University of Minho
School of sciences

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Master thesis
Master's in Molecular Biology, Biotechnology and Bioentrepreneurship in Plants

Work done under the guidance of
Professor Rui Oliveira and Professor Andrea Ševčovicová

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Anexo 3

DECLARAÇÃO

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Analysis of antioxidant and antigenotoxic activities of artichoke (*Cynara cardunculus* L.) natural leaves extracts

Abstract

Artichoke *Cynara cardunculus* is a perennial plant from the Mediterranean region, known since the ancient times for its nutritional value and for its therapeutic applications, as hepatoprotector and diuretic. The increasing interest for the biological properties in food area, triggered its worldwide production and economic valorization. Besides the nutritional value, artichoke leaves are used in the pharmaceutical industry for the production of commercial extracts rich in phenolic compounds, mainly flavonoids and caffeoylquinic acids. These compounds can protect cells against reactive oxygen species (ROS) attack, and are responsible for many pharmacological properties that the plant presents, such as antioxidant activity. In processes such as photosynthesis and respiration, plants and other living organisms produce ROS due to the prominent oxidizing environment taking place in chloroplasts, mitochondria and peroxisomes. When ROS are produced in cells exceeding the antioxidant capacity of antioxidant agents, the balance between each other is affected and a state of oxidative stress is reached. Plants are considered to be a potential natural resource for the treatment of human diseases due to their extensive, rich and complex composition in organic substances. Beyond the importance in recognizing artichoke as a source of phenolic compounds and flavonoids, responsible for different bioactivities, this project aims to assess the biological potential of two different extracts of *C. cardunculus*. Extracts that differ from each other in the polarity of the solvent and, consequently in their chemical composition. Two extracts, aqueous (Aq) and organic (O), were obtained from fresh leaves of artichoke and tested for the antioxidant, antigenotoxic and antimutagenic activities. Firstly, the total phenolic (TPC) and flavonoid (TFC) contents were determined by colorimetric tests. Secondly, the antioxidant capacity was evaluated by the DPPH scavenging activity, reducing power and iron chelating *in vitro* assays, and by flow cytometry *in vivo* assay, using *Saccharomyces cerevisiae* as eukaryotic cell model. Moreover, the genotoxic potential and the antigenotoxic activity were analyzed *in vitro* by DNA topology assay and by comet assay, using human lymphocytes. Finally, the mutagenic potential and the potential antimutagenic activity were assessed by AMES test, using *Salmonella typhimurium*. Considering the results, Aq extract proved to have a higher content in polyphenols (TPC) than O extract. Moreover, Aq extract appears to confer some antioxidant protection that relies on its scavenging mechanism capacity and reducing power. In addition, Aq extract had no iron chelating capacity. The O extract had *in vitro* antioxidant potential by scavenging activity and protected *in vivo* yeast cells against H_2O_2. Furthermore, in DNA topology assay, O extract was antigenotoxic for all the tested concentrations and Aq extract only for the highest concentration. In comet assay, Aq extract was not antigenotoxic for any of the tested concentrations. Moreover, for both assays, none of the extracts was genotoxic. Accordingly to AMES test, none of the extracts was mutagenic or antimutagenic. Such biological capacities valorize even more the consume of artichoke. Moreover, the difference between the biologically active extracts potentiate the use of artichoke for various nutraceutical, cosmetic and clinical applications.

Keywords: artichoke, ROS, antioxidant activity, antigenotoxic activity, Aq extract and Organic extract.

Análise das atividades antioxidante e antigenotóxica de extratos de folhas de alcachofra (*Cynara cardunculus* L.)

Resumo

A alcachofra, *Cynara cardunculus,* é uma planta perene tradicional da região Mediterrânica, conhecida desde da antiguidade pelo seu valor nutricional e pelas suas aplicações terapêuticas, como hepatoprotetora e diurética. O crescente interesse pelas suas propriedades e atividades biológicas na área alimentar, desencadeou a sua valorização económica e um aumento da produção. Para além do valor nutricional, as folhas de alcachofra são usadas na indústria farmacêutica para a produção de extratos comerciais ricos em compostos fenólicos, principalmente flavonóides e ácidos cafeoilquínicos. Estes compostos podem proteger as células contra o ataque de espécies reativas de oxigénio (ROS), e são responsáveis pelas diversas propriedades biológicas que as plantas apresentam, como a atividade antioxidante. Em processos como a fotossíntese e a respiração, plantas e outros organismos produzem ROS devido ao ambiente oxidante proeminente que ocorre nos cloroplastos, mitocôndrias e peroxissomas. Quando a produção de ROS excede a capacidade de resposta dos agentes antioxidantes, o equilíbrio é afetado e um estado de stress oxidativo é gerado. As plantas são consideradas um potente recurso natural para o tratamento de doenças humanas devido à sua extensa, rica e complexa composição em substratos orgânicos. Para além da importância em reconhecer a alcachofra como fonte de compostos fenólicos e flavonóides, responsáveis por diferentes bioatividades, este projeto visa determinar o potencial biológico de dois extratos diferentes de *C. cardunculus*. Extratos que diferem entre si na polaridade do solvente usado e, consequentemente, na composição química. Dois extratos, aquoso (Aq) e orgânico (O), foram obtidos a partir de folhas frescas de alcachofra e testados para as atividades antioxidante, antigenotóxica e antimutagénica. Primeiro, os valores do conteúdo total de polifenóis (TPC) e flavonóides (TFC) foram determinados através de testes colorimétricos. Segundo, a capacidade antioxidante foi avaliada pelos ensaios *in vitro*, atividade de *scavenging* de DNA, poder redutor e quelação de ferro, e *in vivo* através da citometria de fluxo, usando a *Saccharomyces cerevisiae* como modelo celular eucariótico. Depois, o potencial genotóxico e a atividade antigenotóxica foram analisados pelos ensaios, *in vitro*, o ensaio de topologia de DNA e o ensaio cometa, que utilizou linfócitos humanos. Finalmente, o potencial mutagénico e a potencial atividade antimutagénica foram determinados pelo teste de AMES, usando *Salmonella typhimurium*. Considerando os resultados, o extrato Aq mostrou ter um teor em polifenóis (TPC) maior do que o do extrato O, e alguma proteção antioxidante, que se deve à sua capacidade de *scavenging* de radicais e de poder redutor. Este extrato não apresentou capacidade quelante de ferro. O extrato O tem potencial antioxidante *in vitro* por atividade de scavenging e protegeu *in vivo* as células contra o H_2O_2. No ensaio de topologia de DNA , o extrato O foi antigenotóxico para todas as concentrações testadas, enquanto que o extrato Aq foi apenas para a maior concentração usada. No ensaio cometa, o extrato Aq não foi antigentóxico para nenhuma das concentrações testadas. Para ambos os ensaios nenhum dos extratos mostrou ser genotóxico. De acordo com os resultados do teste de AMES, nenhuma dos extratos mostrou ser mutagénico ou antimutagénico. Tais capacidades biológicas valorizam ainda mais o consumo de alcachofra. Adicionalmente, a diferença entre os extratos biologicamente ativos potencia a utilização da alcachofra para diversas aplicações nutrecêuticas, cosméticas e clínicas.

Palavras-chave: alcachofra, ROS, actividade antioxidante, actividade antigenotóxica, extrato Aq e extrato O.

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List of Abbreviations

% RDPPH - % of Reduced DPPH

4NOP - 4-nitro-o-phenylenediame

9-AC - 9-aminoacidine

AF - Autofluorescence

AH - Antioxidant

ALE - Artichoke Leaf Extract

ALT - Alanine transaminase

Aq - Aqueous extract of *C. cardunculus*

ARE -Antioxidant Response Element

AST - Aspartate transaminase

ATP - Adenosine Triphosphate

CAT - Catalase

CHO - Chinese Hamster Ovary cells

cist-Pt - Cisplatin

DCF - 2,7-dichlorofluorescein

DNA - Deoxyribonucleic acid

DPPH - 2,2-diphenil-1-picrylhydrazyl

DPPH-H - 2,2-diphenil-1-picrylhydrazine

EDTA - Ethylene diamine tetraacetate

EMS - ethyl methanesulfanate

F-C - Folin-Ciocalteu

FSC - Forward light scatter

GA - Gallic Acid

GAE - Gallic acid equivalents

GSH-Px - Glutationa peroxidase

GSSG - Glutathione disulfide

GST - glutationa S-transferase

H_2DCF - 2',7'-dichlorodihydrofluorescein

H_2DCFDA - 2',7'-dichlorodihydrofluorescein diacetate

IC_{50} - Half maximal inhibitory concentration

iNOS - Nitric oxide synthase

Keap 1 - Kelch-like ECH-associated protein 1

LB - Lysogeny broth

LDL - Low Density Lipoprotein

LE - Leaf extract

LMP - Low Melting Point

MDA - Malondialdehyde

MPO - Myeloperoxidase

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH - Dihydronicotinamide-adenine dinucleotide phosphate

NMP - Normal Melting Point

Nrf2 - Nuclear factor erythroid 2-related factor

O - Organic extract of *C. cardunculus*

O.D. - Optical density

PBS - Phosphate buffered saline

Q - Quercetin

QE - Quercetin equivalents

RNS - Reactive Nitrogen Species

ROH - Alcohol

ROOH - Lipid peroxide radical

ROS - Reactive Oxygen Species

RT - Room Temperature

S.D - Standard Deviation

SOD - superoxide dismutase

SSC - Side light scatter

TBE - Tris-borate-EDTA

TCA - Trichloroacetic acid

TFC - Total Flavonoid Content

TPC - Total Polyphenolic Content

YPD - Yeast peptone dextrose

List of appendixes

1. Introduction

Globe artichoke (*Cynara cardunculus* var. *scolymus* L. Fiori) is an ancient herbaceous and perennial crop. A member of the Asteraceae family, which includes three taxa: two domesticated forms of globe artichoke (var. *scolymus* L.) and (var. *altilis*), and the wild cardoon (var. *sylvestris*) (Lanteri *et al.*, 2004; Portis *et al.*, 2005). Originally native from Mediterranean and North of Africa regions, artichoke grows naturally in arid, high temperatures and elevated salinity conditions (de Falco *et al.*, 2015; Falleh *et al.*, 2008).

Artichoke cultivation dates to ancient times, when Greek and Roman civilizations used the plant for feeding and also for its therapeutic applications, as hepatoprotector and diuretic (Velez *et al.*, 2012). Back in the Middle Ages, Arabs played an important role in artichoke diffusion throughout Southern Mediterranean and since then it has been spreading all over the world (Ceccarelli *et al*, 2010). Nowadays, the increase demand and consumption of artichoke has led to worldwide production and a remarkable economic growth of the sector. According to FAO, Europe and Africa are the major players in the artichoke market, accounting for 57.45% and 18% of the worldwide production, respectively. Furthermore, Italy is the world's largest artichoke producer with its annual production around 464 tones, followed by Spain, Egypt and Argentina (FAOSTAT, 2016).

Lately, several epidemiological studies revealed that the ingestion of fruits and vegetables, typical of the Mediterranean diet, helps to decrease the risk of cancer and cardiovascular diseases (Ceccarelli *et al*, 2010). The associated benefits are mainly due to the presence of minerals and a high content of antioxidant compounds such as polyphenols, flavonoids, carotenoids and vitamin C, mainly present in artichoke flower heads. Therefore, artichoke can be considered a functional food recognized for its multiple advantages such as blood circulation promotion, mobilization of energies reserves and for its antibacterial, antioxidant and antifungal properties as well (Fratianni *et al.*, 2007).

Besides the nutritional value of the edible flower buds, artichoke provides several non-edible uses such as the leaves used in the pharmaceutical industry for the production of commercial extracts for their rich composition in polyphenols. In addition, the residual parts comprised between 80% and 85% of the aboveground biomass that could be used in biogas production (De Menna *et al*, 2015). Moreover, because of a wide spectrum of potential applications, cardoon

has been widely explored in scientific research and in industry, like in paper pulp and seed oil production. For instance, the flowers have been extensively used in the preparation of cheese due to their aspartic proteases (Fernández *et al.,* 2006; Sarmento *et al.*, 2009).

Currently the applications and the biological capacities of artichoke are still completely unexplored and so as their bioactive compounds. Approaching new analytical chemical studies on artichoke would strongly value its cultivation and encourage further biological studies and applications (Velez *et al.*, 2012).

1.1. Oxidative stress, a problem affecting living organisms

Since photosynthetic organisms started releasing oxygen into the atmosphere, aerobic organisms became susceptible to oxygen reactive species (ROS). In processes such as photosynthesis and respiration, plants and other living organisms, produce these reactive molecules due to the prominent oxidizing environment taking place in chloroplasts, mitochondria, peroxisomes and other parts of the cell (Tripathy & Oelmüller, 2012). Though, when excess oxygen radicals are produced in cells exceeding the capacity of antioxidant agents, the balance between each other is affected and a state of oxidative stress is reached. Imbalance in the redox state generates oxidative stress and the accumulating ROS are harmful for plants and other organisms.

ROS are produced from molecular oxygen in normal cellular metabolism. Based on their activity, reactive species can be classified into free radicals and non-radicals. A molecule containing one or more unpaired electrons and reactive is called free radical. On the other hand, when two free radicals share their unpaired electrons, nonradicals are formed (Birben *et al.*, 2012). Molecular oxygen has an even pair of electrons, and two unpaired electrons O-O in the π^* orbitals of higher energy (Zhang *et al.*, 2017). Activation and reduction of oxygen molecule by the excitation of electrons, as a result of the energy consumed or interaction with transition elements, leads to reactive metabolites production (Yoshikawa & Naito,2002). Due to the highly reactive unpaired electrons, free radicals are normally unstable. Moreover, the new reactive oxygen metabolites are stronger oxidants than the original molecule (O_2). Therefore, the main forms of reactive oxygen species, with physiological significance, are superoxide anion ($O_2^{\cdot-}$), hydroxyl

radical (OH•) and hydrogen peroxide (H_2O_2). The principal endogenous oxidants and some of their reaction equations are present in **Table I**.

The major site for producing superoxide anion is the mitochondrion, the organelle where adenosine triphosphate (ATP) is produced to feed all energy requiring cellular procedures. In the present organelle, electrons are transferred through mitochondrial electron transport chain to reduce oxygen into water. However, approximately 1 to 3% of all the electrons leak from the system and produce $O_2^{\cdot-}$. The $O_2^{\cdot-}$ is formed by the addition of one electron to the molecular oxygen and mediated by NADPH oxidase or xanthine oxidase (reaction **a)** in **Table I**); Miller *et al.*, 1990). Then this radical can be easily converted into hydrogen peroxide by the action of superoxide dismutase (SOD) (**Table I b)**).

Table I. Principal oxygen reactive species, endogenous oxidants and their oxidative reaction equations. Adapted from, Birben *et al.*, 2012 and Nimse & Pal, 2015.

Oxidant	Formula	Oxidative reaction equations
Superoxide radical	$O_2^{\cdot-}$	**a)** $2O_2 + NADPH \longrightarrow 2O_2^{\cdot-} + NADP^+ + H^+$ **b)** $2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$
Hydrogen peroxide	H_2O_2	Xanthine + H_2O + O_2 $\rightleftharpoons$ uric acid + H_2O_2
Hydroxyl radical	OH•	**c)** $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\bullet + OH^-$ **d)** $O_2^{\cdot-} + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2$
Singlet oxygen	$^1O_2$	**f)** $OCl^- + H_2O_2 \longrightarrow {}^1O_2 + H_2O + Cl^-$
Hydroperoxyl radical	HOO•	$O_2^{\cdot-} + H_2O \longrightarrow HOO^{\cdot} + OH^-$
Hypochlorite ion	ClO^-	**e)** $Cl^- + H_2O_2 + H^+ \xrightarrow{MPO} HOCl + H_2O$
Nitric oxide	NO•	**g)** $O_2^{\cdot-} + NO\bullet \longrightarrow ONOO^-$

(SOD - Superoxide dismutase, MPO - Myeloperoxidase)

Hydrogen peroxide freely diffuses across the plasma membrane. This reactive species can be produced by various enzymes, such as xanthine oxidase, amino acid oxidase, NADPH oxidase, and in peroxisomes by consumption of O_2 in metabolic reactions (Birben *et al.*, 2012). ROS are

also generated from $2O_2^{\cdot-}$ and H_2O_2 by the "respiratory burst" and it can be explained by a succession of reactions called Haber-Weiss and/or Fenton reactions. The hydrogen peroxide in the presence of transition metals, like cooper (Cu^{2+}) and iron (Fe^{2+}), is cleaved into OH^- in the Fenton reaction (reaction **c)** in **Table I).** Superoxide itself, by Haber-Weiss reaction can also react with H_2O_2 and originate $OH^{\cdot}$ (reaction **d)** in **Table I**). Hydrogen peroxide can also be formed by the enzyme myeloperoxidase (MPO) located in the neutrophil cytoplasmatic granules (Babior, 2004). In activated neutrophils, H_2O_2 is consumed by MPO. MPO, when chloride ion is present, H_2O_2 is converted into hypochlorous acid (HOCl), a potent oxidant (reaction **e)** in **Table I).** Moreover, singlet oxygen ($^1O_2$) is generated when hypochlorous acid loses a proton in the presence of hydrogen peroxide. Among these free radicals, there is the nitric oxide (NO•), produced by the enzyme nitric oxide synthase (iNOS), an enzyme that synthesizes reactive nitrogen species (RNS) from arginine. Nitric oxid acts as a $O_2^{\cdot-}$ quencher, and together they produce a very strong and versatile oxidant, peroxynitrite ($ONOO^-$) (reaction **g)** in **Table I**) (Nimse & Pal, 2015). The mechanisms mentioned above, show the endogenous sources of ROS. But, ROS can also be produced by exogenous factors, pollutants, smoke, ultraviolet radiations, or drugs (Shahidi & Zhong, 2010).

In high concentrations ROS induce adverse modifications into cell components, like lipids, proteins and DNA (Valko *et al.*, 2006). For instance, hydroxyl radical is the most reactive of ROS and can damage these biomolecules and also initiate lipid peroxidation, by taking one electron from polyunsaturated fatty acids. Furthermore, HOCl in the presence of DNA can induce DNA-protein interactions, producing pyrimidine oxidation products (Kulcharyk & Heinecke, 2001). Therefore, ROS are responsible for the development of many pathological conditions, including cancer, neurological disorders, diabetes, asthma, and so many others (Ercan *et al.*, 2006; Fitzpatrick *et al.*, 2009).

Over time, aerobic organisms have integrated natural defense mechanisms, antioxidant systems, to block harmful effects of ROS. This antioxidant demand can be enzymatic or non-enzymatic. The major enzymatic antioxidants are SODs, CAT (catalase) and GSH-Px (glutathione peroxidase). Superoxide is the mostly produced ROS, which suffers dismutation by SOD, converting $O_2^{\cdot-}$ into O_2 and H_2O. The enzyme works in the presence of metal ions cofactors, such

as cooper, zinc or manganese (Gough & Cotter, 2011). After this, the H_2O_2 produced by action of SOD's or of oxidases, is reduced to water by catalase (**Equation h)**, Park *et al.*, 2009).

Equation h) $2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$

$ROOH + 2GSH \xrightarrow{GSHPx} ROH + GSSG$

GSH-Px can be found in the cytoplasm and in the extracellular space in almost every human tissue. Such as catalase, GSP-Px also converts H_2O_2 into water, and presents strong antioxidant activity against fatty acid hydroperoxides (Arthur, 2001). CAT, SOD and GSH-Px play a synergetic effect in the scavenging of $O_2^{\cdot-}$. Clearly, different expression profiles, subcellular locations, and substrates of the antioxidant enzymes show the complex nature of the ROS biology.

Phenolic compounds are natural non-enzymatic antioxidants that provide an antioxidant response through different mechanisms of action. The beneficial actions of phenolic compounds are mostly attributed to their antioxidant capacity. This activity is associated with free radical scavenging, chelating of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways (Soobrattee *et al.*, 2005). However, the structure and their conjugation with other phenolics, degree of glycosilation/acylation, molecular size and solubility are the most important features to amplify the bioactive response of these compounds (Balasundram *et al.*, 2006). Likewise, the number and configuration of H-donating hydroxyl groups are major structural characteristics for the antioxidant capacity of phenols (Pannala *et al.*, 2001).

Vitamin E (α-Tocopherol) is located in the inner leaflet of cell the membrane, and as an antioxidant acts as a 'chain braker' during lipid peroxidation in cell membranes and also in other lipid particles comprising LDL (low-density lipoprotein) (Nimse & Pal, 2015). Basically, vitamin E donates electron to peroxyl radical (LOO•), which is produced during lipid peroxidation (**Equation i)**).

Equation i) $LOO\bullet + \alpha\text{-Tocopherol-OH} \longrightarrow LOOH + \alpha\text{-Tocopherol-O}\bullet$

The products of this reaction are relatively stable to not initiate lipid peroxidation (Morlière *et al.*, 2012; Stocker *et al.,* 1991). It is important to note that despite of the scavenging capacity of

vitamin E for lipid peroxyl radicals (LOO•), both *in vivo* and *in vitro* systems, this molecule revealed to not be efficient scavenger of OH• and alkoxyl radical (OR•) (Niki, 2014). Similarly to vitamin E, vitamin C also donates electrons to lipid radicals in order to terminate the lipid peroxidation chain reaction.

Bioflavonoids are reported to exert multiple biological effects regarding free radical scavenging activity. This is responsible for its protection against DNA damage, mainly induced by hydroxyl radicals. They can also chelate metal ions, such as copper and iron, forming a flavonoid-metal ion complex and subsequently preventing the generation of ROS and consequent DNA cleavages (De Souza & Giovani, 2004; Zhou *et al.*, 2001). Flavonoids use the 3- or 5-hydroxyl or hydroxyl groups in *ortho* position, in the B-ring, to form complexes with metal ions (Miguel, 2011). Flavanols, for example, are inhibitors of lipid peroxidation, since they reduce lipid oxidation. They reduce oxidative modifications of lipid membranes by preventing the entrance of oxidants in the bilayer and consequently lipid oxidation in the hydrophobic membrane matrix (Verstraeten *et al.*, 2003). Furthermore, the antioxidant capacity of this class is also related to the number of hydroxyl groups present. Quercetin is a well-known flavonol that protects oligonucleotides of the DNA from oxidative stress induce by OH• (Krishnamachari *et al.*, 2002).

Phenolic acids such as hydroxycinnamates, can prevent LDL oxidation and related diseases. Interestingly, *in vitro* studies using LDL as an oxidizing substrate showed that hydroxycinnamic acids have higher antioxidant activity when compared to the corresponding hydroxybenzoic acids (Natella *et al.*, 1999). The hydroxylation and methylation of the aromatic ring are normally associated to its antioxidant activity. The antioxidant efficiency on the human LDL oxidation *in vitro*, decreases in the following order: caffeic acid, sinapic acid, ferulic acid and *p*-coumaric acid. For instance, caffeic acid has a *o*-dihydroxy group in the phenolic ring enhancing the antioxidant activity (Meyer *et al.*, 1998). The free-radical scavenging mechanism is very similar to that of flavonoids due to their ability to donate a hydroxyl hydrogen and resonance stabilization of the resulting antioxidant radicals.

In the next chapter, it is explained how these compounds of natural origin are classified according to their structures and sizes. The knowledge of the chemical composition of a plant or extract is the essential basis for its exploration and application, and may later relate to the bioactive effects either known or not.

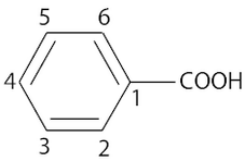
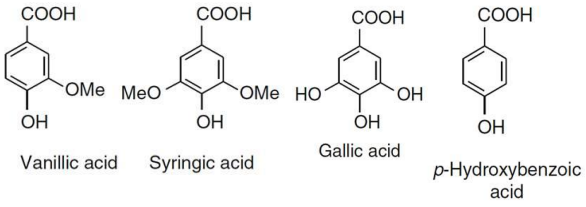
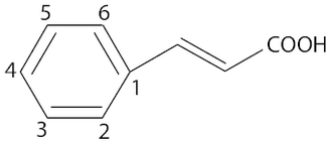
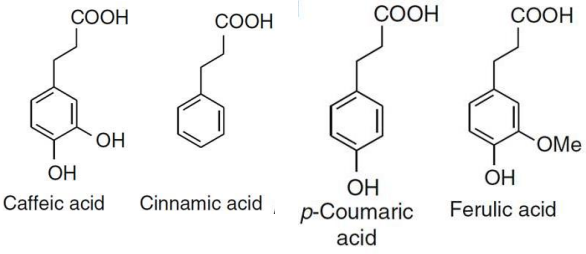
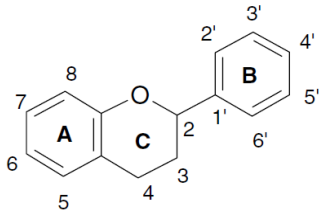
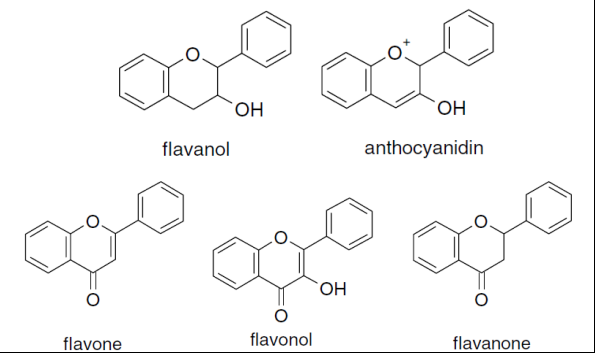
1.2. Phytochemicals

Plants have been used since antiquity for the treatment of human diseases, due to their extensive, rich and complex composition in organic substances. The isolation of such compounds is essential for obtaining better knowledge of their range of bioactivities and for the development of new drugs. It is known that around twenty-five thousand species of plants are used worldwide in the pharmaceutical industry (Queiroz *et al.*, 2015). The economic factor has increased the appreciation and utilization of artichoke, since plants can produce by themselves chemical compounds with biological properties. Therefore, the costs of synthesis and production are reduced and the research of new drugs is valued (Yuan *et al.*, 2016). The application of medicinal plants has become an alternative therapeutic resource used by the medical community, but it is important to assure the efficacy and safety of their biological properties. Since many plants have toxic substances in their composition and therefore can be harmful when some concentrations are administrated (Jiménez *et al.*, 2003).

Synthesized and accumulated in higher plants, phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways (Randhir *et al.*, 2004). These compounds can protect the cells against free radicals attack and tissue injuries (Magnani *et al.*, 2014). Since they are one of the most common groups of phytochemicals, they play an important role in plants growth, reproduction and protection against pathogens, predators and UV radiation. In addition, they define the color and sensory characteristics of fruits and vegetables (Alasalvar *et al.*, 2001).

The polyphenolic structure comprises aromatic rings, phenols, bearing one or more hydroxyl substituents, and range from simple phenolic molecules (phenolic acids) to highly polymerized compounds (flavonoids) (Lin *et al.*, 2016). Thus, there are many different structural possibilities that result in a wide range of phenolic compounds in nature, making it difficult to categorize phenolic compounds into several classes. Although the attempt to categorize phenolic compounds into simple phenolics and polyphenols revealed to be insufficient since there are huge structural differences between polyphenols. So, this category is comprised by flavonoids, coumarins, stilbenes, lignans and lignins. Of these, phenolic acids and flavonoids are considered the main dietary phenolic compounds, as figured in **Table II** (King & Young, 1999).

Table II. General classification of phenolic acids and polyphenolic compounds such as flavonoids.

	Class	Subclass	Derivates
Phenolic compounds	Phenolic acids	Hydroxybenzoic acids 	
		Hydroxycinnamic acids 	
	Polyphenolic	Flavonoids 	

In edible plants, phenolic acids appear as conjugated esters or glycosides esters or glycosides. Their role in plants is not yet very clear, but it is known that they are associated with many functions such as, nutrient uptake, protein synthesis, enzymatic activity, photosynthesis and structural components (Maobe *et al.*, 2012). These phenols have a phenolic ring and at least one organic carboxylic acid. The number of carbon units of the lateral chain attached to the phenolic ring determines whether they are hydroxybenzoic acids, structure C_6-C_1 (aromatic ring C6 linked to one-carbon side chain), or hydroxycinnamic acids, with the structure C_6-C_3. The basic skeleton is the same, the difference can be found in the number and positions of the hydroxyl groups in the aromatic ring (Goleniowski *et al.*, 2013). Hydroxybenzoic acids characterized by the structure C_6-C_1 include gallic, *p*-hydroxybenzoic, vanilic and syringic acids. Variations in the structures relies in the hydroxylation and methylation of the aromatic ring. On the other hand,

hydroxycinnamic acids present a three-carbon side chain linked to the aromatic ring (C_6-C_3). The most common ones are caffeic, ferulic, *p*-coumaric and cinnamic acids (Balasundram *et al.*, 2006). In nature, chlorogenic acid (5-O-caffeoylquinic acid), a caffeic acid esterified with quinic acid, is the most common one (Goleniowski *et al.*, 2013).

Flavonoids, considered the largest group of plant phenolic compounds, are widely distributed in fruits and vegetables. The low molecular weight compounds present C_6-C_3-C_6 configuration and their structure consist of two C_6 units of phenolic nature, ring A and ring B, joined by a 3-carbon bridge in the form of a heterocyclic ring, ring C (**Table II**). The two aromatic rings have different origins, ring A derives from the acetate/melavonate pathway while the ring B derives from phenylalanine through the shikimate pathway (Bohm, 1998; Merken & Beecher, 2000). Due to variations in substitution patterns to ring C, flavonoids can be further divided into various classes such as flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols and anthocyanidins. The substitutions in ring A and ring B, which include oxygenation, alkylation, glycosylation, acylation and sulfation, are responsible for the variety of compounds within each class of flavonoids. In plants, the majority of these compounds exist as glycosides. Antioxidant activity and other biological activities depend on both structural difference and the glycosylation patterns (Balasundram *et al.*, 2006; Tsao, 2010).

Tannins, however, have relatively high molecular weight and can be divided in hydrolysable and condensed tannins. The former are esters of gallic acid, while the latter are polymers of polyhydroxyflavan-3-ol monomers, also known as proanthocyanidins (Porter, 1989). Depending on the interflavanic linkages, condensed tannins can be divided into two classes by taking into consideration the A-ring oxidation pattern. A-type are those in which monomers are linked through C_2-O-C_7 or C_2-O-C_5 bonding, and B-type are those in which C_4-C_6 or C_4-C_8 are common (Tsao, 2010).

1.2.1. Chemical composition of Artichoke

C. cardanculus leaves are mainly known in folklore for their therapeutic potential as diuretic, cholorectic, antioxidant and hepatoprotective (Fratianni *et al.*, 2007). Despite the traditional use, the therapeutic potential of artichoke is poorly developed and so its application as source of bioactive compounds. So, a pharmacological research for the use of these bioactive compounds would value its cultivation (Velez *et al.*, 2012). Therefore, studies have been performed in order to quantify and qualify the bioactive compounds of artichoke (Schütz *et al.*, 2004; Wang *et al.*, 2003; Zhu *et al.*, 2004).

The phenolic composition of plants is strongly influenced by biological factors (genotype, organ and ontogeny), as well as environmental (temperature, salinity, water stress, and light intensity) conditions. In addition, different development stages of plant leaves show a variation in polyphenolic content, generally they increase from the vegetative to the productive stage (de Falco *et al.*, 2015; Lombardo *et al.*, 2010).

The chemical composition of *C. cardunculus*, it is known for having a high content in polyphenolic compounds, mainly flavonoids and caffeoyliquinic acids. Caffeoyliquinic acids such as cynarin are present both in heads and leaves, however cynarin is more predominant in heads (Sonnante *et al.*, 2010). Leaves, on the other hand, have a higher content in flavonoids, such as flavones, apigenin, quercetin and luteonin and phenolic acids including gallic, sinapic, ferulic, chlorogenic, vanilic and *p*-coumaric acids (Falleh *et al.*, 2008). Therefore, the phenolic content is generally higher in leaves than in heads (Negro *et al.*, 2012).

Terpenes are a wide and diverse class of organic compounds. Within terpenes there are sesquiterpenes and triterpenes, main classes of lipophilic components, present in cultivated artichoke. Unlike triterpenes, sesquiterpenes are present in high quantities in leaves. Cynaropicrin is the principal sesquiterpene in artichoke leaves, that shows several biological effects (Ramos *et al.*, 2013). More details about classes and sub-classes of phenolic compounds and classes of terpenes present in artichoke are described in **Table III**.

Table III. Main phenolic compounds and terpenes in artichoke composition.

Class	Sub-class	Main derivatives	References
Phenolic acids	Hydroxycinnamates	Sinapic acid, caffeic acid, cumaric acid, cynarin, ferulic acid, chlorogenic acid	(de Falco *et al.*, 2015; Fratianni *et al.*, 2007; Negro *et al.*, 2012; Schütz *et al.*, 2004)
	Hydroxybenzoic acids	Vanilic acid, gallic acid, syringic acid	
Flavonoids	Flavanones	Narirutin, naringerin	(de Falco *et al.*, 2015; Fratianni *et al.*, 2007; Negro *et al.*, 2012; Schütz *et al.*, 2004)
	Flavone	Cynaroside, luteolin, apigenin	
Terpenes	Sesquiterpenes	Aguerin A and B, cynaropicrin	(de Falco *et al.*, 2015)
	Triterpenes	Cyanarasaponins, lupeol, taraxasterol acetate	

The polarity of these compounds influences their ability to easily cross the cell membrane, where they can exert antioxidant activity. In addition, the polarity of the extraction solvents used influence which compounds will be present, resulting in different extraction yields. So, the solubility of phenolic compounds depends on the type of solvent used and the degree of phenolics polymerization (Gálvez *et al.*, 2005).

1.3. Biological activities

Since artichoke is a plant rich in bioactive compounds, it makes it a target of interest due to its biological properties and activities (Williamson & Holst, 2008). Artichoke leaves extract's pharmacological properties are attributed mainly to polyphenolic compounds, specially inulin (Salem *et al.*, 2015). Studies already performed have proved that artichoke is a powerful bioactive and pharmacological source, highlighting the hepatoprotective, diuretic, cholorectic, antioxidant and anticarcinogenic activities (**Tabela IV**; Velez *et al.*, 2012).

Table IV. Secondary metabolites of artichoke and some of their biological activities.

Class of compounds	Bioactive compounds	Bioactivities and properties	References
Phenolic acids	**Caffeoylquinic acids** Chlorogenic acid; Cynarin;	Hepato protective, Antioxidant, Diuretic, Anticarcinogenic, Anti-HIV, Choleretic	(de Falco *et al.*, 2015; Negro *et al.*, 2012; Salem et al., 2015)
Flavonoids	**Flavones** Apigenin; Luteolin; Cynaroside	Cholerectic, Antioxidant, Antimicrobial, Inhibition of lipid peroxidation, Inhibition of multidrug-resistance, Hepato protective, Antibacterial, Vasorelaxant	(de Falco *et al.*, 2015; Negro *et al.*, 2012; Salem *et al.*, 2015)
Terpenes	**Sesquiterpenes** Grosheimin; Cynaropicrin	Cytotoxic, Antiphotoaging agent, Anti-hyperlipidemic, Inhibition of thoracic aorta contraction	(de Falco *et al.*, 2015)
	Triterpenes α and β amyrin; Taraxasterol; Taraxasterol	Antimutagenic, Anti-inflammatory, Anticarcinogenic, Inhibitor of skin tumor	

Artichoke has been used in the treatment of liver diseases, due to their antioxidant and hepatoprotective properties. These physiological effects may be due to the action of certain compounds, such as cynarin, caffeic acid and luteolin (De Queiroz *et al.*, 2016). These extracts can conduct an antioxidant response by inhibition of plasma MDA (Malondialdehyde) production, LDL oxidation, ROS production, HMG-CoA reductase and therefore decrease the endogenous cholesterol synthesis that occurs in the liver. (Jafari-Dehkordi & Seidkhani-Nahal, 2011)

Liver is the most metabolically complex organ in the body. It is involved in nutrient metabolism, secretion, storage and detoxification of endogenous and exogenous substances (Ghosh *et al.*, 2011). For being a fundamental organ for biotransformation and metabolism, it can be affected by the reactive oxygen species produced as by-products of its normal metabolism and detoxification reactions. In response, liver activates its extensive antioxidant mechanism, which allows liver to protect cells against oxidation. However, in case of oxidative stress, artichoke's antioxidant compounds can mediate hepatoprotective activity against the oxidative

stress, promoting the redox balance in liver cells (Baranisrinivasan *et al.*, 2009; Miccadei *et al.*, 2008).

In the presence of a molecule like the aldehyde acrolein, glutathione (GSH), a complex responsible for the antioxidant response gets depleted, inducing ROS formation and consequently oxidative damage. Acrolein is a cyclophosphamide metabolite, synthesized in 1958, which is very common in food and water and is considered as a pollutant. Some studies showed that the presence of acrolein in liver cells can lead to hepatoxicity (Tong *et al.*, 2017). On the other hand, cynarin and caffeoylquinic acid, present in artichoke, can efficiently protect HepG2 cells against acrolein-induced toxicity. The protective effect is mediated through ROS scavenging, inhibition of GSH depletion, and is associated with the activation of the Nrf-2-antioxidant response element pathway, **Figure 1.**

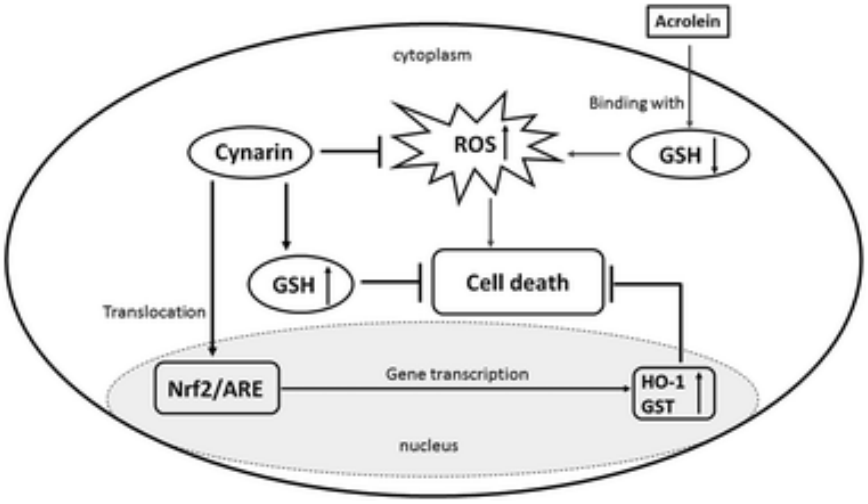


Figure 1. The cynarin-mediated hepatoprotective response against acrolein in liver cells (Tong *et al.*, 2017).

Under physiologic conditions, Nrf2 (nuclear factor erythroid 2-related factor), a major regulator of cellular redox balance, binds to kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm. Under stress, cynarin would display the activation of Nrf2, through Keap 1(kelch-like ECH-associated protein 1) modification or by Nrf2 phosphorylation (Li *et al.,* 2015). The activated Nrf2 translocates into the nucleus and interacts with antioxidant response element (ARE), stimulating the expression of cytoprotective genes, including antioxidant enzymes such as GST (glutathione S-transferase) and phase II detoxifying enzymes. The GST enzyme is responsible for GSH active formation and both are powerful antioxidants that inhibit cell death by stopping ROS

formation. Therefore, cynarin displays a combined protection response, inhibiting GSH depletion by acrolein and activating Nrf-2-antioxidant response element pathway (Tong *et al.*, 2017).

Hyperchlolesterolemia is a disease provoked by the accumulation of LDL, which increases the cholesterol levels and lead to cardiovascular diseases. Traditional treatments use inhibitors of HMG-CoA reductase. The enzyme responsible for producing cholesterol in the mevalonate pathway. When HMG-CoA is phosphorylated the enzyme becomes inactive and stops cholesterol synthesis. Since some drugs used for treatment are associated with carcinogenic potential, artichoke leaves extract (ALE) have been suggested as a different pharmacological alternative (De Queiroz *et al.,* 2016). Moreover, the flavonoid luteolin exhibited an inhibitory effect on cholesterol biosynthesis (Salem *et al.*, 2015). Previous studies showed artichoke to protect mice hepatocytes cultures affected by oxidative stress, caused by H_2O_2. In addition, its extracts are able to inhibit LDL oxidation and reduce intracellular ROS production, in endothelial and monocytes cell cultures, caused by the oxidized LDL (Zapolska-Downar *et al.*, 2002). In another study, mice pre-treated with artichoke were injected intraperitoneally with CCl_4 (carbon tetrachloride). Results showed a significant decrease in ALT (Alanine transaminase) and AST (Aspartate transaminase) activities due to an increase in GSH-Px activity. Their activity is requested when the liver is injured. Therefore, in pre-treated mice artichoke extracts were able to protect hepatocytes liver cells against the damage caused by the oxidant CCl_4. In addition, the levels of vitamin E, vitamin C and the activity of enzymes such as SOD and GST remain steady, contrarily to those treated with CCl_4 (Mehmetçik *et al.*, 2008). Artichoke is believed to have antioxidant activity responding with free-radical scavenging mechanism, mainly due to its composition in cynarin, chlorogenic and flavonoids. These compounds can also respond by metal ion chelating mechanisms (Pérez-García *et al.*, 2000).

Beyond antioxidant activity ALE natural phytochemicals can also have other protective properties like antimutagenic and antigenotoxic activities (Miadokova *et al.*, 2008). Traditionally, the use of different isolated natural compounds in health treatments, due to their pharmacological and therapeutic properties, can bring some concern on their performance. Instead of being used in traditional healing, natural compounds are now used as part of health promotion or disease prevention strategies, so acute treatment has been replaced by chronic exposure. This does not necessarily mean that compounds are unsafe, but that safety in modern

world cannot be assumed (IARC, 2002). Moreover, in most countries herbal medicines and their relatives are introduced into the market without any mandatory or toxicological evaluation. For example, a few herbs are capable of producing undesirable or adverse reactions some of which could cause serious injuries (Ekor, 2014). Therefore, it is important to have a rational use of them by carrying scientific studies to validate their efficacy and safety, since they can present mutagenic and genotoxic effects (Carvalho *et al.*, 2011). Additionally, the research developed will allow to understand the mechanisms of action associated, such as inhibition of genotoxic effects, antioxidant action, enzyme modulation, gene expression, modulation of cell proliferation, apoptosis and signal transduction (Čipák *et al.*, 2003; Dajas *et al.*, 2003; Ebeler, 2004; Miadoková *et al.*, 2002).

Mutagen is an agent that causes a direct or indirect genetic change in DNA, resulting in a mutation. These genetic alterations are retained in somatic cells, and in germinal cells who are passed on to further generations. Meanwhile, agents that damage DNA resulting in cell death or reversible mutations are genotoxins. However, all mutagens are genotoxic, but not all genotoxins are mutagens (Nagarathna *et al.*, 2013). Nevertheless, despite several flavonoids possess antimutagenic activity, some of them have also shown to be genotoxic in a variety of eukaryotic and *in vivo* systems (Snyder and Gillies, 2002). Chlorogenic acid, an antioxidant phenolic acid, can also act as pro-oxidant (Cao *et al.*, 1997). Furthermore, Sotibrán and others (2011) demonstrated that this phenolic acid neither induced oxidative stress in *D. melanogaster* nor protect DNA against paraquat-induced oxidative stress lesions.

A variety of natural agents, called antimutagens can act by inhibiting or preventing mutagens to induced DNA damage or they can activate repair mechanisms (Jacociunas *et al.*, 2012). Some studies have indicated flavonoids, such as apigenin and luteolin, to have some antigenotoxic and antimutagenic activities (Miadokova *et al.*, 2008). The frequency of DNA damages induced by alkylating agent ethyl methanesulfonate (EMS), in Chinese hamster ovary cells (CHO), was assessed by comet assay when treated with *C. scolymus* leaf extract (LE; Jacociunas *et al.*, 2012). The antigenotoxicity evaluation was performed by pretreatment, simultaneous treatment, and post-treatment. Only simultaneous treatment showed significant reductions when compared to lesions induced by EMS alone. But, interestingly, there was not a dose-response correlation, since the lowest concentrations proved to be more protective than higher ones. On the other

hand, the same extract presented some genotoxic effects, inducing DNA damages in eukaryotic cells when higher doses were applied (Zan, 2008). Therefore, lower concentrations applied were the least genotoxic and the most protective against EMS effects. The genotoxicity of *C. scolymus* LE could be explained by pro-oxidant effects related to the higher concentrations used. Other studies, with *D. melanogaster* in the presence of EMS proved ALE to be antigenotoxic due to their chemical composition. Especially, flavonoids that are also thought to have an important role in cancer therapy. For example, in the treatment of different melanomas, apigenin helps the inhibitory effect caused by the drug cisplatin (cist-Pt). This drug is often used in cancer therapy and known to have different side effects. The extract of *C. carduncul*us, establish a synergetic effect by enhancing the cytotoxic/cytostatic effect of the cytostatic drug cis-Pt. Therefore, apigenin potentiated the inhibitory effect of cis-Pt melanoma growth and made the cancer therapy more efficient (Miadokavá *et al.*, 2006).

2. Aim

Beyond the importance of recognizing artichoke as a source of different bioactivities due to their composition in phenolic compounds and flavonoids, this project aims to assess the biological potential of two different extracts of *C. cardunculus*. Since Aqueous (Aq) extract and Organic (O) extract differ from each other in the polarity of the solvent and, consequently in chemical composition. The various properties and activities of each extract are very important, since it allows the application of these extracts in different pharmaceutical and cosmetic products. Moreover, the determination of chemical content allows the establishment of a direct relation between extracts biological activities and their phenolic and flavonoid content, proving the bioactivity of those compounds. Therefore, in order to evaluate the biological properties of artichoke extracts and to determine their total phenolic and flavonoid contents, the following methodologies were applied for both extracts:

- TPC and TFC colorimetric analysis;

- Antioxidant activity evaluation by, *in vitro*, DPPH scavenging activity, reducing power assay, iron chelating assay, and by *in vivo* flow cytometry with a redox-sensitive fluorescent probe;

- Genotoxic potential and antigenotoxic activity, *in vitro*, analysis by DNA topology assay and by comet assay, using human lymphocytes;

- Mutagenic potential and antimutagenic activity evaluation, *in vivo*, by AMES test with bacteria *S. typhimurium.*

This work will valorize the *C. cardunculus* as a powerful edible ingredient and also as a bioactive plant with an important role in further applications, on scientific studies and health treatments.

3. Materials and Methodologies

3.1. Extract preparation

The biological material selected for these experiments was provided by the Earth Essences company, in Póvoa do Lanhoso, Portugal. Extracts were prepared as described by Sampaio (2017), using *Cynara cardunculus* leaves as biological material. First, 5 g of new fresh leaves were boiled for 10 min in 50 mL of water, followed by a vacuum filtration. Thereafter, the filtrate was extracted with ethyl acetate and two phases were formed, the aqueous below and the organic phase on top. The organic phase was collected, and the solvent was evaporated followed by lyophilization, obtaining the organic extract. While, the aqueous phase was extracted two more times with ethyl acetate, then the excess of after was removed with the addition of a desiccant and subsequent lyophilization. The organic phases resulting from these two extractions were further added to the first organic phase, before the rotary evaporator. Therefore, two extracts were obtained, the organic extract (O) and the aqueous extract (Aq). Moreover, through the 50 mg/mL of stock solution for each extract (50 mg of solid extract diluted in 1 mL of water or 70% ethanol) different dilutions were prepared, water for Aq extract and ethanol 70% for O extract, to be used in the following assays.

3.2. Colorimetric analysis of total phenolic and flavonoid content

3.2.1. Quantification of total phenolic compounds (TPC)

The total phenolic content was determined using the Folin-Ciocalteu (F-C) method adapted to a 96-well microplate (Blainski, Lopes & De Mello, 2013). Briefly, samples (10 µL) were mixed with 100 µL of F-C reagent (1:10, dH2O) and left for 5 min at room temperature (RT). Thereafter, 80 µL of Na_2CO_3 (1M) was added and the mixture was then incubated at RT in the dark for 60 min. The blanks were prepared for the different extracts with the respective solvents, water and/or 70% ethanol, for a final volume of 190 µL. Controls were prepared with 100 µL of F-C, 80 µL of Na_2CO_3 reagents, and 10 µL of the respective solvent. Gallic acid (GA) was used as a standard, and different concentrations (25, 50, 100, 250, 500 and 1000 µg/mL) were prepared in 70% ethanol for the elaboration of a calibration curve. The absorbance was measured at 760 nm, using the equipment Synergy™ HTX Multi-Mode Microplate Reader. The results of total

phenolics TPC were expressed as equivalents of GA, GAE mg/g of extract, and calculated by the following equation.

$$\textbf{Equation j)}\ TPC = C_{GAE} \times \frac{V}{M}$$

Where C_{GAE} is the concentration of extract in GA equivalents, from the GA calibration curve (µg/mL), V is the volume of extract (mL), and M is the mass (g) used in the preparation of the artichoke extracts.

3.2.2. Quantification of total flavonoids compounds (TFC)

The total flavonoids content (TFC) was evaluated using an adapted 96-well microplate method, based on flavonoid-aluminum chloride complex formation. Firstly, 100 µL of $AlCl_3$ (20 mg/mL) were mixed with 100 µL of each sample. Then, the mixture was incubated for 60 min in the dark at RT. The blanks were prepared for the different extracts with the respective solvents, water and/or 70% ethanol, for a final volume of 200 µL. Moreover, controls were made with 100 µL of $AlCl_3$ and 100 µL of the sample solvent. The measurements were performed in a microplate reader, Synergy™ HTX Multi-Mode Microplate Reader, at 420 nm. Quantification of TFC was based on the calibration curve, generated with different concentrations of standard quercetin (Q) (5, 10, 25, 50, 100 and 250 µg/mL), dissolved in 70% ethanol. Total flavonoids content was expressed as equivalents of Q, QE mg/g of extract, and calculated by the following equation.

$$\textbf{Equation m)}\ TFC = C_{QE} \times \frac{V}{M}$$

Where C_{QE} is the concentration of extract in QE, from the Q calibration curve (µg/mL), V is volume of extract (mL), M is mass of extract of artichoke (g).

3.3. Determination of antioxidant activity

3.3.1. *In vitro* 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

Initially, a DPPH solution in 100% ethanol (0.039 mg/mL) was prepared. Extract samples (15 µL) and controls (containing extract solvent instead of extract), were mixed with 285 µL of the DPPH (1:10) diluted solution. The blank was made with 100% ethanol and 15 µL of extract

solvent. Gallic acid was prepared with 70% ethanol (0.1, 1, 5, 10, 25, 50, 100, 500 and 1000 µg/mL) and used as a standard. Then, the samples were incubated for 60 min in the dark at RT. The scavenging activity was measured at 517 nm, using the microplate reader Synergy™ HTX Multi-Mode Microplate Reader. The DPPH scavenging activity was calculated using the following equation.

$$\textbf{Equation n)}\ \%\ \text{Reduced DPPH} = \left(\frac{\text{Abs C-Abs S}}{\text{Abs C}}\right) \times 100$$

Where, Abs S is the absorbance of sample and Abs C is the absorbance of control. The percentage of reduced DPPH was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of sample necessary to cause 50% of reduced DPPH.

3.3.2. *In vitro* reducing power assay

Briefly, 100 µL of both extracts were mixed with 500 µL of 200 mM phosphate buffer pH 6.6 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4), followed by 500 µL of 1% (w/v) potassium ferricyanide. After vortexing, the mixture was incubated with a shaker at 50 °C for 20 min. Posteriorly, 500 µL of 10% TCA (w/v) was added and then the mixture was centrifuged at 3000 rpm, for 10 min at 4 °C. Then, to 500 µL of the supernatant 500 µL of dH_2O_2 and 100 µL of 0.1% $FeCl_3$ (w/v) were added. Gallic acid solutions were prepared with 70% ethanol (10, 25, 50, 75, 100, 250, 500, 750 and 1000 µg/mL) and used as a standard. Blanks were prepared with the respective extract solvent, while the controls were made with extract solvent plus phosphate buffer and potassium ferricyanide. After all, the absorbance was measured spectrophotometrically at 700 nm using a UV-Vis Genesys 20 spectrophotometer. A higher absorbance of the mixture is correlated with greater reducing power.

3.3.3. *In vitro* iron chelating assay

First, the following mixtures were prepared, 900 µL of dH_2O, 100 µL of extract (50, 100, 250, 500, 750 and 1000 µg/mL), 50 µL of 2 mM $FeCl_2$ and 200 µL of 5 mM ferrozine. Then, the solution was mixed and left in the dark for 10 min. Blanks were made with the respective extract solvent, and the control was ferrozine, $FeCl_2$ and the respective extract solvent. Ethylene diamine tetraacetate 2 mM (EDTA) (50, 100, 250, 500 and 1000 µg/mL) was used as a

standard. The absorbance of the solution was measured spectrophotometrically at 562 nm, in UV-Vis Genesys 20 spectrophotometer. In the presence of other complexing agents, the $FeCl_2$-ferrozine complex is not formed causing a decrease in the red color intensity of complexes. Therefore, the iron chelating capacity is calculated by the percentage of inhibition of $FeCl_2$-ferrozine complex, using the following equation.

$$\textbf{Equation o)}\ \%\ \text{of Inhibition} = \left(\frac{\text{Abs C-Abs S}}{\text{Abs C}}\right) \times 100$$

Where Abs C corresponds to the absorbance of control and Abs S is the absorbance of the sample. The percentage of inhibition was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of the sample necessary to cause 50% of inhibition.

3.3.4. *In vivo* flow cytometry assay

Firstly, the pre-culture of BY4741 yeast strain (genotype *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) from a stock culture in solid YPD broth (0.1% glucose, 0.05% yeast extract, 0.1% bacto-peptone and 0.1% agar, in 100 mL of dH_2O_2), was prepared by scraping some yeast cells from the yeast solid YPD culture into 5 mL of liquid YPD medium (0.1% glucose, 0.05% yeast extract and 0.1% bacto-peptone in 100 mL of dH_2O_2), under aseptic conditions. The pre-culture was left to grow overnight in the orbital shaker at 30°C, 200 rpm. Thereafter, the pre-culture was diluted with fresh YPD medium to an O.D of 0.1, read spectrophotometrically, and then cells were incubated at 30°C for 4 hours until an O.D of 0.4-0.8. Then, cells were harvested by centrifugation 5000 rpm, for 2 min at 4°C, Eppendorf 5810 centrifuge, and washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The suspension cell was diluted with PBS to an O.D of 0.02, and 500 µL were removed for later reading of the autofluorescence (AF). Meanwhile, 100 µL of 50 mM H_2DCFDA were added to 10 mL of cell suspension, then incubated for 60 min in the dark at 30 °C. Later, the suspension was once again centrifuged at 5000 rpm, for 2 min at 4°C, washed and resuspended in PBS. The cell suspension was then divided into 450 µL aliquots, and cells were incubated with 25 µL of 0.1 M H_2O_2 and 25 µL of extract. Positive controls (C+) were prepared with 450 µL of cell suspension, 25 µL of 0.1 M H_2O_2 and 25 µL of the respective extract solvent. Negative controls (C-) were performed the same way, but instead of H_2O_2, cells were incubated with the respective extract

solvent (50 µL). Then, samples were incubated in orbital shaker for 20 min, in the dark at 30 °C, and finally analyzed by CytoFlex in LX Beckman Coulter cytometer, using a 525/40 BP fluorescence channel at 488 nm.

3.4. Determination of genotoxic potential and antigenotoxic activity

3.4.1. *In vitro* DNA topology assay

The genotoxic and antigenotoxic potential of the extracts were assessed whit DNA topology assay using the plasmid pBR322 (0.05 µg/µL), and evaluated in the presence and absence of the $FeSO_4$, respectively. For the genotoxicity, each sample was prepared for a final volume of 10 µL with 0.5x TBE buffer (0.9 M Tris-HCl, 0.9 M H_3BO_3, 0.2 M EDTA, pH 8), 1 µL of pDNA, and Aq extract (50, 100, 500 and 1000 µg/mL) or O extract (10, 25, 50 and 100 µg/mL). For the antigenotoxicity potential, each sample was prepared as before and 1 µL of 2 mM $FeSO_4$ was added. The negative control was made as the genotoxic samples, without the extract, while positive control was made as the antigenotoxic samples, without the extract. Then, samples were mixed with 2 µL of loading dye solution (60% glycerol, 10 mM Tris-HCl, 60 mM EDTA, 0.03% bromophenol blue and 0.03% xylene cyanol FF) and applied on 1% agarose gel in 0.5x TBE, containing 10 µL of 10000x in water GelRed (Biotium), an ultra-sensitive and stable fluorescent nucleic acid dye for staining DNA in agarose gels, allowing bands visualization. The gel was submitted to electrophoresis at 75 V for 45 min and after gel image was captured by VWR GenoSmart.

3.4.2. *In vitro* Comet Assay

The alkaline comet assay was performed as described by Silva and others (2000), with some modifications, using human lymphocytes. The blood donor was a non-smoker female, aged 22, and the blood was collected by a capillary puncture. The blood was immediately divided into 30 µL aliquots with 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7,4) and kept on ice for 30 min. Later, 100 µL of Histopaque 1077 (Sigma) were gently added to each aliquot, in order to separate lymphocytes from the whole blood, and centrifuged at 5000 rpm, for 3 min at 4°C. Lymphocytes were carefully removed and added again to an aliquot with 1 mL of fresh PBS, followed by another centrifugation, in the same

conditions as before. The supernatant was discarded to isolate lymphocytes, and the pellet resuspended in 140 µL of 1.5% low melting point (LMP) agarose dissolved in PBS.

The glass slides were each covered with 0.5% normal melting point (NMP) agarose at about 50°C and kept at RT for 5 min to allow agarose to solidify. Thereafter, the lymphocyte and LMP suspension, were separated into two different drops on the precoated slides with NMP, and kept at 4°C for 10 min. To each slide, 1 mL of extract (10, 25, 50 and 100 µg/mL for O extract, or 50, 100, 250 and 500 µg/mL for Aq extract) was applied, then covered and kept at 4°C for 60 min. Two different slides of each concentration were prepared, in order to evaluate the genotoxic and antigenotoxic potentials, in the presence and absence of H_2O_2, respectively. For genotoxicity, extracts and C- slides were kept in PBS 1 hour, while for antigenotoxicity, extracts and C+, after 55 min, were subjected to a 10 mM H_2O_2 treatment, for 5 min. Thereafter, slides were washed with PBS and immersed in a freshly prepared cold lysing solution (2.5 M NaCl, 130 mM EDTA, 10 mM Tris, pH 10), to which was added 1mL of 1% Triton X-100, and let for 60 min at 4°C.

The slides were then placed in a horizontal electrophoresis tank filled with fresh electrophoresis solution (10 M NaOH, 200 mM EDTA, pH 13), just enough to cover all the slides. First, the slides were left for 20 min at 4ºC, to allow the unwinding of the DNA. Then, electrophoresis was initiated in the dark, at 4°C for 30 min, at 25 V and between 260 and 320 mA. Subsequently, the slides were washed for 5 min in PBS, then further 5 min in cold sterilized water and then they were left to dry for 24 hours. DNA was labelled with 10 µL of 10000x in water GelRed and covered with coverslips, to be analyzed on an Olympus BX51 fluorescence microscope with 200x magnification. The CometScore software was used to evaluate the DNA migration, comet tail length, of lymphocytes cells.

3.5. Determination of mutagenic potential and antimutagenic activity

3.5.1. *In vivo* AMES test

Three different histidine-dependent strains of *S. typhimurium* were selected for this assay, in order to assess the mutagenicity and antimutagenicity potentials of both extracts. These strains have in common a mutation in a gene of the histidine operon. Different strains have different types of mutation, while TA97 and TA98 share the same frameshift kind mutation, TA100 is susceptible to base-pair substitution mutation, **Table V.** The antimutagenicity was evaluated in the

presence of mutagens, capable of reverting the mutation in the histidine operon. The mutagens used were, sodium azide (NaN_3) for the TA100, 4-nitro-*O*-phenylenediame (4NOP) for TA98, and 9-aminoacridine (9-AC) for the TA97(Mortelmans & Zeiger, 2000).

Table V. Sequence of DNA targets of the *S. typhimurium* TA97, TA98 and TA100 strains, the histidine mutations and the reversion event (Mortelmans & Zeiger, 2000).

Strain	Histidine mutation	DNA target	Reversion event
TA97	hisD6610	-C-C-C-C-C-C-	Frameshift
TA98	hisD3052	-C-G-C-G-C-G-C-G-	Frameshift
TA100	hisG46	-G-G-G-	Base-pair substitution

The extracts were evaluated for the mutagenicity, by treating *S. typhimurium* strains only in the presence of the extracts. Whereas, for the antimutagenicity, the bacteria were co-treated with each extract and the respective mutagen. The C- was prepared with bacteria only, while C+ was prepared with bacteria and the respective mutagene (Tejs, 2008). First, 100 µL of each *S. typhimurium* strain, cultured for 10-12 hours at 37°C with stirring in LB medium broth (1% bacto tryptone, 0.5% bacto-yeast extract, 1% NaCl) were mixed with 3 mL of top agar (0.09 M NaCl, 0.018 M agar). Then to evaluate the mutagenic effect of both extracts, 50 µL of Aq or O extracts (1, 10 and 100 µg/mL) were added and the mixture was poured in minimal glucose agar plates (11.6 mM Na_3 citrate, 0.9 mM $CaCl_2$, 1.66 mM $MgSO_4$, 25 mM NH_4NO_3 and 36.8 mM KH_2PO_4). In order to evaluate the antimutagenic effect for TA97, TA98 and TA100 strains, 100 µL 9-AC, 50 µL of 4NOP and 20 µL of NaN_3 (50 µg per plate) were, respectively, added to the test tube mixture and then poured to the minimal glucose agar plates. The plates were left to dry for 10 min and after incubation at 37°C for 48h, the cells were counted.

3.6. Statistical Analysis

Statistical analysis was conducted using the GraphPad Prism 5 software. Data are shown as the mean values ± SD from at least three independent experiments ($n \geq 3$). In all cases, P-values lower than 0.05 were considered as statistically different, and different letters are used to identify significant differences between columns. Firstly, the significant differences of TPC and TFC results for both extracts were analyzed by t-test analysis. For the antioxidant activity, the DPPH scavenging assay was analyzed by t-test analysis and the reducing power capacity was analyzed by the comparison of slope, in order to test the differences between each treatment. Moreover, iron chelating assay results were analyzed by 1-Way ANOVA analysis of variance followed by Tukey's Multiple Comparison Test. Furthermore, for the flow cytometry, comet assay and AMES test, results were evaluated by 1-Way ANOVA analysis of variance followed by Dunnett's Multiple Comparison Tests. In the genotoxicity experiments, LE treatments were compared in relation to the negative control. In the antigenotoxic/antimutagenic evaluation, treatments were compared to the positive control to observe the modulatory effects of the *C. cardunculus* LE. Likewise, flow cytometry results were analyzed and the antioxidant capacity was compared to the positive control.

Due to the lack of biological material of O extract, it was not possible to determine its TFC value, iron chelating properties and the antigenotoxic potential by comet assay. Moreover, the range of concentrations used was determined considering possible toxicity of the O extract for concentrations higher than 100 µg/mL.

4. Results and Discussion

4.1. Total Phenolic (TPC) and Flavonoids (TFC) Content

TPC and TFC determination were carried out by colorimetric analysis, the Folin-Ciocalteau for polyphenolic compounds, and a flavonoids-$AlCl_3$ complex based-method for the flavonoids. In the presence of polyphenols, Folin reagent forms a blue complex that can be quantified spectrophotometrically at 760 nm (Blainski *et al.*, 2013). An increase in absorbance is then correlated with a higher polyphenolic content. In the same way, flavonoids also form a complex with $AlCl_3$, of yellow color that absorbs at 420 nm (Andersen & Markham, 2015). Therefore, an elevated flavonoid content is detected by a rising absorbance. Gallic acid, a strong antioxidant, was used as a standard in the quantification of polyphenols while quercetin was used for flavonoids. The total polyphenolic and flavonoid content, respectively in equivalents of GA and Q, are presented in **Table VI**.

Table VI. Total polyphenolic and flavonoid content (mean ± S.D) results for *C. cardunculus* Aq and O extracts, expressed in mg GAE/g extract and mg QE/g extract, respectively.

	TPC (mg GAE/g extract)	TFC (mg QE/ g extract)	% TFC/TPC
Extract	(mean±S.D)	(mean±S.D)	
Aq	33.11±7.87	8.15±0.032	24.61
O	15.69±6.86	-	-

The Aq extract showed a TPC (33.11±7.87 mg GAE/g extract) higher than O extract (15.69±6.86 mg GAE/g extract). Moreover, Aq extract presented a TFC (8.15±0.032 mg QE/g extract) which is about 4 times smaller than the TPC value. Therefore, 24.61% of all polyphenols are flavonoids. In addition, according to the t-test analysis, the values of TPC and TFC for the Aq extract were significantly different (P=0.0154).

The chemistry of extracts depends on the polarity of the solvent, time and temperature of the extraction, and on the chemical composition of the raw material (Rajbhar *et al.*, 2015). Mainly, solvent´s polarity influences the composition of both extracts in TPC and TFC, where more polar solvents, like water, appear to be more efficient in polyphenolic extraction (**Table VI**). Moreover, during the first extraction, dry leaves of artichoke were boiled in water to dissolve the

phenolic compounds of artichoke leaves, which limits at first the dissolution of some non-polar compounds. Therefore, the TPC of O extract is smaller, because it came originally from the Aq extract. However, after the ethyl acetate extraction, non-polar compounds are mainly found in the organic phase. Ethyl acetate polarity allows the dissolution of molecules with less polarity, such as isoflavones, flavones, flavanones, methylated flavones and flavonols. Contrarily, flavonoid glycosides are better extracted with water mixtures (Andersen & Markham, 2005). Besides the polarity of the solvent, the TPC of Aq extract is also influenced by the two extractions with ethyl acetate, since this solvent probably helped the dissolution of some more polyphenols, who were already present in the extract. Once the extension of the extraction time, hence the use of two solvents, with different polarities, allows a better dissolution of different phenolic compounds (Guiné *et al.*, 2017). The polarity of the solvent used influences the chemical composition of the extracts, which is then translated into different responses and mechanisms of action.

4.2. Antioxidant activity

4.2.1. *In vitro* DPPH scavenging activity

Developed by Blois, in 1958, DPPH scavenging assay is a simple and sensitive method that measures the scavenging capacity of natural antioxidants. In the presence of antioxidants, the nitrogen atom of DPPH is reduced by receiving a hydrogen atom, forming the DPPH-H (2,2'-diphenyl-1-picrylhydrazine, as represented in **Fig 2**. The measurement of absorbance at 517 nm, the wavelength which DPPH absorbs, allows the determination of the scavenging capacity of extract. Therefore, free radical scavenging capacity is based on DPPH discoloration, from purple to yellow, since the percentage of reduced DPPH (% RDPPH) increases with the decrease in absorbance (Kedare & Singh, 2011).

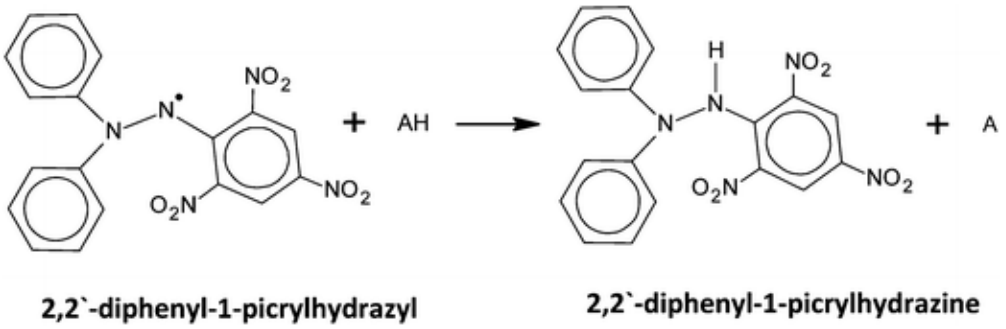


Figure 2. DPPH reducing reaction in the presence of an antioxidant (AH).

The percentage of reduced DPPH for the standard GA, O and Aq extracts were analyzed for different concentrations, as shown in **Fig. 3**. Regarding the resulting equations, slope decreased by the order of GA, O extract and Aq extract. Aq extract slope was significantly different from zero, with P<0.0001, which means that the extract exhibited scavenging activity (**Fig. 3a**). Although the O extract slope was significantly different from zero (P=0.0114), the difference is less significant than for the Aq extract (**Fig. 3b**). The standard GA was significantly different from zero since P=0.0081 (**Fig. 3c**).

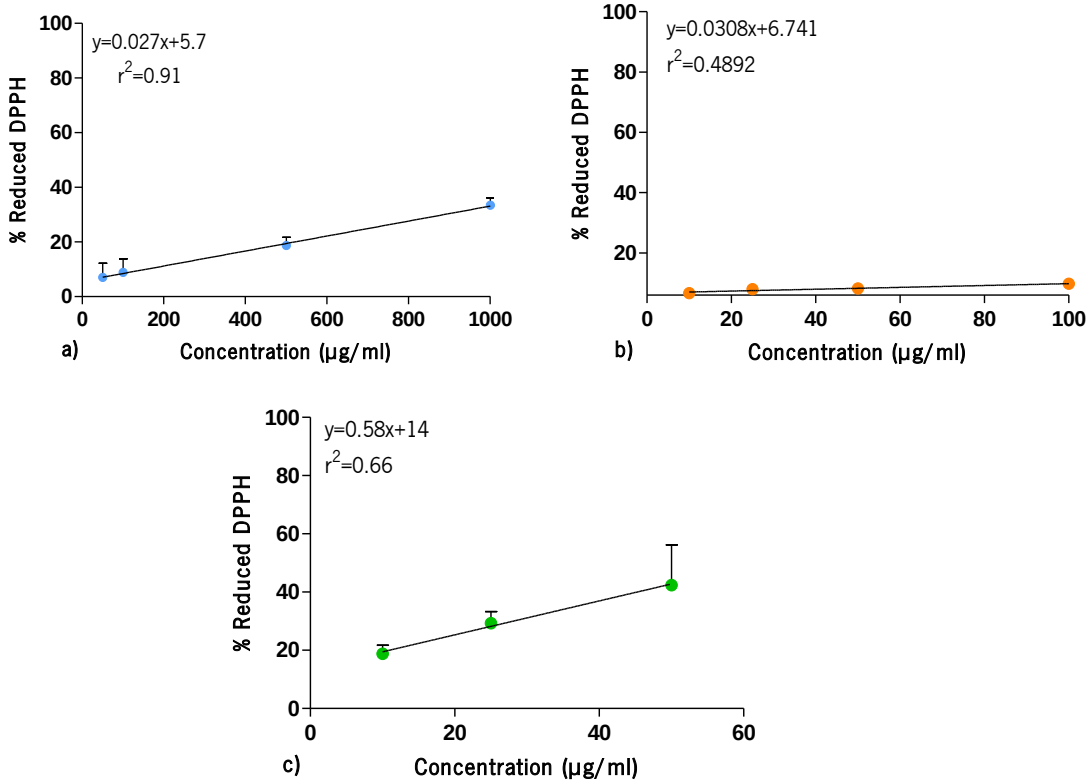


Figure 3. *In vitro* DPPH scavenging activity of *C. cardunculus* aqueous (Aq) and organic (O) extracts, and of standard gallic acid (GA) with the respective linear regression equation and associated error: **(a)** Aq extract (50 100, 500 and 1000 µg/mL), **(b)** O extract (10, 25, 50 and 100 µg/mL) and **(c)** GA (10, 25 and 50 µg/mL). The percentage results of reduced DPPH are representative of three independent replicates, n=3.

The IC_{50} was calculated through the calibration equations of GA and Aq and O extracts, which is defined by the concentration needed to reduce 50% of DPPH in solution (**Table VII**). According to the t-test analysis, there are significant differences between Aq extract and the standard and also between O extract and the standard, both with P<0.0001. The GA IC_{50} value

showed to be much lower than the IC_{50} of both extracts, where Aq extract presented the highest result. Therefore, these results suggest that O extract showed a higher scavenging activity than Aq extract. Besides Aq extract higher percentage of reduced DPPH, the concentrations used for this extract were higher than those used with O extract.

Table VII. IC_{50} (mean ± S.D) results for *C. cardunculus* aqueous (Aq) and organic (O) extracts, and the standard gallic acid (GA), expressed in mg/mL.

Samples	IC_{50} (mg/mL)
	mean±S.D
GA	0.06±0.01
Aq	1.61±0.07
O	1.40±0.02

According to the IC_{50}, O extract shows higher DPPH scavenging activity. Even though, Aq extract higher TPC value, O extract might possess chemical compounds with higher scavenging capacity than those presented by the Aq extract. Probably due to the presence of hydroxyl groups in phenols, once their number and position in the aromatic core influence the antioxidant capacity (Benhammou *et al.*, 2013). However, there are studies which report that aqueous extract of ALE is the most suitable solvent for extraction of phenolic compounds (da Silva Oliveira *et al.*, 2014). Although the scavenging capacity presented by the Aq extract, it is important to highlight that these results are at high concentrations that could be, possibly, toxic *in vivo* (Zan *et al.*, 2013).

In previous studies with artichoke, aqueous extract showed DPPH scavenging activity, in a dose-dependent manner. However, the extraction procedure used was different, extracts were only extracted with water and were performed with dry leaves of *C. scolymus* (Ben Salem *et al.*, 2017).

4.2.2. *In vitro* reducing power activity

This assay allows the evaluation of the reducing capacity of antioxidant extracts (Vijayalakshmi & Ruckmani, 2016). The complex formed by potassium ferricyanide, trichloroacetic acid and ferric chloride can be reduced in the presence of an antioxidant, through donation of an electron **Fig. 4**. This reduction is noticed at 700 nm by the green-blue complex formation, when ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}) (Jayanthi & Lalitha, 2011). A higher absorbance at 700 nm corresponds to a higher number of complex formation, which itself translates into a greater reducing power capacity.

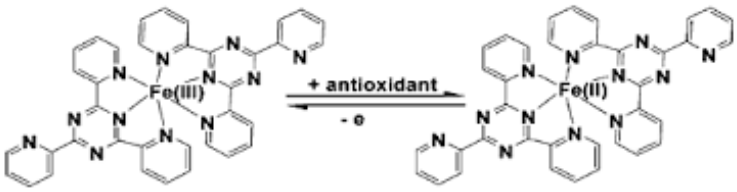


Figure 4. Reduction of the potassium ferricyanide, trichloroacetic acid and ferric chloride (3+) complex in the presence of an antioxidant, resulting into the potassium ferrocyanide, trichloroacetic acid and ferrous chloride (2+) complex.

Both extracts were tested for a range of concentrations between 50 and 1000 µg/mL, **Fig. 5**. The Aq extract and the standard GA slope were significantly different from zero, once $P<0.0001$ and $P=0.0008$, respectively. On the other hand, O extract was not significantly different from zero, with $P=0.7636$, **Fig. 5b**. Moreover, the slopes decreased by the order of GA, Aq extract and O extract. Therefore, Aq extract presented some reducing power capacity, though O extract did not presented.

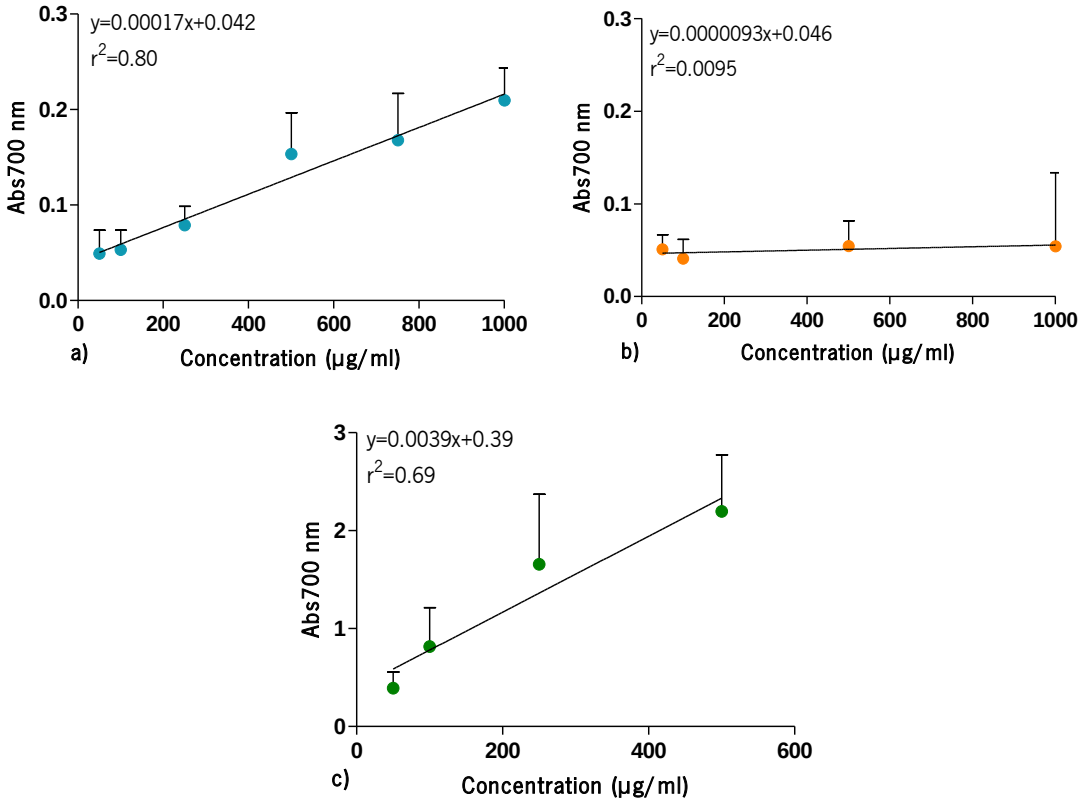


Figure 5. Reducing power capacity of *C. cardunculus* aqueous (Aq) and organic (O) extract, and of standard gallic acid (GA) with the respective linear regression equation and associated error: **(a)** Aq extract (50, 100, 250, 500, 750 and 1000 µg/mL), **(b)** O extract (50, 100, 500 and 1000 µg/mL), and **(c)** GA (50, 100, 250 and 500 µg/mL). The results of absorbance are representative of three independent replicates, n=3.

The reducing power activity presented by Aq extract could be related with its higher polyphenolic content since polar compounds have hydroxyl group (-OH), allowing the reduction of the ferrous-complex by donating an hydrogen. However, O extract compounds did not showed reducing power capacity, suggesting that they have less hydroxyl groups, polar groups.

4.2.3 Iron chelating capacity

The ferrous iron chelating assay allows determining the ability of natural extracts in chelating transition metal ions, such as ferrous ion. Antioxidants compete with ferrozine for ferrous ions, resulting in a decrease of the red ferrozine-Fe^{2+} complex formation. Therefore, the chelating capacity of extracts is given by a decreasing in the red color intensity of complexes at 562 nm (Yusof *et al.*, 2013). This was determined by the percentage of inhibition of ferrozine-Fe^{2+} complex formation, that decreases with higher antioxidant concentrations (**Equation o)**).

For each concentration, the percentage of inhibition of ferrozine-Fe^{2+} complex for Aq extract showed to be much lower when compared to EDTA (**Fig. 6**). The standard EDTA, did inhibited the formation of ferrozin-Fe^{2+} complex by chelating the ions Fe^{2+}, in a dose-dependent manner. Moreover, the chelating capacity is significant different for all the concentrations used, with $P<0.0001$.

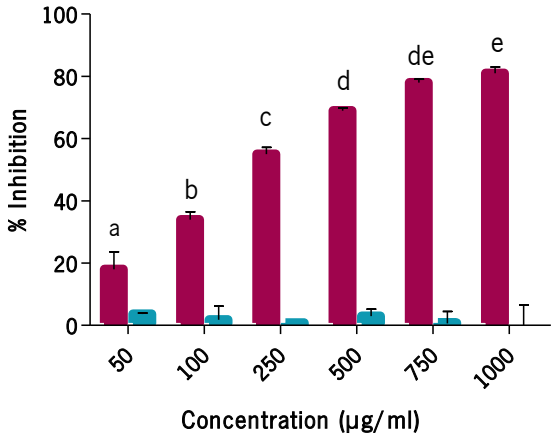


Figure 6. Chelating capacity of *C. cardunculus* aqueous (Aq) extract, and the respective standard EDTA. The percentage (%) of inhibition of ferrozine-Fe^{2+} complex, at 700 nm, was calculated for: EDTA (purple) vs Aq extract (blue) (50, 100, 250, 500, 750 and 1000 µg/mL). The results (mean±S.D), n=3, of EDTA were statistical analyzed by the 1-Way ANOVA analysis, followed by Tukey's multiple comparison test, which allows the evaluation of differences between different concentrations of extract. Different letters are used when there are significant differences.

Considering the Aq extract, the low values of chelating capacity and the lack of constancy between the results, suggests that the chelating activity is practically absent (**Fig. 6**). According to the results, the Aq extract, probably, does not have iron chelating capacity which is commonly associated with presence of flavonoids and their aromatic rings.

4.2.4 *In vivo* flow cytometry

Flow cytometry allows the measurement of optical and fluorescence characteristics of a single cell in a fluid stream, when it passes through a light source (Macey, 2010). Size, granularity and fluorescence features are used to evaluate and differentiate the cells in a heterogeneous population. The essential principle of flow cytometry is related to light scattering and fluorescence emission, that occurs when a light beam strikes the moving particles. Light scattering is divided into forward light scatter (FSC) which is proportional to cell size, and side light scatter (SSC) which is proportional to cell granularity. These parameters are affected by the membrane, nucleus, granularity and shape of cell. Fluorescence emission is associated with fluorescence probe, which is proportional to the number of fluorescent probe bound to the cell (Adan *et al.*, 2017). The 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) is widely used for detecting intracellular ROS and oxidative stress. This probe is cell-permeable and hydrolyzed by intracellular esterases, resulting in 2,7- dichlorodihydrofluorescein (H_2DCF), which is retained in the cell. In the presence of ROS, is oxidized to a fluorescent product, 2,7-dichlorofluorescein (DCF) with maximum excitation and emission spectra of 495 nm and 529 nm respectively (Kalyanaraman *et al.*, 2012).

The *S. cerevisiae* (strain BY4741) was the eukaryotic model cell used, that presents by itself antioxidant defense mechanisms such as antioxidant enzymes in response to ROS. In order to evaluate the antioxidant activity of the extracts, yeast cells were treated with different concentrations of Aq and O extracts, in the presence of H_2O_2. The fluorescence intensity of *S. cerevisiae* cells is proportional to the intracellular oxidation, therefore cells treated with an oxidant agent will emit higher fluorescence than untreated cells (**Fig. 7**). Moreover, in the presence of an antioxidant extract, the fluorescence decreases.

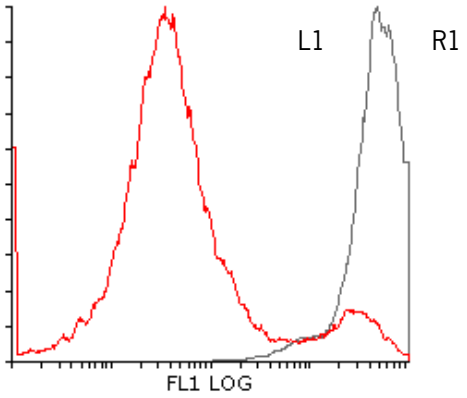


Figure 7. Flow cytometry histogram of *S. cerevisiae* negative (C-) and positive (C+) controls. The histogram presents the number of events with the fluorescence (PL1 LOG), for: C- (red) only with *S. cerevisiae* cells, and for the C+ (grey), which presents *S. cerevisiae* cells and H_2O_2. For the C+, the cells were treated with H_2O_2 (10 mM), in the dark for 20 min. L1 and R1 represents, respectively, the % of cells with lower and higher fluorescence than C+.

The percentage of cells, with higher fluorescence than C+ (R1) and with lower fluorescence than the C+ (L1) for O extract, are shown in **Table VIII**. It's possible to see the differences between the negative and the positive controls, with low and high fluorescence respectively. Therefore, the cells alone in the C- are not oxidized and that the H_2O_2 solution worked perfectly. The fluorescence was significantly different from the C+, for the 25 and 50 µg/mL of O extract and also for C-. Although, for lower and higher concentrations, the differences of fluorescence were not significant.

The percentage of cells with lower fluorescence than the C+, increases in a dose-dependent manner. Moreover, the results between tested concentrations were significantly different (P=0.0009). For concentrations higher than 5 µg/mL, the percentage of cells with less fluorescence than the positive control was higher than 50, which suggests that the extract has some antioxidant activity. However, for lower concentrations tested (1 and 0.1) the extract did not exhibited any effect. So the range of concentrations which cause some protective effect can be found between 5 and 100 µg/mL.

Table VIII. *In vivo* flow cytometry results for the % of cells with lower and higher fluorescence than C+. *S. cerevisiae* cells were first treated with 50 mM of H_2DCFDA, for 1 hour in the dark at 30°C, and then treated with organic (O) extract (0.1, 1, 5, 10, 25, 50 and 100 µg/mL), and H_2O_2 (10 mM), 20 min in the dark at 30°C. The fluorescence was analyzed by flow cytometry at 488 nm, using the CytExpert software. The results (mean ±S.D), n=3, were evaluated by 1-way ANOVA analysis, followed by Dunnett post-test to determine the significant differences (***) between the different concentrations of extract compared to the C+.

Sample	% cells with less fluorescence than C+	% cells with more fluorescence than C+
C- EtOH 70%	99.95±0.04 (**)	0.05±0.04
C+ EtOH 70%	50.58±1.50	49.42±1.50
O100	70.18±9.16 (ns)	29.82±9.16
O50	84.05±24.18 (**)	15.95±24.18
O25	80.53±11.72 (*)	19.47±11.72
O10	66.35±8.68 (ns)	33.65±8.68
O5	55.21±5.91 (ns)	44.79±5.91
O1	47.72±12.00 (ns)	52.28±12.00
O 0.1	39.79±9.73 (ns)	60.21±9.73

The percentage of cells with less and more fluorescence than C+, for the Aq extract, was not significantly different between tested concentrations (P=0.0132; **Table IX**). On the other hand, the differences of fluorescence between the two controls were significant (**), and the positive control showed higher fluorescence. Regarding Aq extract, there was no dose-dependent effect and the percentage of cells with higher fluorescence than the C+ was always higher than 50. Moreover, the standard deviations were too high. Therefore, this extract was not able to protect *S. cerevisiae* cells from oxidative stress, and may be increasing the effect of H_2O_2 working as a pro-oxidant.

Table IX. *In vivo* flow cytometry results for the % of cells with lower and higher fluorescence than C+. *S. cerevisiae* cells were first treated with 50 mM of H_2DCFDA, for 1 hour in the dark at 30ºC, and then treated with aqueous (Aq) extract (0.1, 1, 10 and 25 µg/mL), and H_2O_2 (10 mM), 20 min in the dark at 30ºC. The fluorescence was analyzed by flow cytometry at 488 nm, using the CytExpert software. The results (mean ±S.D), n=3, were evaluated by 1-way ANOVA analysis, followed by Dunnett post-test to determine the significant differences (***) between the different concentrations of extract compared to the C+.

Sample	% cells with less fluorescence than C+	% cells with higher fluorescence than C+
C- H_2O	99.86±0.09 (**)	0.14±0.09
C+ H_2O	47.70±5.09	52.30±5.09
Aq25	31.61±9.46 (ns)	68.39±9.46
Aq10	39.69±51.95 (ns)	60.31±51.96
Aq1	28.70±19.51 (ns)	77.30±19.51
Aq0.1	22.27±16.16 (ns)	77.73±16.16

Both extracts have compounds with *in vitro* antioxidant properties, although only the O extract presented antioxidant activity in living cells. The presence of more hydrophobic compounds in the organic extract, allowed an intracellular response by the extract due to the permeability of its compounds. Contrarily, the polarity of Aq extract prevents its compounds to operate inside the cells, more hydrophilic compounds. For that reason, the Aq extract showed *in vitro* antioxidant activity, by scavenging activity and reducing power, but was not able to protect in living *S. cerevisiae* cells. Moreover, the antioxidant activity of O extract, was explained by the direct interaction between the extract compounds and ROS, and probably not by the activation of the endogenous antioxidant mechanisms of the cell. Once in this experiment, yeast cells were co-treated with the extract and H_2O_2, preventing a higher exposition time of cells to H_2O_2 and the interaction between H_2O_2 and the extract alone.

4.3 Genotoxicity potential and antigenotoxic activity

4.3.1 *In vitro* DNA Topology Assay

DNA Topology assay allows the *in vitro* evaluation of genotoxic and antigenotoxic potentials of tested compounds by detection of different electrophoretic mobilities of topological forms of DNA. This method is based in the reducing ability of $FeSO_4$ to induce structural damages in pDNA. The damages caused by $FeSO_4$ induce different arrangements in the DNA conformation, leading to modifications in the migration pattern during agarose gel electrophoresis (Franke *et al.*, 2005; Lee *et al.*, 2012). The pDNA can take three different conformations: supercoiled DNA or form I (native conformation); form II (relaxed DNA) that results from single-strand DNA breaks; or form III (linear DNA) generated by double-strand breaks (**Fig. 8**).

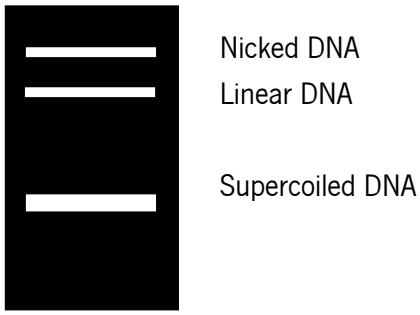


Figure 8. The migration of different conformations of DNA, under an electric field from negative pole (above) to positive pole (below). In crescent order of migration: form I (supercoiled DNA), form II (linear DNA), and form III (nicked DNA).

The undamaged topological forms, such as form I, migrate faster than those forms with DNA strand breaks, as forms II and III (De Mattos *et al.*, 2015). For the evaluation of the genotoxicity and antigenotoxicity of both extracts, two different controls were prepared. The C-, with pDNA only, to use in the genotoxic potential, while the C+, where pDNA was treated with $FeSO_4$ (2 mM), to use as control for the antigenotoxic activity.

Regarding the genotoxicity evaluation, since the migration pattern was the same for an extract sample and for the C-, means that the extract alone was not harmful to pDNA. Accordingly, the mobility of O extract (10, 25, 50 and 100 µg/mL) and Aq extract (50, 100, 500 and 1000 µg/mL) was the same as C- (**Fig. 9a**). For the antigenotoxicity, the migration pattern of Aq extract (50, 100 and 500 µg/mL) was the same as the C+, where prevail the pDNA II and III

forms. The highest concentration, however, showed a high mobility as the C-. Moreover, the O extract presented the same migration pattern as the C-, for all the concentrations used (**Fig. 9b**).

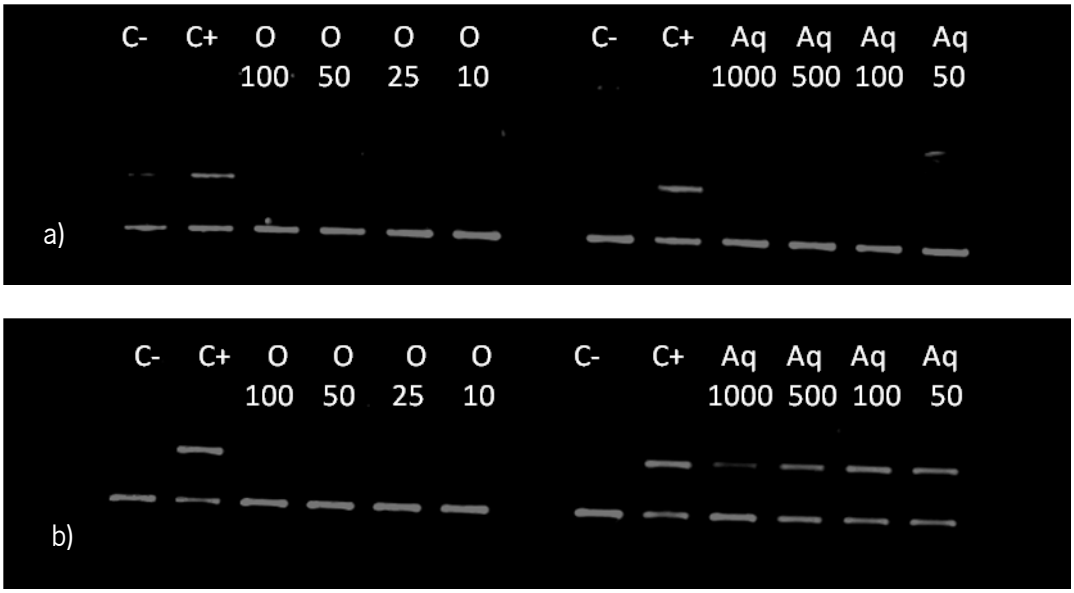


Figure 9. *In vitro* DNA topology images of the mobility of *C. cardunculus* aqueous (Aq) and organic (O) extracts, and their respective negative (C-) and positive (C+) controls. For the genotoxicity **(a)**, pDNA was treated with Aq extract (50, 100, 500 and 1000 µg/mL) and O extract (10, 25, 50 and 100 µg/mL). For the antigenotoxicity **(b)**, pDNA was treated with Aq and O extract, same concentrations, and $FeSO_4$ (2 mM). The C-, pDNA only, was used as the control for the genotoxicity, while the C+, with pDNA and $FeSO_4$, was used as the control for antigenotoxicity. The 1% agarose gel was stained with GelRed (10 000x in water), and the electrophoresis ran for 45min at 75 V.

Considering the concentrations used, the results suggest that neither of the extracts were genotoxic. Once, the high mobility was associated with undamaged pDNA. Bearing the antigenotoxic potential, the extracts behave differently. Aqueous extract, 1000 µg/mL, seems to protect the DNA against $FeSO_4$ induced damage. The antigenotoxic activity presented by this extract could be explained by its phenolic composition, which had reducing power and scavenging properties. Moreover, the extracts could, probably, be antigenotoxic for higher concentrations. In addition, the compounds of O extract were capable to protect DNA, for all the extract concentrations, suggesting that O extract was antigenotoxic. Once more, the antigenotoxic activity relies on the TPC and TFC values of the extracts, and their polarities. Probably, due to the

presence of some non-polar compounds in O extract, such as flavonoids, the antigenotoxic activity was influenced. Some flavonoids which can protect DNA through hydrogen bond interactions, suggesting that those compounds could form a protective lipophilic shield around plasmid DNA, preventing DNA to interact with $FeSO_4$ (Horvathova *et al.*, 2014). Additionally, this activity cannot be explained by the flavonoids chelating capacity, since the extract did not show any chelating capacity.

4.3.2. *In vitro* Comet Assay

Comet Assay was used to detect DNA damage induced by H_2O_2, in human lymphocyte cells, and also to examine the effects of an antioxidant agent responding to an oxidative stress (Kushwaha *et al.*, 2011). After the extraction and isolation of the lymphocytes, these were treated with different concentrations of Aq and O extract, for the genotoxicity. Whereas, for the antigenotoxicity, the pre-incubated lymphocytes were then treated with H_2O_2. Negative control (lymphocytes only) and positive control (lymphocytes and H_2O_2) were prepared for the genotoxicity and the antigenotoxicity, respectively. Once lymphocytes were lysed, they were subjected to an electric field of electrophoresis. Where undamaged DNA remained at the maximum level of folding (nucleolus), achieving a smaller displacement in the gel, like C-. However, in the presence of H_2O_2, DNA unfolds, achieving a higher electrophoretic migration, which is very similar to a comet tail, like the C+. Therefore, the tail length is proportional to DNA damages (Najafzadeh & Anderson, 2016).

The tail lengths of comets, in the presence of the extracts only, were statistically analyzed and all the samples were compared to the C- (**Fig. 10**). First, the C- showed to be significantly different from the C+, and C+ presented comets with the higher tail length. The reduced tail length of C- comets, showed that lymphocytes were intact and good to use. Moreover, the tail length mean of comets for both Aq (P=0.0.0132) and O extract (P=0.0020), were not significantly different from the C-. In addition, the comet tail length was dose-dependent for the Aq extract (**Fig. 10a**), and also for the O extract except for the 25 µg/mL (**Fig. 10b**).

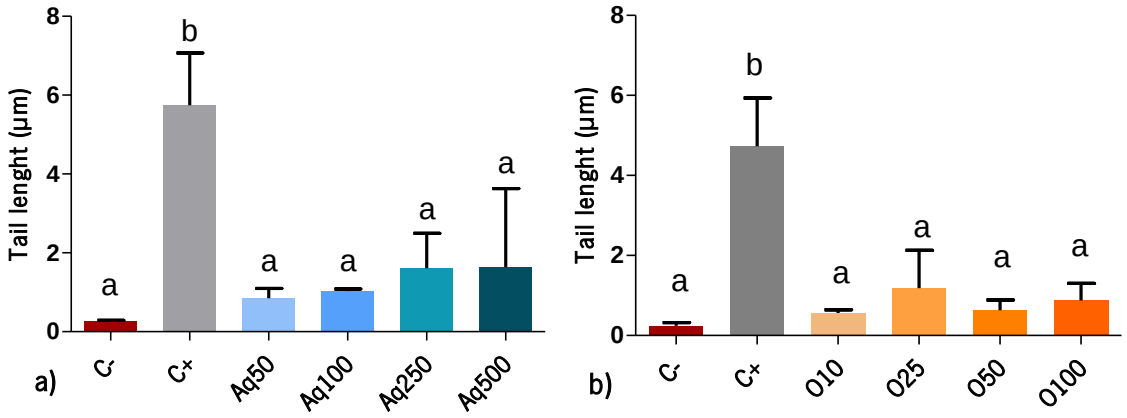


Figure 10. *In vivo* comet assay using human lymphocytes for the genotoxicity of *C. cardunculus*, **(a)** aqueous (Aq) and **(b)** organic (O) extracts, and their respective negative (C-) and positive (C+) controls. Lymphocytes were treated with Aq extract (50, 100, 250 and 500 µg/mL) and O extract (10, 25, 50 and 100 µg/mL), for 1 hour, and the C-, lymphocytes only, was used as control. The electrophoresis was at 25V for 30 min, and then the comets were dyed with GelRed (10 000x diluted in H_2O) and visualized with 200x magnification. The results (mean±S.D), n=3 where 1 replicate corresponds to 20 lymphocytes measured, were evaluated by 1-way ANOVA analysis, followed by Dunnett's post-test to determine the significant differences between the different treatments and the C-. Different letters are used when there are significant differences.

Comparing both extracts with the C-, for the tail length, there are no significant differences, therefore none of the extracts induced DNA damages. Which means that, the results suggest that Aq and O extracts are not genotoxic.

To evaluate the Aq extract antigenotoxicity, lymphocyte cells were pre-treated with the extracts and then with H_2O_2, and the tail length of comets are shown in **Figure 11**. Once more, C- and C+ were different, and C+ presented the higher tail length. The tail lengths of Aq extract were to high comparing to C-, suggesting that the extract was not able to protect lymphocytes against genotoxic effect of H_2O_2.

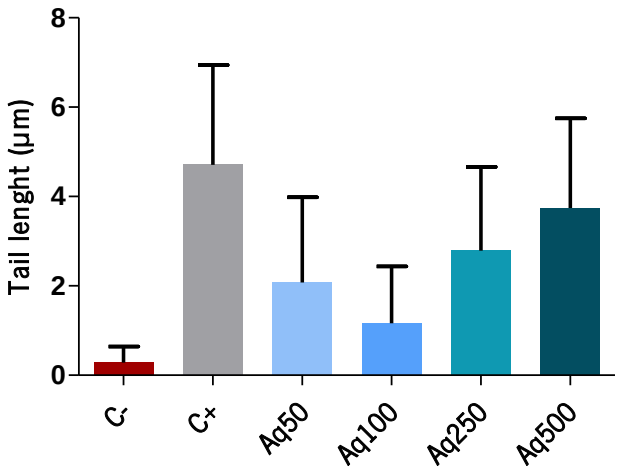


Figure 11. *In vivo* comet assay using human lymphocytes for the antigenotoxicity of *C. cardunculus* aqueous (Aq) extract, and the respective negative (C-) and positive (C+) controls. The lymphocytes were pre-treated with Aq extract (50, 100, 250 and 500 µg/mL) for 55 min, and pos-treated with H_2O_2 (10 mM) for 5 min. The C+, lymphocytes and H_2O_2, was used as control. The electrophoresis was at 25V for 30 min, and then the comets were dyed with GelRed (10 000x diluted in H_2O) and visualized with 200x magnification. The results shown were n=1, where one replicate corresponds to 20 lymphocytes measured.

The results are in accordance with the data shown in DNA topology assay, none of the extracts showed to be genotoxic for the concentrations used, and Aq extract was not antigenotoxic whether in the presence of $FeSO_4$ or H_2O_2. Therefore, scavenging activity and reducing power were not involved the response of Aq extract.

In previous studies, considering *C. scolymus* aqueous extract, results showed that leaf extracts did not presented genotoxicity in peripheral blood cells (Zan *et al.*, 2013).

4.4. Mutagenicity potential and Antimutagenic activity

4.4.1. *In vivo* AMES Test

The AMES test is a test normally used to determine the mutagenic potential of new chemicals and drugs, but also allows the determination of antimutagenic activity of extracts. This test works with three different histidine-requiring *S. tymphimurium* mutant strains, each one carrying different mutations in various genes in the histidine operon. The TA98 and TA97 have in common the same kind of mutation, additions and deletions of 1 or more bases (but not multiple of 3), the frameshift mutations, while TA100 mutation is a base-pair substitution. Therefore, when *S. tymphimurium* strains are cultured on a medium without histidine, there are no colony growth. However, in the presence of a mutagen which can revert the mutation in histidine genes, genes function is restored, and consequently the number of colonies spontaneously increases. Thus, only those mutagens that revert to histidine dependence are able to form colonies.

In order to study the mutagenic effect of extracts, *S. tymphimurium* was treated alone with the extracts. Hence, extracts were compared to the C-, which was prepared only with *S. tymphimurium.* When the number of colonies of the extract is similar to the C-, means the extract is not mutagenic. For all the studied strains, the standard deviations values were too high, especially in the case of strains TA97 and TA98, hindering future conclusions. The differences between C- and C+, for the strains TA97 (P=0.475) and TA98 (P=0.4815), were not significant (**Figure 12**). Moreover, the number of *Salmonella* colonies had no concentration-dependent relation, for any of the extracts. Therefore, it was not possible to determine whether the extracts exert a mutagenic effect on these strains. However, the differences between C- and C+ were significantly different for the TA100 strain (P=0.0190; **Fig. 12c**). In addition, the number of colonies for all the concentrations used, Aq and O extracts, were similar to the C-, but had no concentration-dependent manner and the standard deviations were too high. Therefore, was not possible to determine if the extracts were mutagenic or not for the *S. tymphimurium* TA100 strain. However, other studies did not demonstrate mutagenic results with leaves from *C. cardunculus* in *Salmonella typhimurium* (Miadokova *et al*., 2008).

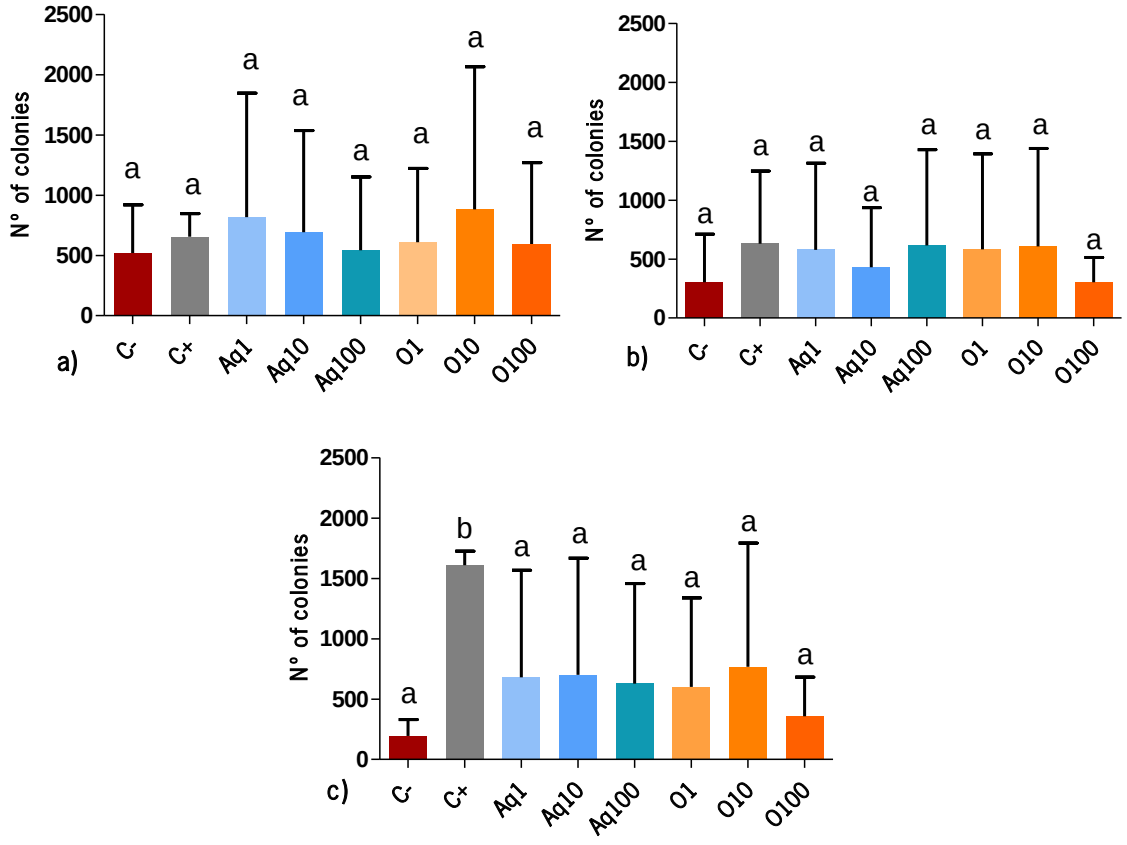


Figure 12. *In vivo* AMES test, using *S. tymphimurium,* for *C. cardunculus* aqueous (Aq) and organic (O) extracts, and the respective negative (C-) and positive (C+) controls. The bacteria cells were treated with Aq and O extracts (1, 10 and 100 µg/mL), C- was only the bacteria, and C+ was bacteria, extract and the respective mutagen. The genotoxicity was determined for: **(a)** TA97 strain, **(b)** TA98 strain and **(c)** TA100. The results (mean±S.D), n=3, were evaluated by 1-way ANOVA analysis, followed by Dunnett's post-test to determine the significant differences between the different treatments and the C-. Different letters are used when there are significant differences.

For the antimutagenic potential, S. *tymphimurium* cells were co-treated with the extracts in the presence of the mutagens, 9-AC for the TA97, 4NOP for the TA98, and NaN_3 for the TA100 (Mortelmans & Zeiger, 2000). The extracts were compared with C+, which was *S. tymphimurium* and mutagen (**Fig. 13**). The differences between C- and C+ for the strains TA97 (P=0.3633) and TA98 (P=0.1386), were not significantly different, therefore was not possible to determine the antimutagenicity of these extracts. Although, there was significant differences between the C- and C+ for the TA100 strain, the number of colonies was higher than the C-, for both extracts

(P=0.0025; **Fig. 13c**). Moreover, for both extracts, there was no significant differences between any concentrations tested. Considering these differences, the high value of the standard deviations, and the higher number of colonies, we could say that none of the extracts are antimutagenic for the TA100 strain. Therefore, the extracts were not able to stop mutagen of revert histidine gene mutation.

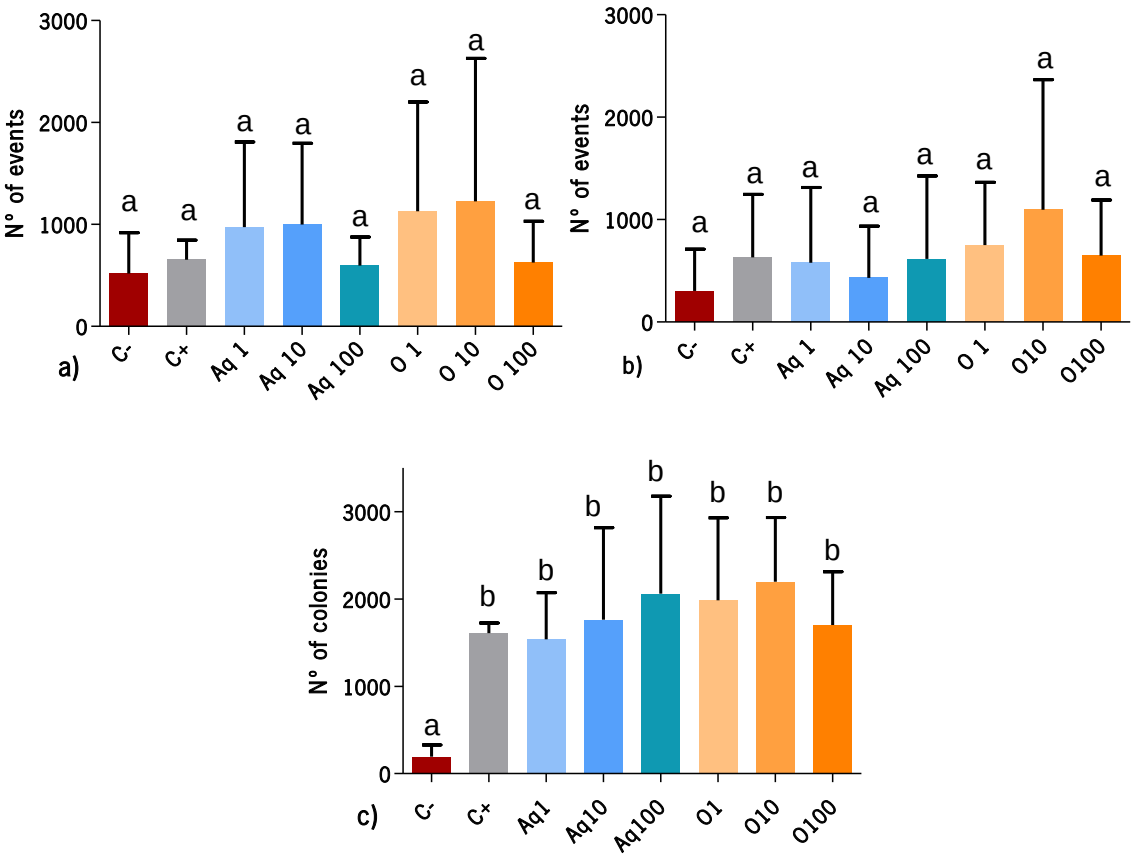


Figure 13. *In vivo* AMES test, using *S. tymphimurium,* for *C. cardunculus* aqueous (Aq) and organic (O) extracts, and the respective negative (C-) and positive (C+) controls. The bacteria cells were treated with Aq and O extracts (1, 10 and 100 µg/mL), C- was only the bacteria, and C+ was bacteria, extract and 50 µg of the respective mutagene per plate (100 µL of 9-AC, 50 µL of 4NOP and 20 µL of NaN_3). The antigenotoxicity was determined for: **(a)** TA97 strain and 9-AC mutagen, **(b)** TA98 strain and 4NOP mutagen, and **(c)** TA100 strain and NaN_3 mutagen. The results (mean±S.D), n=3, were evaluated by 1-way ANOVA analysis, followed by Dunnett's post-test to determine the significant differences between the different treatments and the C+. Different letters are used when there are significant differences.

Since it was not possible to ascertain the mutagenic and antimutagenic potential of the extracts, in the presence of *S. tymphimurium* TA97, TA98 and TA100 strains, further testing should be done. In order to determine the activities of these extracts and possibly relate them with the TPC and TFC values.

5. Conclusion and future perspectives

Considering these experimental results, natural *C. cardunculus* leaves extracts, showed different biological properties in response to cellular oxidative stress. The various *in vitro* and *in vivo* assays present different results, considering mainly the extracts compositions and the polarity of the solvents used during the extraction procedure.

First, the colorimetrical analysis revealed that water was the best solvent for the extraction, which corresponds to a higher polyphenolic content. Suggesting that Aq extract composition is mainly polar, and that O extract presents more affinity to less polar compounds, such as some flavonoids. Therefore, the composition of the extracts is extremely important to understand the exposed results. Regarding the antioxidant capacity, Aq extract showed *in vitro* antioxidant activity, by scavenging activity and reducing power, but was not able to protect in living *S. cerevisiae* cells. Furthermore, results suggest that chelating activity of Aq extract is not relevant. Moreover, O extract exhibited *in vitro* antioxidant potential by scavenging activity but not by reducing power, and was able to protect *S. cerevisiae* from oxidative stress. This could be explained by the presence of hydrophobic compounds, in O extract, since they can permeate the cell membrane and disclose a greater antioxidant response than Aq extract.

DNA topology assay results are in accordance with comet assay results, since none of the extracts were genotoxic. Moreover, in DNA topology, O extract was antigenotoxic for the tested concentrations, while Aq extract just presented antigenotoxic effects for the highest concentration. Therefore, the underlying mechanisms of action could be explained by the presence of some non-polar compounds which are not in the Aq extract. In addition, Aq extract did not protected lymphocyte cells against H_2O_2 genotoxic effect, besides its antioxidant activity *in vitro*. Finally, the mutagenic potential and the antimutagenic potentials of extracts, by AMES test, for three different *S. tymphimurium* strains were only able to be assess with the TA100 strain. As a result, none of the extracts were mutagenic, and none were able to inhibit the mutagen reversion in histidine operon.

The knowledge of the chemical composition of extracts is extraordinarily important because it allows ascertaining which compounds are present in the extract and which biological activities are associated with them. Therefore, is extremely imperative to perform a chemical analysis of these extracts to achieve new and better acknowledgment about the species *C. cardunculus*.

Moreover, in order to verify that O extract *in vivo* antioxidant activity and *in vitro* antigenotoxic activity, were influenced by the presence of flavonoids, it would be interesting to determine its content in flavonoids. Furthermore, the evaluation of O extract antigenotoxicity, by comet assay, to see if the results support the results showed by DNA topology assay. Hence, there were no significantly differences between C- and C+, for TA97 and TA98 *S. tymphimurium,* and for TA100 the results were inconclusive, therefore AMES test should also be repeated.

The biological properties presented by *C. cardunculus* leaves extracts, valorize even more the consume of artichoke, and the recognition of this plant as a source of bioactivities, such as antioxidant and antigenotoxic. Moreover, the extracts presented different properties that can be potential used in many, nutraceutical, cosmetic and clinical applications. Additionally, it is important to take into account that biological effects of artichoke should be clarified before considering it as a therapeutically useful plant. Therefore, experimental research of this kind becomes an essential tool to characterize artichoke natural extracts, hence the knowledge of its properties and limitations.

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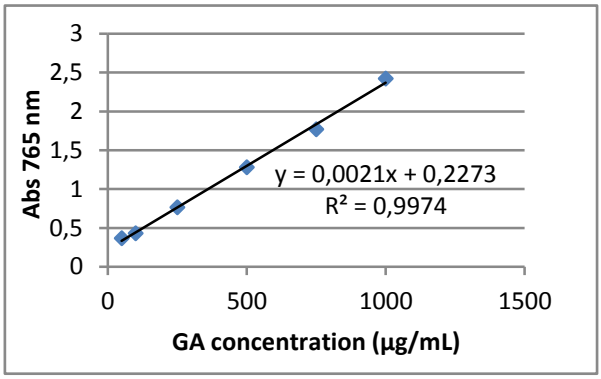
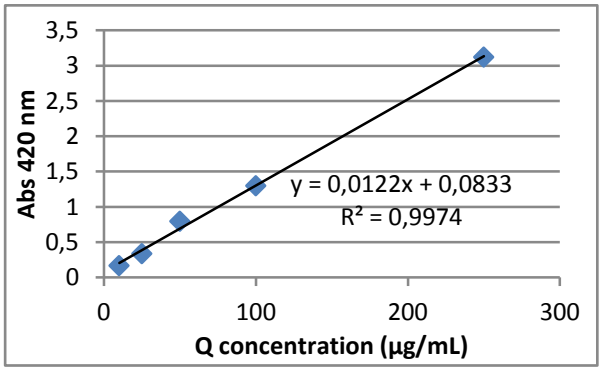
Appendix

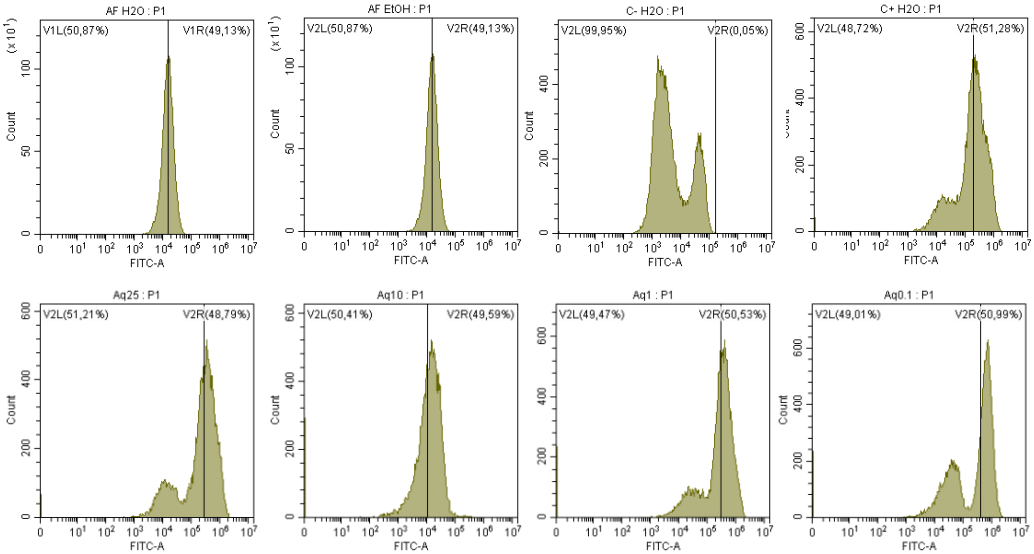
Appendix A. Gallic acid calibration curve.

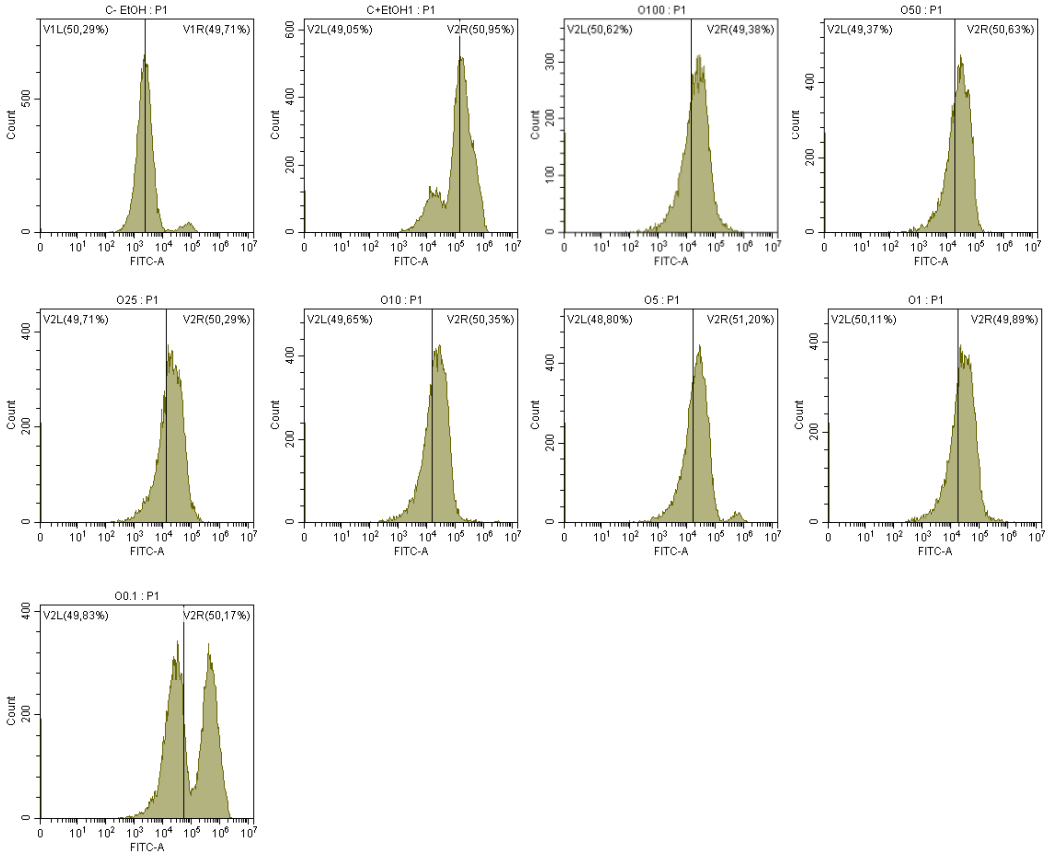


Appendix B. Quercetin calibration curve.

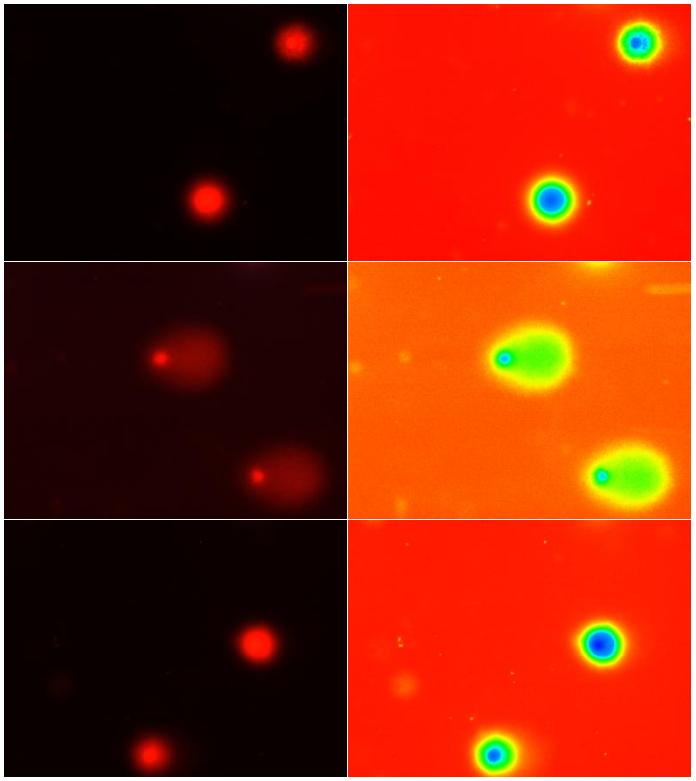


Appendix C. Flow cytometry histograms, by CytExpert software.





Appendix D. Comet assay lymphocytes images, by CometScore.



Genotoxicity

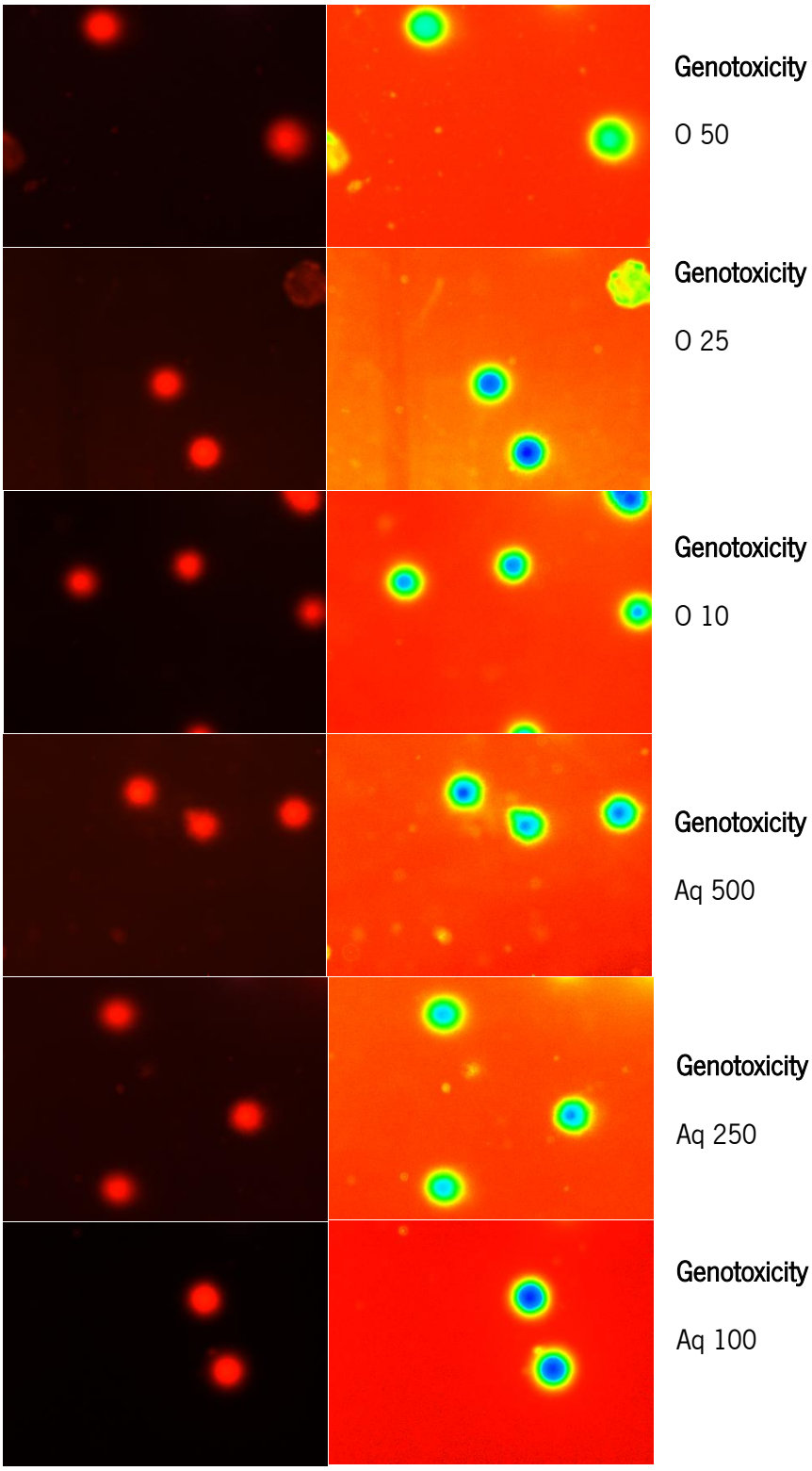
C-

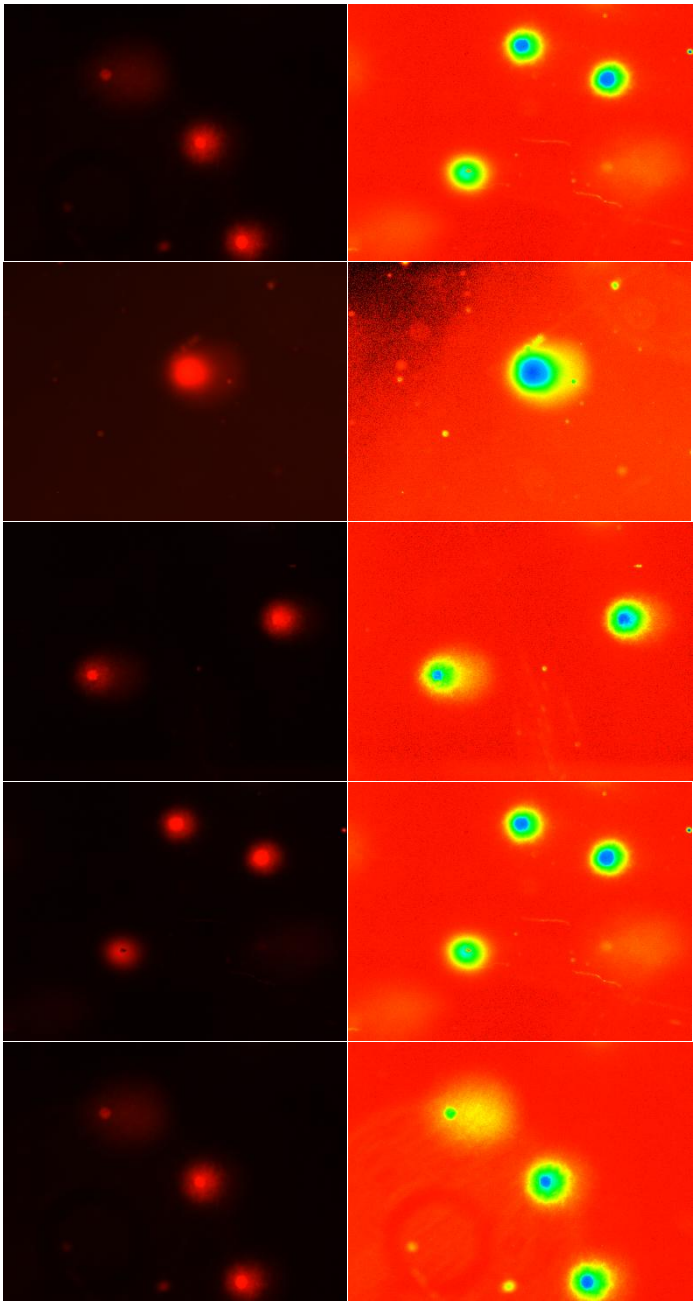
Genotoxicity

C+

Genotoxicity

O 100


Genotoxicity
O 50
Genotoxicity
O 25
Genotoxicity
O 10
Genotoxicity
Aq 500
Genotoxicity
Aq 250
Genotoxicity
Aq 100



Genotoxicity

Aq 50

Antigenotoxicity

Aq 500 + 10 mM H_2O_2

Antigenotoxicity

Aq 250 + 10 mM H_2O_2

Antigenotoxicity

Aq 100 + 10 mM H_2O_2

Antigenotoxicity

Aq 50 + 10 mM H_2O_2