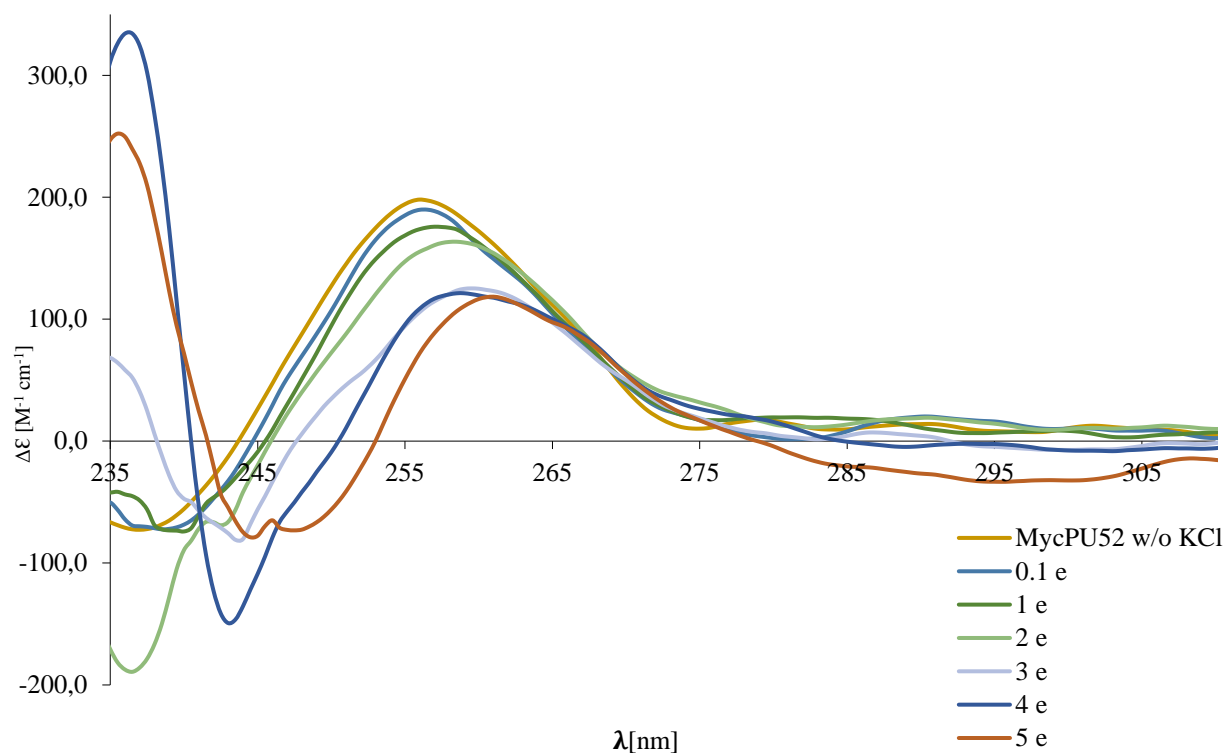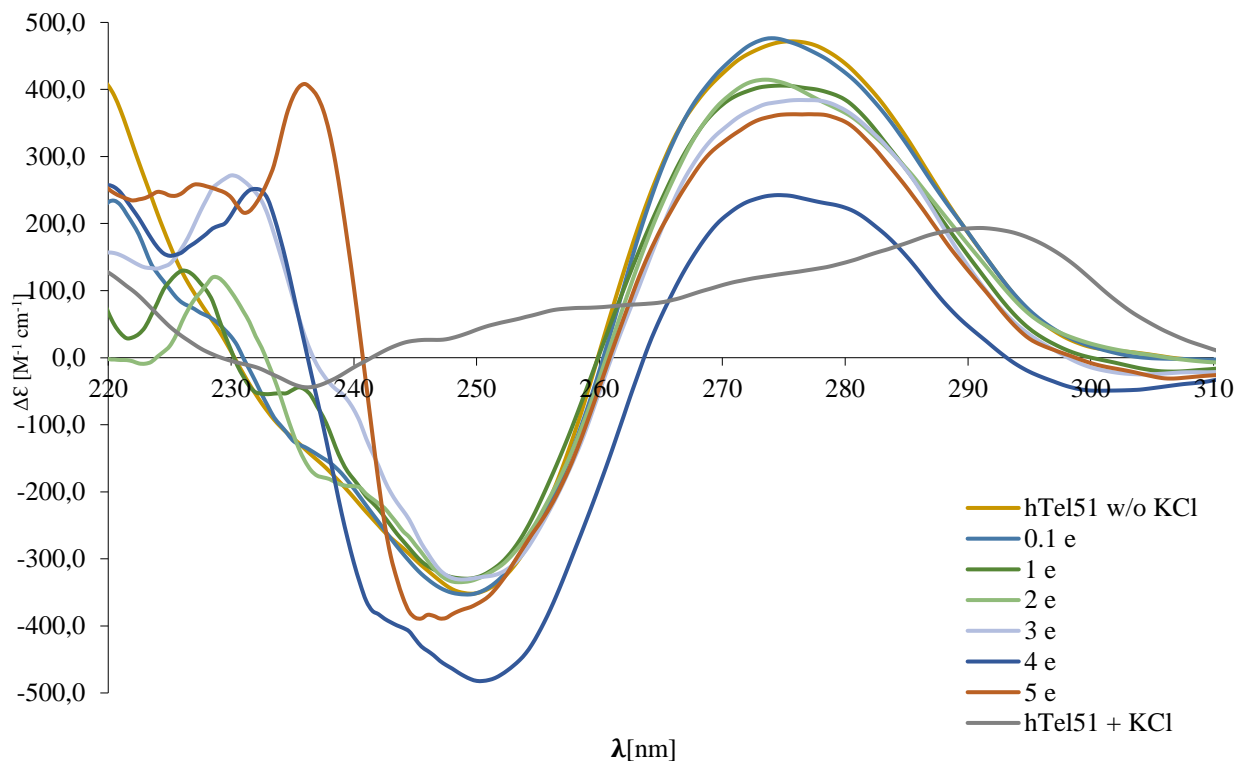
Graph 7. CD spectroscopy of G4 oligonucleotide MycPU52 in addition of gallic acid at increasing concentration from 0.1 to 5 molar equivalents [e].

The affinity of G4-ligands in the presence of G4 structures was confirmed by the ThT competition assay. Nonetheless, with the aid of CD spectroscopy, it was possible to determine the conformation of the respective G4 motifs.

For purpose of a better visualization of peaks shifting or any other relevant change during the examination, the graph of the MycPU52 oligonucleotide with KCl was not attached, because by presenting such wide values, the range of values of the ordinate axis in the graph increases and therefore, the confluence of the graphs plotted at different molar equivalencies would not be easy to examine.

As expected, after the ThT assay, gallic acid did not present any activity linked to the G4 structure, so what was described before can be confirmed by graph 6., where the hTel51 oligonucleotide with antiparallel conformation is indicating that gallic acid does not bind to G-motifs in this system, or the molar equivalence used during the experimental test was not sufficient.

However, regarding the MycPU52 oligonucleotide, there is an insufficient attempt to show a negative peak at 240 nm, but there is a shifting progress around 265 nm while increasing the concentration of the gallic acid. In other words, gallic acid was related to a parallel G-quadruplex formation, which is characteristic conformation to this c-myc oligonucleotide type.
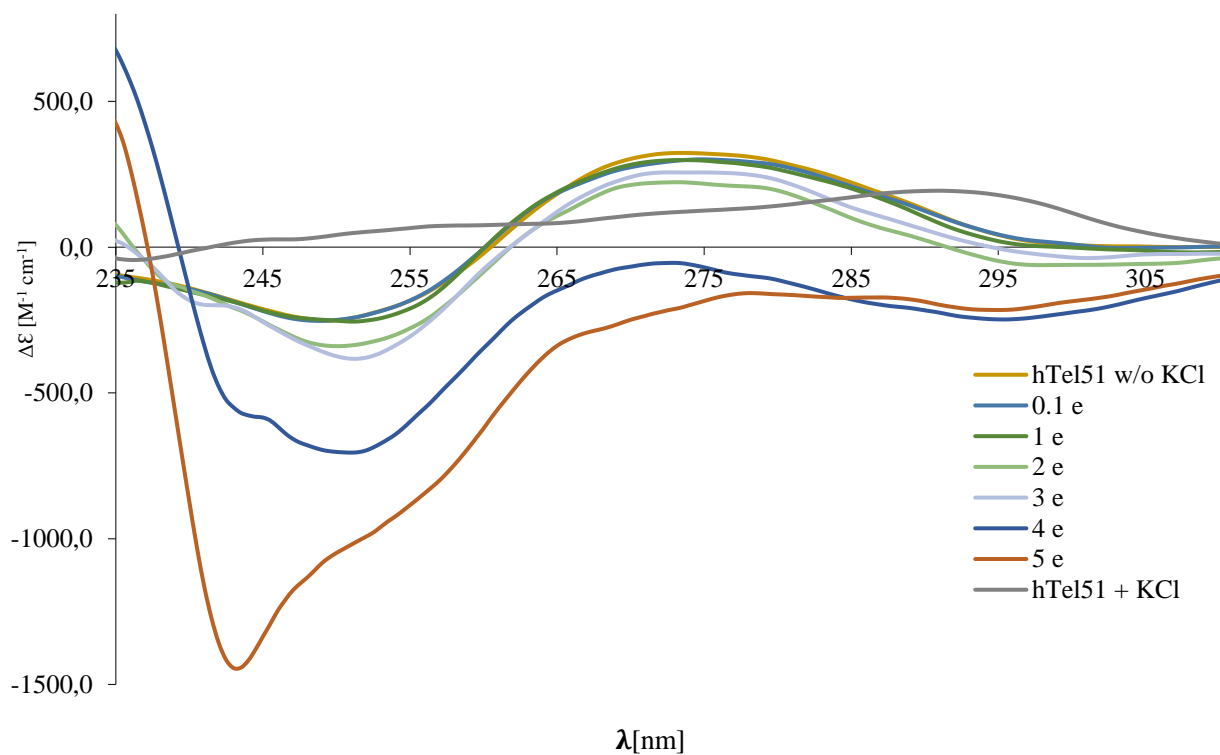
Graph 8. CD spectroscopy of G4 oligonucleotide hTel51 in addition of ellagic acid at increasing concentration from 0.1 to 5 molar equivalents [e].
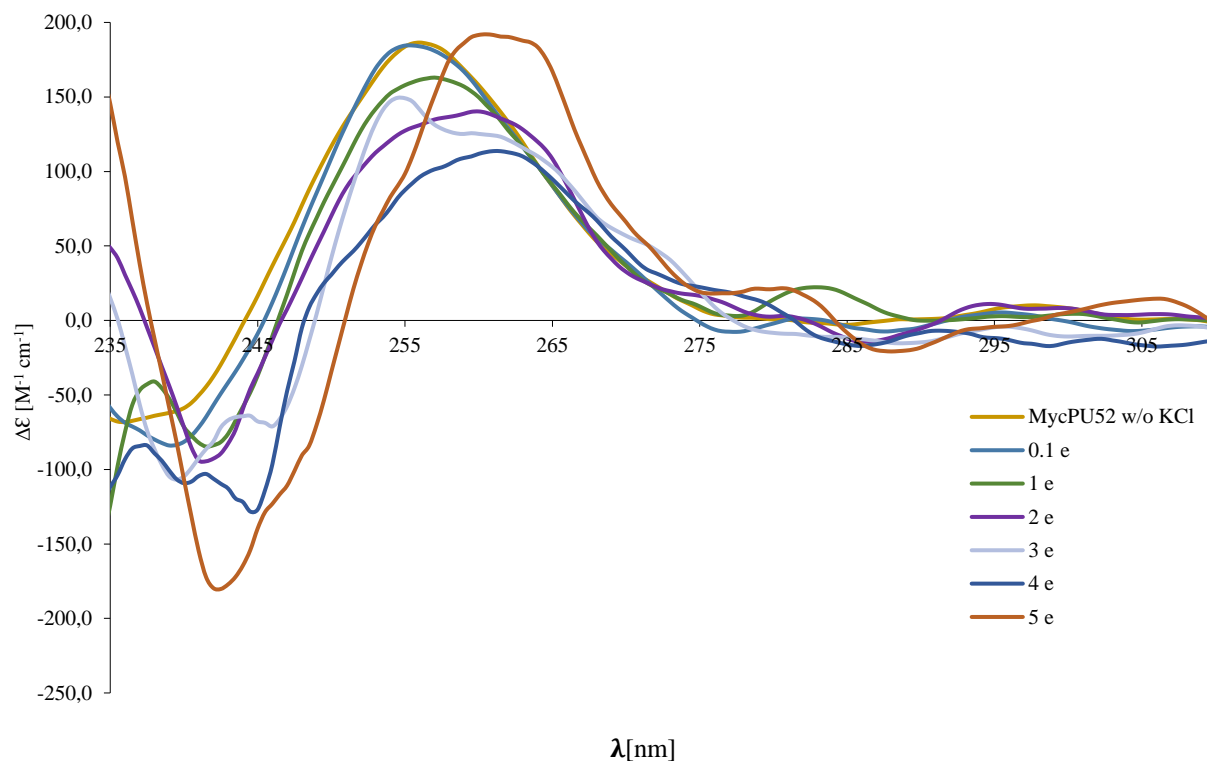
According to the literature and visual confirmation of antiparallel conformation of G4 structures in the hTel51 oligonucleotide in graph 4., the addition of ellagic acid did not interfere with these structures, as it can be seen, where the peak was misplaced at another wavelength in graph 8. However, during the examination of the MycPU52 oligonucleotide trough the CD spectroscopy plotted in the graph 9., a positive shift was observed as the concentration of the ligand was added, which is a similar situation to the already mention gallic acid and therefore presenting a confirmation of the ellagic acid as a G4-ligand.
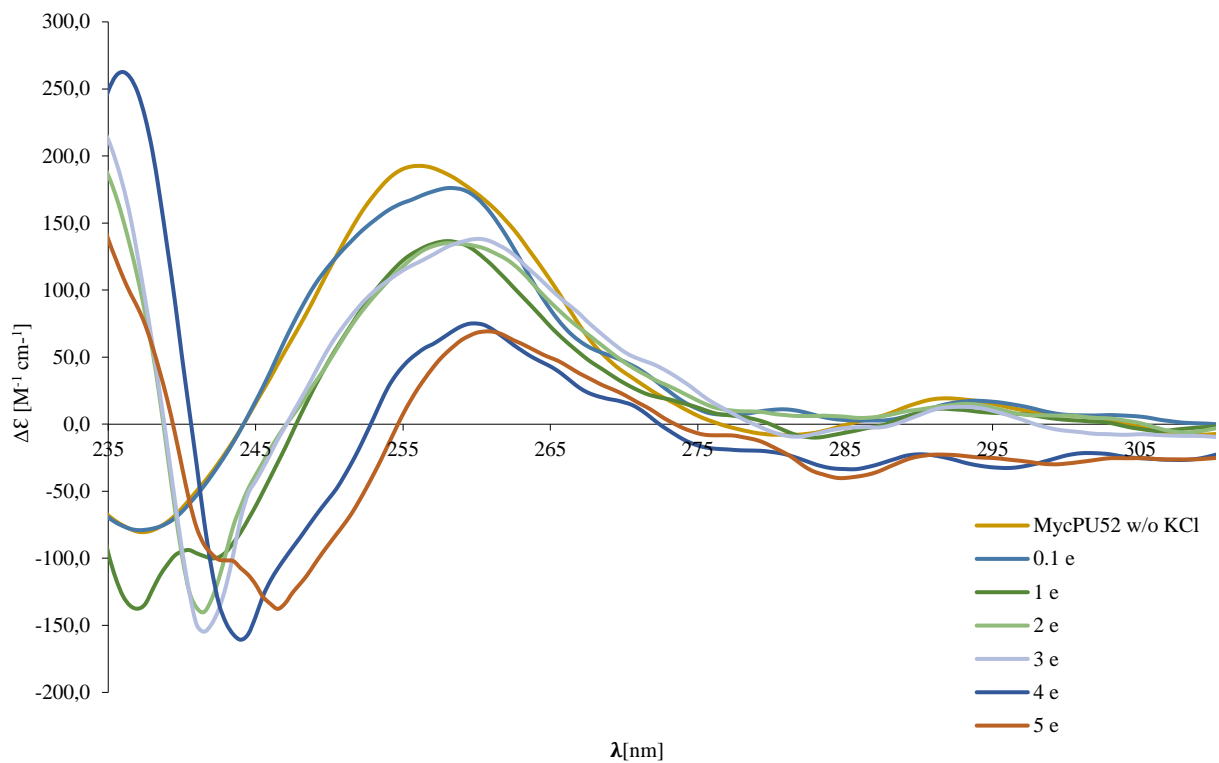
Graph 9. CD spectroscopy of G4 oligonucleotide MycPU52 in addition of ellagic acid at increasing concentration from 0.1 to 5 molar equivalents [e].

Graph 10. CD spectroscopy of G4 oligonucleotide hTel51 in addition of curcumin at increasing concentration from 0.1 to 5 molar equivalents [e].

Graph 11. CD spectroscopy of G4 oligonucleotide MycPU52 in addition of curcumin at increasing concentration from 0.1 to 5 molar equivalents [e].

According to the literature and visual confirmation of antiparallel conformation of G4 structures in the hTel51 oligonucleotide in graph 4., the addition of curcumin did not interfere with these structures, as seen the peak misplaced at another wavelength in graph 10. However, during the examination of the MycPU52 oligonucleotide trough the CD spectroscopy plotted in the graph 11., a positive shift was observed as the concentration of the ligand was added, including an increase peak value around 260 nm.

Regarding the oligonucleotides used for the experiment, it can be inferred that the addition of phenolic compounds to the hTel51 in the absence of KCl did not cause any significant shift of the peaks to a given G4 conformation, therefore, the selected polyphenols do not stabilize its characteristic antiparallel conformation. Nonetheless, these results are not related to the possible ability of G4-ligands to bind to hTel51, as confirmed in the ThT assay, at least for ellagic acid and curcumin.

5.2. Cytotoxicity assay

The tested phenolic compounds, acting as pro-oxidants, generating sufficient levels of reactive oxygen species to induce oxidative stress, and leading to apoptosis of the carcinoma cells [47], showed a dose dependent mechanism in inhibiting the growth of tumor cell lines (MCF-7, breast cancer and H1299, lung cancer), against a lower level of inhibition found in the non-tumoral control cell line (HDF164, human dermal fibroblasts).

Nevertheless, as showed in attachments 2. and 3.; the phenolic compounds seemed to have a positive effect on the cell lines, tumoral and non-tumoral, where at low concentrations, such as 1,56 µM and 3,12 µM for gallic and ellagic acid, the number of living cells were increased thanks to them and in the same way, low concentrations of curcumin, such as 0,15 µM and 0,31 µM, presented the same effect. Therefore, at low concentrations, the chosen phenolic compounds had a stimulating effect, while at higher concentrations, they were more lethal for the cells.

The cytotoxic evaluation of the phenolic compounds by the AlamarBlue assay and the subsequent calculation of the IC_{50} allowed to stipulate which phenolic compound was the most selective substance, the one presenting a high potential for anticancer activity.

A selective substance means that an optimal result is given when the IC_{50}'s for the cancer cell line is lower compared to the IC_{50}'s for the non-cancer cell line. Here the table 3. shows the substance with greater efficiency and with greater selectivity.

The most effective phenolic compound is the one that requires less concentration to inhibit the biological process, cause cell damage or cell death, which in this case corresponds to curcumin, with values lower and/or close to 1 µM; however, its selectivity was not appropriate because in contrast to the cytotoxic evaluation in the non-tumor cell line, it showed the same lethality.

That is why, when making a more detailed analysis of what this IC_{50} really represents, it was found that ellagic acid was the most selective phenolic compound among these three, since the average concentration to inhibit carcinogenic cells was relatively more appropriate regarding the IC_{50} value on non-tumor cells.

Table 3. IC_{50} [µM] results from 72 h treatment of MCF-7, H1299 and HDF cell lines with selected phenolic compounds.

	IC_{50} [µM]			IC_{50} [µg/ml]		
	MCF-7	H1299	HDF164	MCF-7	H1299	HDF164
Gallic acid	51,19	43,79	82,55	8,71	7,45	14,04
Ellagic acid	18,50	15,86	30,72	5,59	4,79	9,28
Curcumin	0,85	1,26	0,79	0,31	0,46	0,29

However, the values for IC_{50} found during the cytotoxicity assay may varied when compared to literature; this could have happened due to confounding factors during the performance of the experiment, such as, the seeding cell number, the concentration or the structural arrangement of the phenolic compound added, the concentration of the reagent added, the incubation period, the supernatant removal, the type of culture media or the cell line used; all this cause limitations during the comparison of results with other investigations, showing changes in the morphology of the cell, altering the cell functionality and system, or even induce of cell death.

This can be notice for example in Rezaei-Seresht et al., 2019 [48], where the IC_{50} value of gallic acid against MCF-7 was 18 µg/ml after 72 h treatment, or the IC_{50} value of ellagic acid against MCF-7 was 0,65 µg/ml after 72 h treatment in Saadullag et al., 2020 [49], or the IC_{50} value of curcumin against MCF-7 was 30,78 µg/ml after 72 h treatment in Khazaei Koohpar et al., 2015 [50].

6. CONCLUSION

A diet rich in fruit and vegetables, along with healthy lifestyles and awareness of the surroundings are considered protective actions against cancer development and also a way to reduce the risk of suffering other inherited diseases. Nonetheless, the lifestyle changes do not have any protector effect in patients within a wide range of cancers or other hereditary diseases, but to help in prevention and carrying of them.

The dietary polyphenols, naturally found in plant-based food does not represent a risk in health when not taken in excess since they are found in suspension or dilution; used during the experimental work are known for their effects anti-tumors by targeting the epigenetic machinery and related mechanisms such as stabilization of G-quadruplexes, especially in parallel conformation, as described during the CD spectroscopy.

These natural phytochemicals have the potential benefit due to its physicochemical properties, including the reverting effects in the epigenetic modifications in tumor suppressor genes, as well as its capacity to counteract or induce the oxidative stress.

Among the mechanisms of participation or interaction in biological and biochemical functions, the chosen phenolic compounds have shown that through stabilization of G4 structures and higher concentrations, a remarkable trail to inhibit or arret damages due to reactive oxygen species and any other innate part or xenobiotics, as to inhibit the reproduction of tumoral cell lines, whereas at low concentrations, a stimulation of the cell reproduction process.

Through *in vitro* experiments, the effect of polyphenols on the G4 secondary structure of DNA was verified, which allowed to probe their ability to stabilize them, specifically in a parallel way. This binding is important because biochemical processes depend on its stability, especially those related to oxidative stress and tumor diseases.

In addition, the selectivity of the evaluated compounds was determined through a *in vivo* cytotoxic assay, indicating that due to its effectiveness in inhibiting biological processes, curcumin has greater strength, but against therapeutic antitumor effects, ellagic acid is more selective.

7. LITERATURE

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8. LIST OF ABBREVIATIONS AND SYMBOLS

O_2^- - Superoxide anion,

[kb] – Binding constant,

[nt] – Nucleotide's length,

·OH - Hydroxyl radical,

$^1O_2$ - Singlet oxygen,

Acetyl-CoA – Acetyl coenzyme A,

ATP – Adenosine triphosphate,

CAT – Catalase,

C-C – Carbon-carbon bond,

CpG – 5'-C-phosphate-G-3' or cytosine nucleotide followed by guanine nucleotide,

Cu – Copper,

DNA – Deoxyribonucleic acid,

DNMT – DNA methyltransferase,

Fe – Iron,

G4 – G-quadruplex, guanine secondary structures DNA,

GC – Guanine-cytosine

GHS – Endogenous antioxidant glutathione,

H19 – Gene, which control the expression of several genes within the imprinted gene network,

H2O2 – Hydrogen peroxide,

HO_2· - Hydroperoxyl,

KRAS – Ki-ras2 Kirsten rat sarcoma viral oncogene,

lncRNA – Long Non-coding RNA,

lncRNA-MBD1 – Specific long noncoding RNA with chromatin-modifying complex MBD1,

miRNA – MicroRNA,

Mn – Manganese,

mRNA – Messenger RNA,

mRNA – Messenger RNA,

ncRNA – Non-coding RNA,

NO - Nitric oxide,

NO_2 - Nitrogen dioxide,

NO_3 - Nitrate radical,

O2 – Oxygen,

O_3 - Ozone,

Q - Semiquinone,

RNA – Ribonucleic acid,

RO· - Alkoxyl,

ROO· Peroxide,

SAM – S-Adenosyl methionine,

Se - Selenium

sncRNA – Short Non-coding RNA,
SOD – Superoxidase dismutase,
Zn – Zinc.

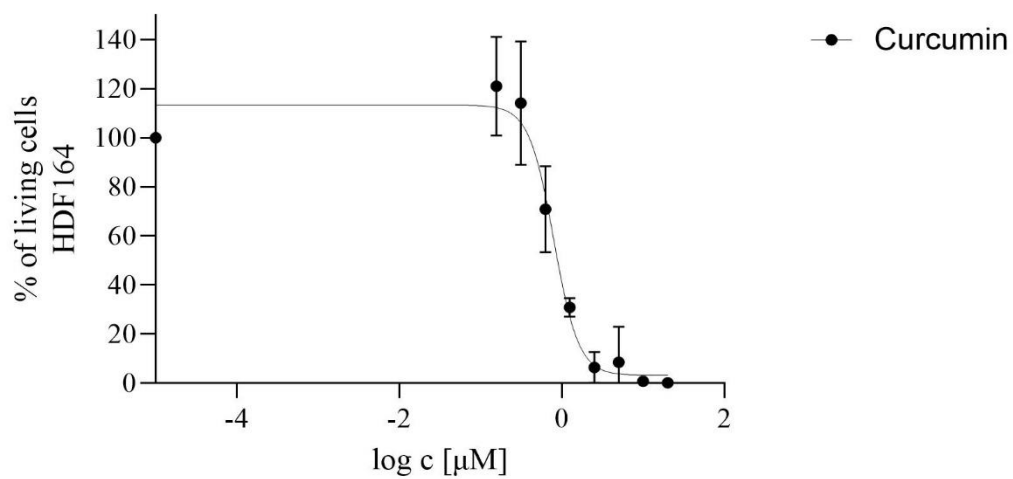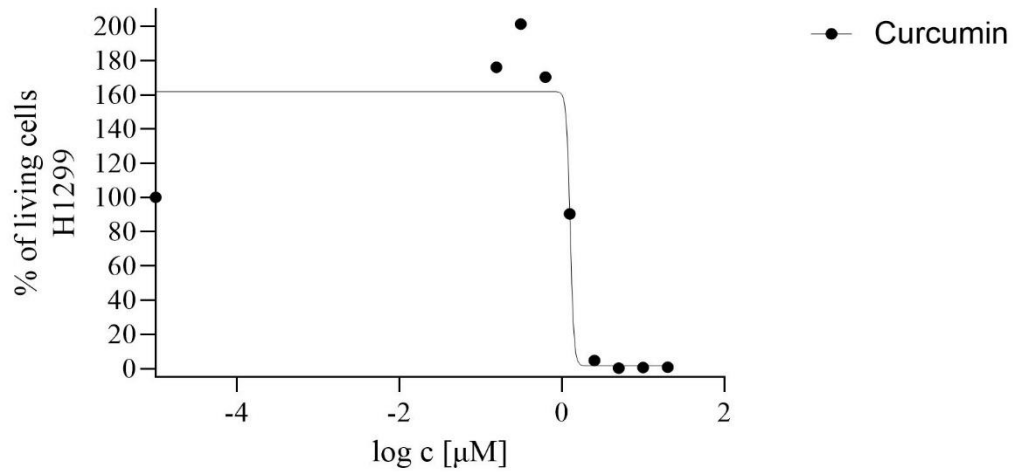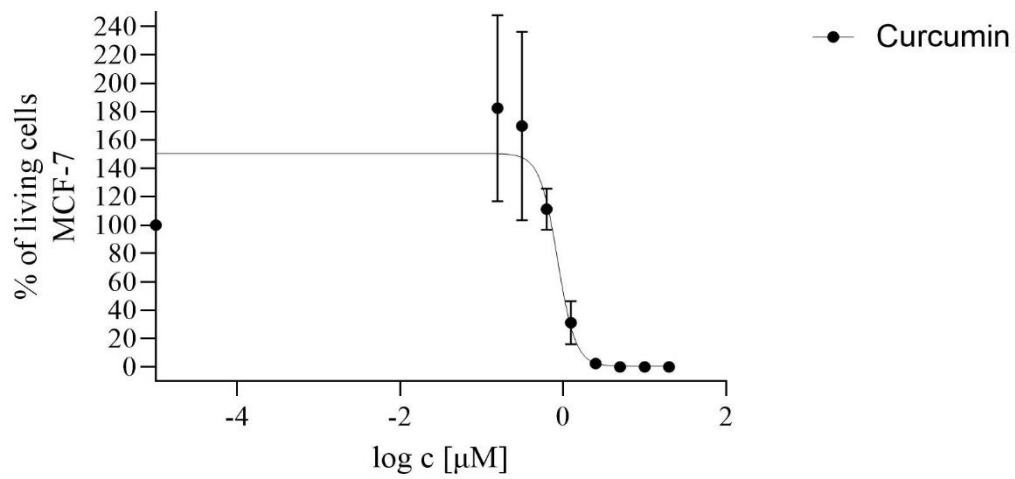# 9. LIST OF FIGURES

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12.ATTACHMENTS

Attachment 1. ThT competition assay for oligonucleotide Random and G4-ligands gallic acid,
ellagic acid and curcumin.

Attachment 2. Dose-dependent cytotoxicity activity of ellagic and gallic acid in different cell lines.

Attachment 3. Dose-dependent cytotoxicity activity of curcumin in different cell lines.