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# Chemical and biological characterization of an aqueous *Sambucus nigra* L. flower extract

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# DECLARAÇÃO

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# RESUMO

Atualmente, há um crescente interesse no uso de recursos naturais como fonte de compostos bioativos, com potenciais benefícios para a saúde. Desde a antiguidade que é recorrente o uso plantas ao nível da medicina tradicional, nomeadamente a flor de sabugueiro (*Sambucus nigra* L.). Recentemente, esta planta tem sido a base de vários produtos nutracêuticos, contudo, ainda não existem evidências científicas claras que fundamentem a sua bioatividade, bem como os mecanismos celulares associados. De acordo com a literatura, os extratos de sabugueiro são constituídos sobretudo por compostos terpénicos voláteis, triterpénicos, esteróis, e compostos fenólicos. É, contudo, importante salientar que a composição de cada uma destas famílias é dependente do método de extração (solvente, temperatura e tempo).

O principal objetivo deste estudo foi a caracterização química de um extrato aquoso de flor de sabugueiro e a sua validação como agente anticancerígeno.

A extração aquosa da flor de Sabugueiro foi realizada a diferentes temperaturas (50, 70 e 90 °C). O extrato aquoso obtido a 90 °C apresentou a atividade anti-radicalar mais elevada (0.157 ± 0.001 mmol TE $g^{-1}$) e uma concentração de compostos fenólicos mais elevada (45.32 ± 2.20 mg CAE $g^{-1}$).

As análises qualitativas e quantitativas de GC-MS e HPLC-MS do extrato aquoso de flor de sabugueiro permitiram identificar 46 compostos, sendo a quercetina e o ácido clorogénico representativos de 86 % da totalidade dos compostos fenólicos identificados na fração hidrofílica, e a naringenina (27.16 %) o composto maioritário na fração lipofílica.

A atividade epigenética do extrato da flor de sabugueiro na desmetilação do *MLH1* foi testada em combinação com o 5-fluorouracil em células do cancro colorretal (RKO), usando a 5-azacitidina como controlo positivo. Os resultados sugerem que a combinação do extrato de sabugueiro com o 5-FU não resulta num efeito sinergético, o que indica que o extrato não tem influência na desmetilação do *MLH1*.

Apesar do extrato da flor sabugueiro ter diversas moléculas com capacidade antioxidante, o extrato não demonstrou proteção ou indução de reparação de danos do DNA após um insulto oxidativo ($H_2O_2$).

Ao nível da atividade antimicrobiana, verificou-se que bactérias gram-positivas são mais suscetíveis à presença deste extrato, nomeadamente, *Staphylococcus aureus* clinicamente isolado e *Staphylococcus epidermidis*.

Em conclusão, verificou-se que o extrato de flor de sabugueiro não possui capacidade de modular atividade epigénica e estimular a proteção/reparação de danos oxidativos. Ao invés, verificou-se que o extrato possui atividade microbiana, nomeadamente contra bactérias gram-positivas.

# ABSTRACT

Nowadays, there is a growing interest on the use of natural resources as sources of bioactive compounds with potential health benefits. Since the antiquity, that is recurrent the use of plants in folk medicine, such as the elderflower (*Sambucus nigra* L.). Recently, this plant has been the basis of several nutraceutical products. Thus, there is not a clear scientific explanation for its bioactivity, as well as the responsible cellular mechanisms. According to the literature, elderflower extracts are mainly composed by volatile terpenic, triterpenic, sterols and phenolic compounds. However, the composition of each one of this families will be dependent of extraction method (solvent, temperature, time).

The main gold of this study was the chemical characterization of an aqueous elderflower extract and its validation as an anticarcinogenic agent.

The aqueous extraction of elderflower was performed at different temperatures (50,70 and 90 °C). The aqueous extract obtained at 90 °C exhibited the highest antiradical activity (0.157 ± 0.001 mmol TE $g^{-1}$) and the highest concentration of phenolic compounds (45.32 ± 2.20 mg CAE $g^{-1}$).

The qualitative and quantitative analysis of GC-MS and HPLC-MS of elderflower aqueous extract allowed the identification of 46 compounds, being quercetin and chlorogenic acid representative of 86 % of the total of phenolic compounds identified in hydrophilic fraction, and naringenin (27.16 %), the major compound in lipophilic fraction.

The epigenetic activity of elderflower aqueous extract on demethylation of *MLH1* was tested in combination with 5-fluorouracil (5-FU) in colorectal cancer cells (RKO), using 5-azacytidine (5-azaC) as a positive control. The results suggest that the combination of elderflower extract with 5-FU did not result in a synergetic effect, which indicates that elderflower extract did not have an influence on *MLH1* demethylation.

Even though, the elderflower extract has several molecules with antioxidant potential, the extract did not exhibit protection or induction of repair of DNA damage after exposure to oxidative stress ($H_2O_2$).

The antimicrobial activity of elderflower extract was evaluated, and it was verified that gram-positive bacteria were the most susceptible to the presence of this extract, namely, *Staphylococcus aureus* clinically isolated and *Staphylococcus epidermidis*.

In conclusion, it was verified that *S. nigra* extract does not have the ability to modulate epigenetic activity and stimulate DNA protection/repair against oxidative stress. Although, this extract exhibits antimicrobial activity, particularly on gram-positive bacteria.

# TABLE OF CONTENTS

**Acknowledgments** III

**Resumo** V

**Abstract** VII

**Table of Contents** IX

**List of Figures** XIII

**List of Tables** XVII

**List of Abbreviations and Acronyms** XIX

**1. Introduction** 3

1.1. Phytochemicals 3

1.1.1. Dietary polyphenols 4

1.1.2. The concept of oxidative stress 5

1.1.3. Antioxidant Activity of Polyphenols 7

1.1.4. Anti-inflammatory Activity of Polyphenols 9

1.2. Oxidative DNA damage and Mismatch Repair Pathway 10

1.2.1. 5-Azacytidine/5-Azacitidine (5-azaC) on Cancer Therapy 11

1.2.2. 5-Fluorouracil (5-FU) on Cancer Therapy 13

1.3. *Sambucus nigra* L. 14

1.3.1. General Considerations 14

1.3.2. Chemical composition and nutritional value 15

1.3.3. Harmful compounds 15

1.3.4. Overview of the *S. nigra* potential health benefits 16

1.3.4.1. Antioxidant Activity 16

1.3.4.2. Anti-inflammatory Activity 18

1.3.4.3. Anti-infective activity 19

1.3.4.4. Activity on colorectal cancer modulation 20

1.3.4.5. Diabetes mellitus 20

1.4. Solid-liquid extraction 21

1.5. Chromatographic-based technologies 21

1.5.1. One dimensional gas chromatography 22

1.5.2. High-performance liquid chromatography (HPLC) 23

1.5.3. Data processing and interpretation 25
1.6. Context and objectives of the thesis 28

**2. Material and Methods 31**

2.1. Extract isolation and characterization 31
2.1.1. Plant material and extraction 31
2.1.2. DPPH scavenging activity and trolox equivalent antioxidant capacity (TEAC) 31
2.1.3. Phenolic content of elderflower aqueous extract 31
2.1.4. GC-MS spectrometry analysis 32
2.1.5. Characterization of HPLC-MS 32
2.2. *In vitro* studies 33
2.2.1. Cell lines and culture conditions 33
2.2.2. Antiproliferative assay in human cells 34
2.2.3. Epigenetic activity of elderflower extract on *MLH1* demethylation 34
2.2.4. Effect of *S. nigra* extract on oxidative DNA damage 35
2.2.5. Comet Assay 35
2.2.6. Cellular Repair Assay 35
2.2.7. Susceptibility Testing 36
2.3. Statistical Analysis 37

**3. Results 41**

3.1. Antioxidant activity and Phenolic Content 41
3.2. Identification and characterization of the compounds on the elderflower extract 42
3.2.1. GC-MS Analysis 43
3.2.2. HPLC-MS Analysis 48
3.3. *In vitro* analysis 54
3.3.1. *In vitro* proliferative activity 54
3.3.2. Epigenetic activity of elderflower extract on *MLH1* demethylation 54
3.3.3. Effects of elderflower extract on oxidatively induced-DNA damage 56
3.3.4. Effects of elderflower extract on repair ability 57
3.3.5. Antimicrobial activity of elderflower aqueous extract 59

**4. Discussion 63**

4.1. Antioxidant activity and Phenolic Content 63

4.2. Identification and characterization of elderflower extract 64

4.3. *In vitro* analysis 66

**5. Conclusions and Future Perspectives 73**

**6. References 77**

**Supplementary Data 97**

Supplementary Data I – GC calibration curve 99

Supplementary Data II – HPLC calibration curve of rutin 100

Supplementary Data III – HPLC calibration curve of chlorogenic acid 101

Supplementary Data IV – Antiproliferative activity by MTT assay 102

Supplementary Data V - Kinetics of attenuation of $H_2O_2$-induced DNA damage on RKO cells 102

Supplementary Data VI - Kinetics of SBs rejoining using $H_2O_2$ 104

Supplementary Data VII – Yield of elderflower aqueous extract 105

Supplementary data VIII – Antimicrobial activity of flavonoids 106

Supplementary Data IX – Absorption of rutin and quercetin 107

Supplementary Data X – List of material 108

# LIST OF FIGURES

**Figure 1.** Phytochemicals classes and some examples of most common compounds of each class. Adapted from Pandey and Rizvi (2009). ____ 4

**Figure 2.** Chemical structure of some common phenolic acids: gallic acid (A) and ferulic acid (B). ____ 5

**Figure 3.** Chemical structure of some common flavonoids: kaempferol (A) and naringenin (B). ____ 5

**Figure 4.** Flavonoid scavenging a radical, leading to the formation of a flavonoid radical or a prooxidant. ____ 7

**Figure 5.** Molecular signaling transductions controlled by ROS molecules. ROS diffused into the cell mitochondria stimulate activations of NF-κB pathway and Nrf2 related pathways. Initiation of NF-κB provokes stimulation of inflammatory transcription factors to upregulate pro-inflammatory molecules (e.g. TNF-a, IL-6, IL-8 and IL-1β). Activation of Nrf2 results in the transcription of antioxidant enzymes (GPx, SOD, CAT, etc). Both endogenous and exogenous antioxidants can scavenge ROS or suppressing NF-kB activated pro-inflammatory signal transduction, thus attenuating oxidative stress. Arrows indicate activation (→), whereas perpendicular lines show inhibition (⊢). ARE, antioxidant response element; BAX, Bcl-2-associated X protein; Maf, transcription factor Maf; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; Nfr2, nuclear factor (erythroid-derived 2)-like 2; $O_2^{\bullet -}$, superoxide anion; P53, tumor protein p53; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor. Adapted from Zhang and Tsao (2016). ____ 8

**Figure 6.** Schematic representation of action of polyphenols as anti-inflammatory agents. COX1, cyclooxygenase-1; COX2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NADPHox, NADPH oxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; pNF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells promoter; ROS, reactive oxygen species; TLR, toll-like receptor. Adapted from Ambriz-Pérez *et al.* (2016). 10

**Figure 7.** Schematic illustration of the mammalian MMR pathway, indicating protein complexes and processes involved in recognition of the mismatch to DNA resynthesis and DNA ligation (Jiricny, 2006). EXO1, exonuclease-1; MutSα, MutSα complex; MutLα, MutLα complex; Pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A. Adapted from Jiricny (2006). ____ 11

**Figure 8.** Schematic representation of reactivation of a silent tumor suppressor gene using 5-azaC. ↑ - overexpression. Adapted from Momparler (2005). 12

**Figure 9.** 5-Fluorouracil (5-FU) metabolism. FUTP metabolite is extensively incorporated into RNA, disrupting normal RNA processing and function. FdUTP can be misincorporated into DNA lead to strand breaks and consequently cell death. Adapted from Longley *et al.* (2003). DHFU, dihydrofluorouracil; DPD, dihydropyrimidine dehydrogenase; FdUDP, 5-fluorodeoxyuridine diphosphate; FdUMP, fluorodeoxyuridine monophosphate; FdUTP, fluorodeoxyuridine triphosphate; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate. 13

**Figure 10.** *Sambucus nigra* L. tree (A), flower (B) and berries (C). 14

**Figure 11.** One-dimensional gas chromatographic system. 23

**Figure 12.** High-performance liquid chromatography system. 25

**Figure 13.** Radical scavenging activity of *Sambucus nigra* L. flower extracts at different isolation temperatures. (A) Radical scavenging activity determined by DPPH assay. (B) Trolox equivalent antioxidant capacity (TEAC) in millimole of trolox equivalents per gram of flower (mmol TE $g^{-1}$). Values are the mean of three independent extraction flasks ± SD. Asterisks denote significant differences (*$p < 0.05$). 41

**Figure 14.** Phenolic content of *Sambucus nigra* L. flower extract at different isolation temperatures expressed in caffeic acid equivalents (CAE). Values are the mean of three independent extraction flasks ± SD. Asterisks denote significant (**$p < 0.01$). 42

**Figure 15.** HPLC-PDA profile chromatogram of aqueous extract of *S. nigra* flowers. Time of retention is expressed in minutes. Compounds are signed by numbers as stated in Table 6 and Table 7. 48

**Figure 16.** Effect on cellular viability of elderflower extract, 5-azaC and 5-FU. The cells were pre-incubated during 48h with 5-azaC (2 μM) or with aqueous elderflower extract (10 μg $mL^{-1}$ and 20 μg $mL^{-1}$) before 5-FU (10 μM) treatment for 48h. The percentage of cell viability was calculated by the absorbance relative to the value detected for the control cells (untreated cells), that was defined as 100% of cell viability. For each condition were performed three independent experiments and

data are expressed as mean ± SD. Asterisks represent significant difference (**$p < 0.01$; ***$p < 0.001$). 55

**Figure 17.** Effects of 24 and 48h of pre-treatment with luteolin (20 μM) and elderflower extract (200 and 400 μg $mL^{-1}$) on oxidative DNA damage induced by 75 μM $H_2O_2$ (5 min, on ice) in RKO cells. [+] - compound present; [-] - compound absent; () – percentage of protection regarding to the respective control. Results are expressed as mean ± SD, of at least three independent experiments. Asterisks represent significant difference (**$p < 0.01$). 57

**Figure 18.** Extent of repair of 75 μM $H_2O_2$-induced damage in RKO cells after pre-incubation during 24 and 48 hours with elderflower aqueous extract (200 and 400 μg $mL^{-1}$) and 20 μM L-7-G followed by 5 minutes (A) and 10 minutes (B) of recovering time. [+] - compound present; [-] - compound absent. Results are expressed as mean ± SD, of at least three independent experiments. Asterisks represent significant difference (***$p < 0.001$). 58

# LIST OF TABLES

**Table 1.** Relevant finding using *in vitro* and *in vivo* assays regarding to antioxidant activity using elderflower and elderberries extracts. 17

**Table 2.** Inflammation process involving the activation of monocytes and/or macrophages caused by pathogens or harmful stimulus when exposed to elderflower or elderberries formulations. ↓ - downregulation; ↑ - activation/stimulation. (Harokopakis *et al.*, 2006; Ho *et al.*, 2017; Olejnik *et al.*, 2015). 19

**Table 3.** Data pre-processing, pre-treatment, identification and quantification, processing, post-processing, validation and interpretation (Goodacre *et al.*, 2007). 26

**Table 4.** The compounds identified in elderflower dichloromethane extract by GC/MS analysis, and their identified biological activities in literature. 44

**Table 5.** Composition of elderflower dichloromethane extract (μg $g^{-1}$ of flower) with the respective relative standard deviation (RSD). 47

**Table 6.** Phenolic compounds identified in *S. nigra* flower hydrophilic extract and corresponding $MS^n$ fragmentation profiles, and their identified biological activities in literature. 50

**Table 7.** Concentration of the polyphenolic compounds in elderflower aqueous extract (μg $g^{-1}$ of flower) determined by HPLC. 53

**Table 8.** $IC_{50}$ values for elderflower extract on different cell lines expressed in mg $mL^{-1}$. 54

**Table 9.** MBC and MFC performance of elderflower aqueous extract against pathogenic organisms. MBC and MFC values are presented in mg $mL^{-1}$. Results are expressed as mean of at least three independent experiments. 60

# List of Abbreviations and Acronyms

| | |
|---|---|
| **1D-GC** | One-dimensional chromatography |
| **5-azaC** | 5-Azacytidine/5-Azacitidine |
| **5-FU** | 5-Fluorouracil |
| **ANOVA** | Analysis of variance |
| **Caco-2** | Human colon adenocarcinoma cell line |
| **CAE** | Caffeic acid equivalents |
| **CAT** | Catalase |
| **$CO_2$** | Carbon dioxide |
| **COX2** | Cyclogenease-2 |
| **DSBs** | Double stranded DNA breaks |
| **EI** | Electron ionization |
| **ESI** | Electrospray ionization |
| **EXO1** | DNA endonuclease |
| **FdUMP** | Fluorodeoxyuridine monophosphate |
| **FdUTP** | Fluorodeoxyuridine triphosphate |
| **FUTP** | Fluorouridine triphosphate |
| **GC-MS** | Gas chromatography-mass spectrometry |
| **GPx** | Glutathione peroxidase |
| **GSH** | Glutathione |
| **$H_2O_2$** | Hydrogen peroxide |
| **HCT-116** | Human colon cancer cell line |
| **HIV-1** | Human immunodeficiency virus |
| **$HO^{\cdot}$** | Hydroxyl radicals |
| **HPLC-MS** | High pressure liquid chromatography-mass spectrometry |
| **HT29** | Human colorectal adenocarcinoma |
| **$IC_{50}$** | The half maximal inhibitory concentration |
| **IL** | Interleukins |
| **L-7-G** | Luteolin-7-glucoside |
| **LCFAs** | Long chain fatty acids |
| **MBC** | Minimum bactericidal concentration |

| | |
|---|---|
| **MCFAs** | Medium chain fatty acids |
| **MFC** | Minimum fungicidal concentration |
| **MMR** | Mismatch repair pathway |
| **MS** | Mass spectrometry |
| **NCTC 2544** | Human keratinocyte cell line |
| **NMR** | Nuclear magnetic resonance |
| **NO** | Nitric oxide |
| **Nrf2** | Nuclear factor erythroid-related factor |
| **$O_2^-$** | Superoxide anion |
| **PCNA** | Proliferating cell nuclear antigen |
| **PI3K** | Phosphatidylinositol-3-kinase |
| **PPARγ** | Peroxisome proliferator-activated receptor |
| **QR** | Quinone reductase |
| **RAW 264.7** | Murine macrophage cell line |
| **RKO** | Human colon carcinoma cell line |
| **RNS** | Reactive nitrogen species |
| **ROS** | Reactive species of oxygen |
| ***S. nigra*** | *Sambucus nigra* L. |
| **SBs** | Stranded DNA breaks |
| **SD** | Standard deviation |
| **SDA** | Sabouraud dextrose agar |
| **SDB** | Sabouraud dextrose broth |
| **SOD** | Superoxide dismutase |
| **TE** | Trolox equivalents |
| **TEAC** | Trolox equivalent antioxidant capacity |
| **TMS** | Trimethylsilyl |
| **TNF-α** | Tumor necrosis factor |
| **TS** | Thymidylate synthase |
| **TSA** | Trypic soy agar |
| **TSB** | Trypic soy broth |
| **UV-Vis** | Ultra-violet-visible spectrophotometer |

# Introduction

# 1. Introduction

Historically, natural products have provided an endless source of medicine. Plant products – as parts of foods or botanical portions and powders – have been used with variable success to cure and prevent diseases. Recently, there is a growing interest in so-called functional foods. In this context, phytochemical compounds stand out due to their health-promoting and/or medical properties (Wildman, 2016).

There is abundant evidence that a diet rich in plant derivatives is associated with lower risk of disease development, such as cardiovascular and inflammatory diseases (Guimarães *et al.*, 2013; Sá *et al.*, 2013). This may be attributed to the numerous bioactive substances present in plants, both of low molecular weight, like phenolic compounds, and high molecular weight, as is the case of polysaccharides. From a vast array of medicinal plants, *Sambucus nigra* L. (*S. nigra*) has been the subject of considerable interest due to their heterogenous mixture of phytochemicals, specially from the family of polyphenols. The study of its properties is of extreme importance to society since they can be integrated in many fields, including cosmetics and pharmaceutical industries.

## 1.1. Phytochemicals

Phytochemicals are bioactive compounds present in plants, generally produced as secondary metabolites. These molecules play a crucial function in protecting plants against some environmental stresses (hordes of bacteria, fungi, insects, and other predators) (Dixon, 2001). The most bioactive phytochemicals on humans are phenolics, carotenoids, alkaloids, saponins, glucosinolates, cyanogenic glycosides and terpenes (**Figure 1**) (Mann & Truswell, 2017). Many of these natural products are assumed to have health promoting properties (Pandey & Rizvi, 2009).

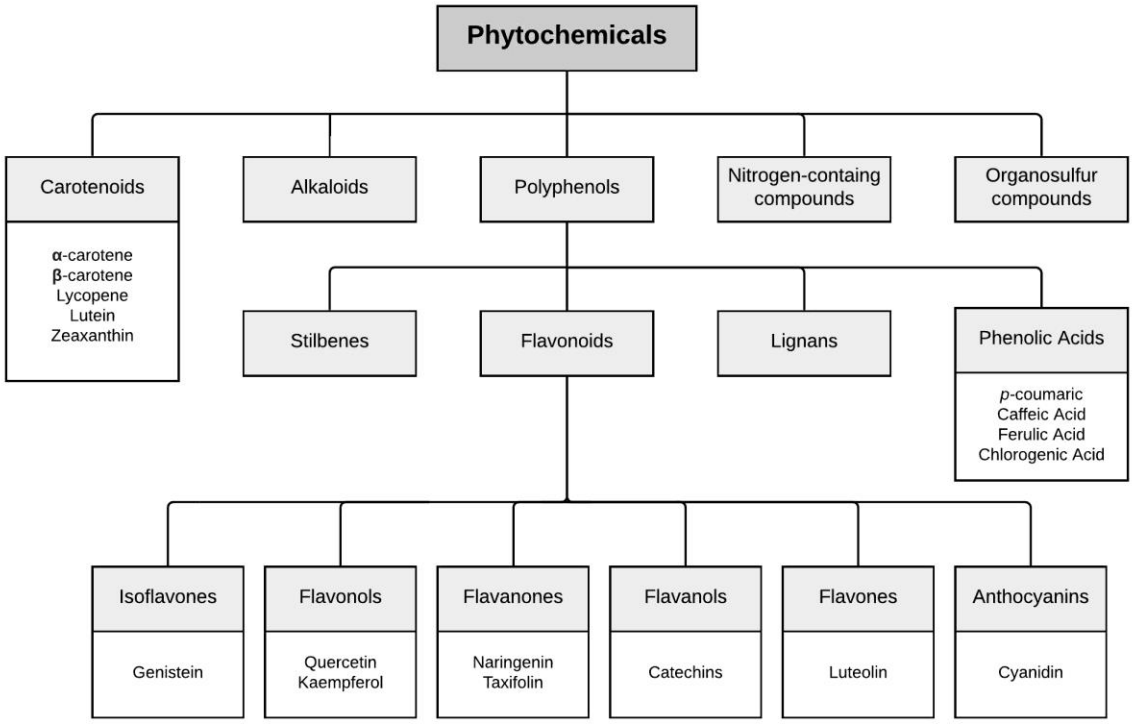


**Figure 1.** Phytochemicals classes and some examples of most common compounds of each class. Adapted from Pandey and Rizvi (2009).

### 1.1.1. Dietary polyphenols

Dietary polyphenols, found in fruits, vegetables, grains, tea and essential oils, are one of the most important class of natural antioxidants and chemopreventive agents found in human diet. There is currently evidence that dietary phenolic compounds can improve human health by lowering risk an preventing the onset of degenerative diseases including cancers, cardiovascular diseases and metabolic disorders (Scalbert *et al.*, 2005). Phenolic compounds can be categorized into several sub-groups according their chemical structure, although, those usually found in plant derivatives can be separated into three main sub-groups: phenolic acids, flavonoids and non-flavonoids (Tsao, 2010). Phenolic acids are hydroxyl derivates of aromatic carboxylic acids which have a single phenolic ring (**Figure 2**).

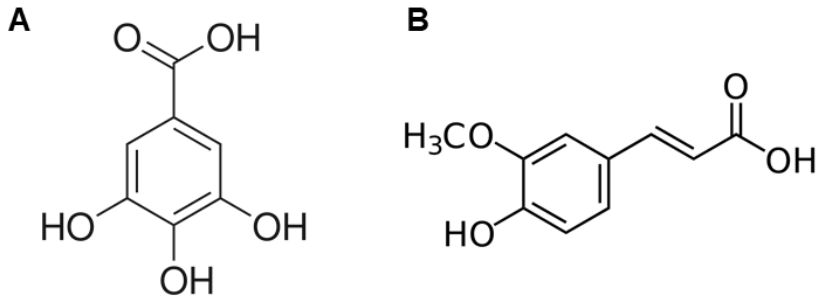


**Figure 2.** Chemical structure of some common phenolic acids: gallic acid **(A)** and ferulic acid **(B)**.

Flavonoids contain two phenolic rings (Ring A and B) linked by three carbon bridges to an oxygenated heterocycle (Ring C) as shown in **Figure 3**.

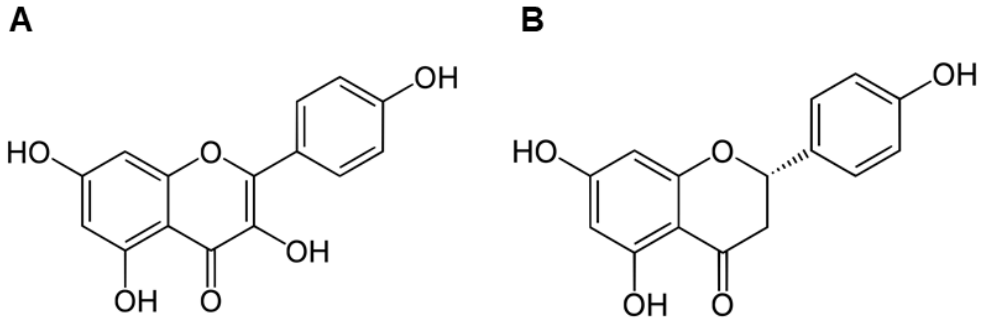


**Figure 3.** Chemical structure of some common flavonoids: kaempferol **(A)** and naringenin **(B).**

The antioxidant and anti-inflammatory properties and also other biological functions of polyphenols have been principally attributed to their particular chemical structure. The aromatic ring feature and the highly conjugated system with several hydroxyl groups make them good electron or hydrogen atom donors, capable of neutralize free radicals and other reactive oxygen species (ROS) (Zhang & Tsao, 2016).

### 1.1.2. The concept of oxidative stress

Oxidative stress is described as the loss of balance between the production/exposure to reactive oxygen/nitrogen species (ROS/RNS) and the organisms ability to counteract their action by the antioxidative protection systems (Koopman *et al.*, 2010). It occurs due to an enhanced ROS/RNS formation or due to decay on the antioxidant protective capacity of an organism.

ROS can have opposite effects in the human body, operating as signaling molecules or harmful agents. They are generated as a result of aerobic metabolism (e.g. oxidative metabolism, β-oxidation of fatty acids me, induction of cytochrome P450 and respiratory burst of neutrophils during inflammatory response) or due to exposure to environmental elements (e.g. UV light, ionization radiation, chemical compounds, heavy metals) (Ames *et al.*, 1993; Cooke *et al.*, 2003; Halliwell, 1996). In homeostatic conditions, ROS are originated endogenously in controlled levels, being crucial for cell survival, differentiation and apoptosis (Bartosz, 2009). In contrast, an disproportion between ROS production and the activity of antioxidant mechanisms, in favor of the former, can lead to oxidative damage of lipids, proteins and DNA (Balaban *et al.*, 2005). Free radical-induced damage induced oxidative stress has been confirmed as a contributor to the pathogenesis and pathophysiology of many chronic conditions such as neurodegenerative diseases (Parkinson, Alzheimer, Huntington's disease and amyotrophic lateral sclerosis), emphysema, cardiovascular and inflammatory diseases, cataracts and cancer (López-Alarcón & Denicola, 2013; Maulik *et al.*, 2013; Sies, 1985).

The most important ROS include superoxide anion ($O_2^-$) and hydroxyl radicals (HO·), and non-radical molecules such hydrogen peroxide ($H_2O_2$) generated by endogenous and exogenous sources (Slupphaug *et al.*, 2003). The radical HO· is defined as the main ROS responsible for damaging DNA once it is highly reactive and can directly damage DNA (Kryston *et al.*, 2011). $O_2^-$ and $H_2O_2$ do not have the ability to damage DNA directly, but the former can be transformed by the action superoxide dismutase (SOD) into $H_2O_2$, that, in the presence of divalent ions (e.g. $Fe^{2+}$ and $Cu^{2+}$), can originate HO· via Fenton reaction **(Equation 1)** (Cabiscol *et al.*, 2000).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \qquad \textbf{(Equation 1)}$$

Therefore, the DNA-damaging outcome of non-reactive ROS, like $H_2O_2$, is mediated by HO· which can lead to base oxidation and strand breaks (Iyama & Wilson, 2013). The most frequent DNA damage product of oxidative stress responsible for induction of carcinogenesis mutations is the modified guanine base 8-oxoG, being result of the addition of HO· to the C8 position of the guanine ring, which is after incorporated by the action of DNA polymerase during DNA synthesis (Barzilai & Yamamoto, 2004).

During evolutionary course, organisms have developed several antioxidant defense systems that are utilized to counterbalance the deleterious effects produced by oxidative stress. Presently, it is well-known that cells exposed to oxidants express stress-induced genes or genes encoding antioxidant defenses (Evans *et al.*, 2004). The counteracting mechanisms can be categorized into enzymatic and

non-enzymatic antioxidants. The antioxidant enzymes are located in the cell and include catalase, SOD, glutathione transferase, glutathione peroxidase, among others (Birben *et al.*, 2012). The enzymatic reactions are capable to eliminate oxygen radicals and their products, lowering the damage caused by oxidative stress. The non-enzymatic antioxidants are mainly of dietary source, involving tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids and polyphenols (Brieger *et al.*, 2012). Consequently, diets rich in antioxidant compounds provide an increased antioxidant defense/response, defending cellular machineries from damaging effect of excessive levels of ROS, and are linked with an inferior risk to develop degenerative diseases (Ames *et al.*, 1993).

### 1.1.3. Antioxidant Activity of Polyphenols

A progressive and irreversible escalation of oxidative damage by ROS exerts severe influence on critical aspects of the biology (Maulik *et al.*, 2013). Dietary phenolics possesses a good scavenging activity, having the ability to neutralize free radicals by donating an electron or a hydrogen atom. Namely, phenolic acids and flavonoids have an effective radical scavenging activity, although, the metal chelating ability and reducing power can differ depending on their structural properties (Perron & Brumaghim, 2009). However, when a phenolic molecule loses an electron or when it acts as a reducing agent, the molecule itself becomes a radical, but a relatively stable one, becoming a prooxidant (Bouayed & Bohn, 2010) (**Figure 4**). Consequently, polyphenols can be a double-edge sword: on one hand, when consumed correctly in the form of food or functional food, they are strong antioxidants, counteracting the excessive oxidative stress; on the other hand, they can exhibit prooxidant activity when used in high doses such as by administration of supplements (Bouayed & Bohn, 2010). The polyphenols ingested are digested by colonic microflora producing bioactive molecules that may interfere in the regulation of gut microbiota or be uptake by colonic epithelia leading to systemic antioxidant and anti-inflammatory effects (Nicholson *et al.*, 2012).

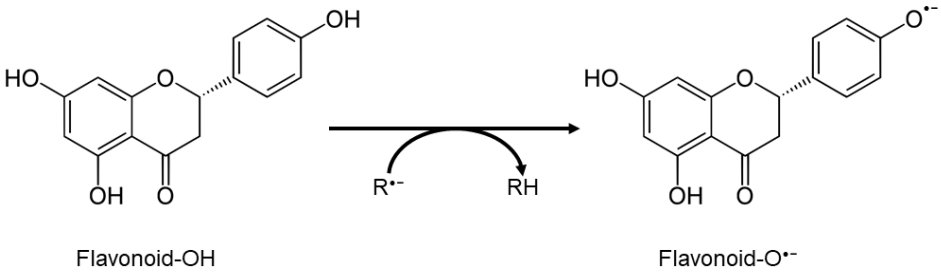


**Figure 4.** Flavonoid scavenging a radical, leading to the formation of a flavonoid radical or a prooxidant.

The ability of dietary polyphenols to restore redox homeostasis and prevent systemic or localized inflammation by enhancing the activity of SOD, catalase (CAT) and glutathione peroxidase (GPx). It has been demonstrated that the expression of these antioxidant enzymes is controlled by a key transcription factor nuclear factor erythroid-related factor (Nrf2). Nfr2 is triggered by the presence of ROS at cellular level, being translocated into the nucleus. There, it regulates the expression of antioxidant-responsive elements (ARE)-mediated of numerous genes encoding the above cited antioxidant enzymes (**Figure 5**) (Kansanen *et al.*, 2013).

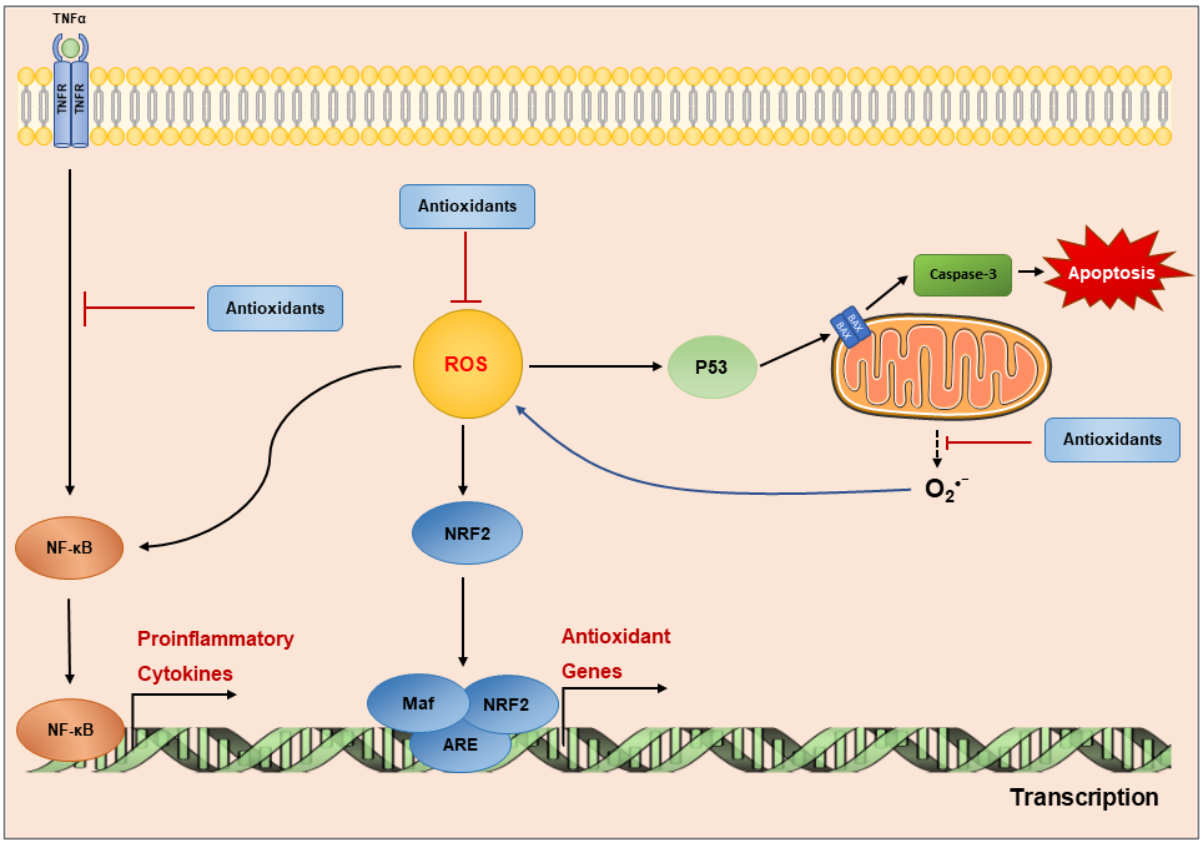


**Figure 5. Molecular signaling transductions controlled by ROS molecules.** ROS diffused into the cell mitochondria stimulate activations of NF-κB pathway and Nrf2 related pathways. Initiation of NF-κB provokes stimulation of inflammatory transcription factors to upregulate pro-inflammatory molecules (e.g. TNF-a, IL-6, IL-8 and IL-1β). Activation of Nrf2 results in the transcription of antioxidant enzymes (GPx, SOD, CAT, etc). Both endogenous and exogenous antioxidants can scavenge ROS or suppress NF-kB activated pro-inflammatory signal transduction, thus attenuating oxidative stress. Arrows indicate activation (→), whereas perpendicular lines show inhibition (⊢). ARE, antioxidant response element; BAX, Bcl-2-associated X protein; Maf, transcription factor Maf; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; Nfr2, nuclear factor (erythroid-derived 2)-like 2; $O_2^{\bullet-}$, superoxide anion; P53, tumor protein p53; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor. Adapted from Zhang and Tsao (2016).

### 1.1.4. Anti-inflammatory Activity of Polyphenols

In the course of inflammation, macrophages can be activated through the detection of a pathogen endotoxin, such as lipopolysaccharide (LPS), via macrophages toll-like receptor (TLR). This action activates a signaling pathway that promotes the release of NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor responsible for the activation of transcription of inflammatory mediator genes, such as interleukins, TNF-α and prostaglandins (PGs); inflammatory enzymes such as inducible nitric oxide synthase (iNOS) responsible for the synthesis of NO; and cyclooxygenases (COXs). TLR signaling also triggers the generation of reactive oxygen species (ROS) (Friedman & Hughes, 2002; Kumar *et al.*, 2017). Overexpression of these mediators, as happens in chronic inflammation, might lead to the occurrence of several chronic diseases.

Polyphenols might exert anti-inflammatory properties especially through radical scavenging activities, regulation of cellular events, and modulation of enzyme action of arachidonic acid metabolism (phospholipase A2, COX) and arginine metabolism (NOS), as well as modulation of proinflammatory molecules production (**Figure 6**) (Ambriz-Pérez *et al.*, 2016).

Molecular mechanisms of polyphenol anti-inflammatory activities comprise inhibition of enzymes related with proinflammatory properties such as COX2, LOX, and iNOS, inhibition of NF-κB and the activating protein-1 (AP-1), activation of phase-II antioxidant detoxifying enzymes, and activation of mitogen activated protein kinase (MAPK), protein kinase-C, and nuclear fact erythroid 2-related factor (Santangelo *et al.*, 2007).

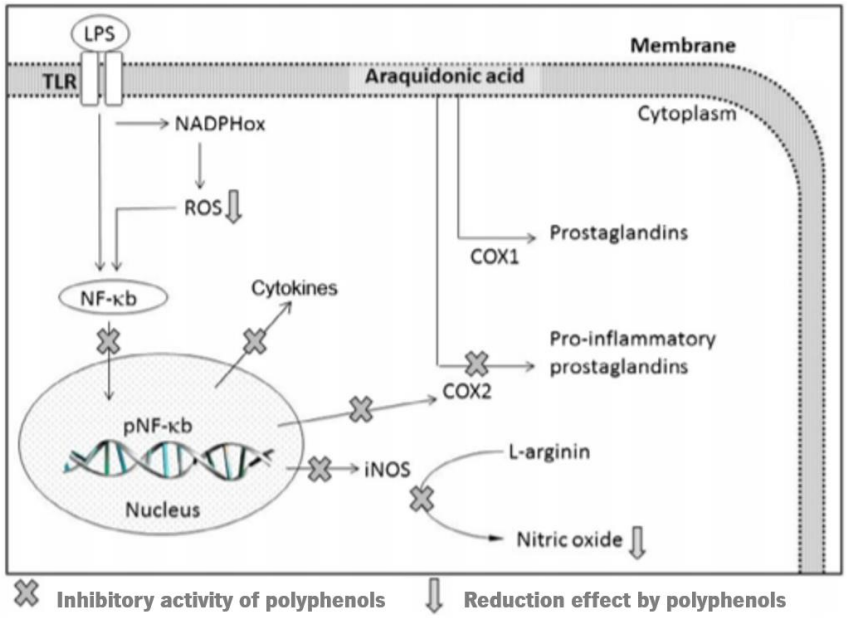


**Figure 6. Schematic representation of action of polyphenols as anti-inflammatory agents**. COX1, cyclooxygenase-1; COX2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NADPHox, NADPH oxidase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; pNF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells promoter; ROS, reactive oxygen species; TLR, toll-like receptor. Adapted from Ambriz-Pérez *et al.* (2016).

## 1.2. Oxidative DNA damage and Mismatch Repair Pathway

A significant consequence of oxidative stress is DNA damage, leading to genomic instability. DNA damage induced by ROS involves structural changes due to single- or double- stranded DNA breaks (SBs), alteration of purine, pyrimidine or deoxyribose; and development of DNA crosslinks through oxidation, depurination, methylation and deamination reactions.

To respond to these lesions, cells have DNA repair mechanisms such as the mismatch repair pathway (MMR) (**Figure 7**). MMR pathway starts when MutSα including Msh2 and Msh6 binds to a mismatch, followed by binding of MutLα [Mlh1 and Pms2]. Later, a proliferating cell nuclear antigen (PCNA) along with an accessory protein (RFC), triggers DNA endonuclease (EXO1), which guided by the MutS/MutL travels to the clamp. The elimination of daughter-strand DNA starts in the direction of the mismatch and then beyond it. Once the incongruity is removed, the activity of EXO1 is blocked by MutL, stopping DNA excision. After this procedure, repair is completed by correct DNA synthesis and ligation by the action of DNA polymerase and a DNA ligase, respectively.

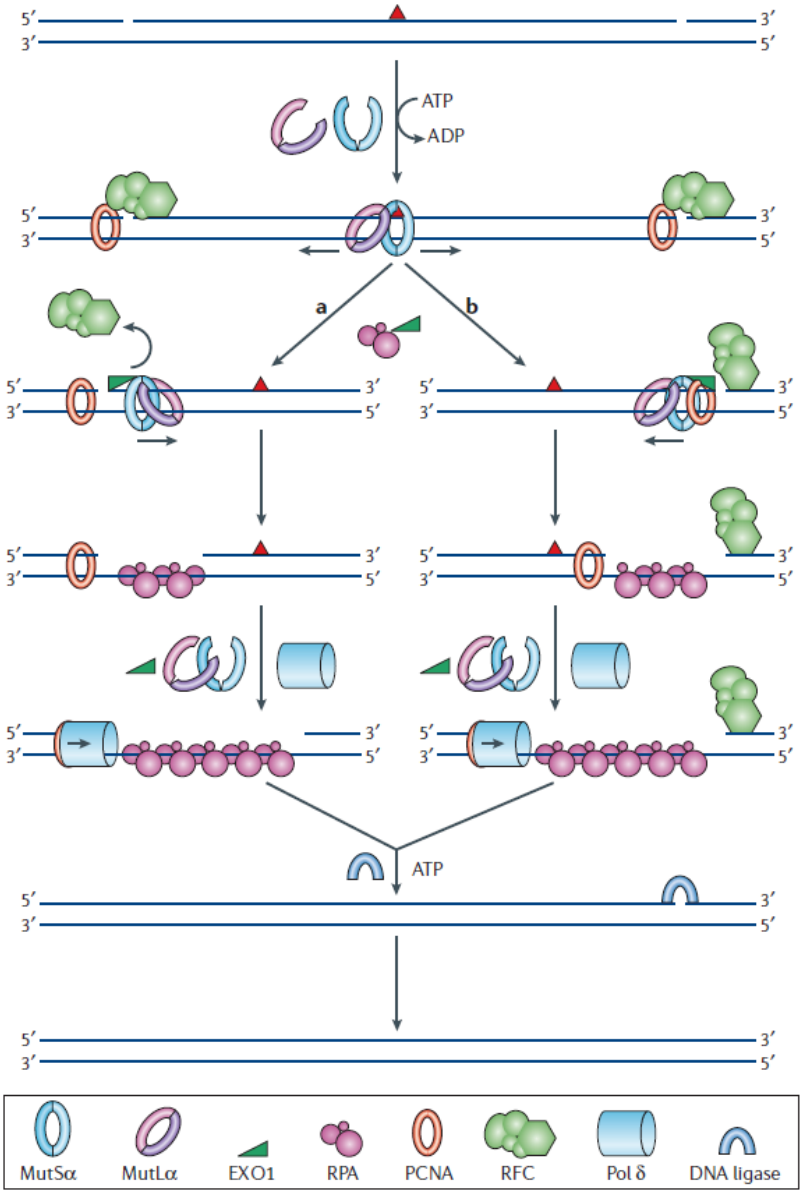


**Figure 7.** Schematic illustration of the mammalian MMR pathway, indicating protein complexes and processes involved in recognition of the mismatch to DNA resynthesis and DNA ligation (Jiricny, 2006). EXO1, exonuclease-1; MutSα, MutSα complex; MutLα, MutLα complex; Pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A. Adapted from Jiricny (2006).

### 1.2.1. 5-Azacytidine/5-Azacitidine (5-azaC) on Cancer Therapy

DNA methylation is a heritable epigenetic mark which involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by the action of DNA methyltransferases (DNMTs). Most DNA methylation is essential for normal development, and it plays an important role in a number of key processes including genomic imprinting, X-chromosome inactivation, and suppression of repetitive

element transcription and transposition. When dysregulated, this process contributes to diseases like cancer by the inactivation tumor suppressor genes such as *MLH1* (Jones & Laird, 1999). The molecular mechanism of silencing gene expression appears to be due to the binding of 5-methylcytosine proteins to the methylated promoter, which blocks the action of transcription factors (Jones *et al.*, 1998).

5-azaC (also known as Dacogen or Vidaza) is an analogue of cytidine ribose nucleoside. It is considered a prodrug and it has two main mechanisms of antineoplastic action. First, its ability to incorporate 5-azacetidine triphosphate into RNA which causes a disruption of nuclear and cytoplasmatic RNA metabolism with subsequent inhibition of protein synthesis (Li *et al.*, 1970). Second, its ability to inhibit DNA methylation, by trapping DNA methyltransferases (DNMTs), leading to global demethylation as cells divide. DNA methylation refers to the addition of a methyl group to the cytosine residue with a high frequency of CG dinucleotides that are typically located in proximity of gene promoters. The degree of methylation of CpG islands plays a role in the control of gene transcription. Usually, fully methylated sites are associated with suppression of gene expression, while hypomethylated or unmethylated CpG islands are linked to active transcription. Forming a tight-binding complex 5-azaC irreversibly binds to DNA methyltransferases, which inhibits its progression along the DNA duplex, resulting in intracellular depletion of the enzyme. Consequently, unmethylated DNA can lead to the transcription of previously quiescent genes (Jones & Taylor, 1981; Taylor & Jones, 1982). 5-azaC demethylation mechanism is shown in **Figure 8**.

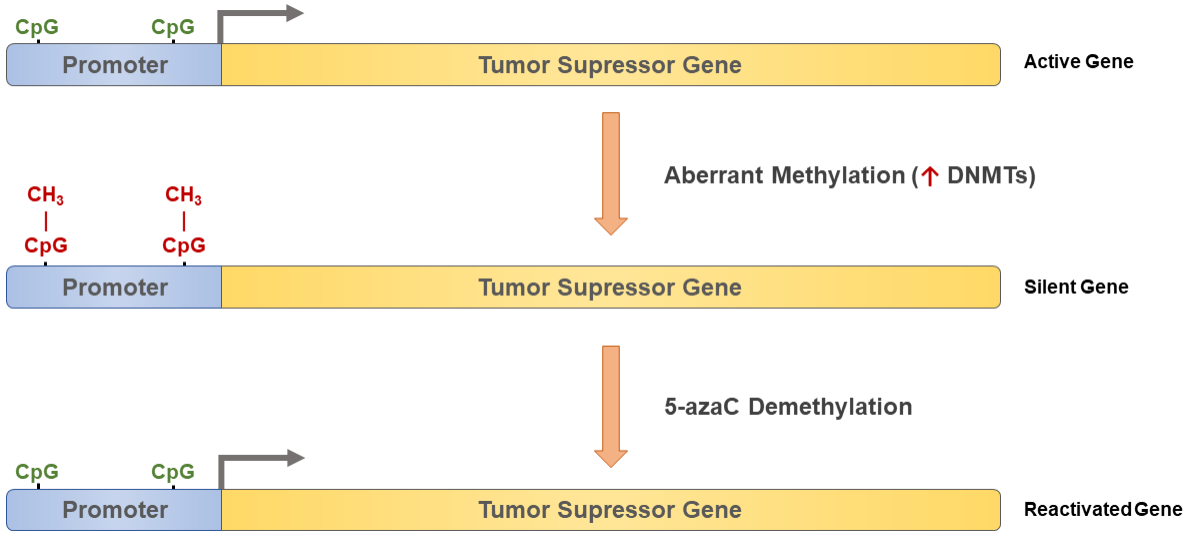


**Figure 8.** Schematic representation of reactivation of a silent tumor suppressor gene using 5-azaC. ↑ - overexpression. Adapted from Momparler (2005).

### 1.2.2. 5-Fluorouracil (5-FU) on Cancer Therapy

The action of anticancer agent classified as antimetabolite drugs, happens through the inhibition of essential biosynthetic processes and/or by being incorporated into macromolecules, such as DNA and RNA, inhibiting the cells normal function. The fluoropyrimidine 5-FU does both (Grem, 2000).

For the last 70 years, the fluoropyrimidine 5-fluorouracil (5-FU) has been positioned in the first line as chemotherapy agent of various cancers, including colorectal, head, neck and breast cancer (Grem, 2000; Toloudi *et al.*, 2015). 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. It rapidly enters the cell using the same facilitated transport mechanism as uracil (Wohlhueter *et al.*, 1980). 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) — these active metabolites disrupt RNA synthesis and increase DNA damage. It results in cell growth arrest and apoptosis (**Figure 9**) (Longley *et al.*, 2003).

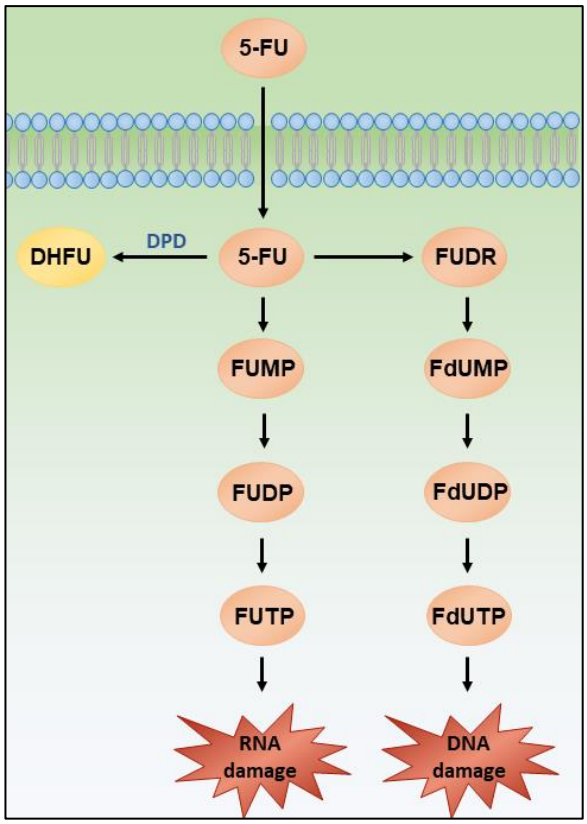


**Figure 9. 5-Fluorouracil (5-FU) metabolism.** FUTP metabolite is extensively incorporated into RNA, disrupting normal RNA processing and function. FdUTP can be misincorporated into DNA lead to strand breaks and consequently cell death. Adapted from Longley *et al.* (2003). DHFU, dihydrofluorouracil; DPD, dihydropyrimidine dehydrogenase; FdUDP, 5-fluorodeoxyuridine diphosphate; FdUMP, fluorodeoxyuridine monophosphate; FdUTP,

fluorodeoxyuridine triphosphate; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate.

However, the response rates of 5-FU for advanced colorectal cancers is less about 10%, when given as a single agent, and its bioavailability is also limited (rapid degradation to DHFU by DPD) (Diasio & Harris, 1989; Giacchetti *et al.*, 2000). Furthermore, 5-FU induces severe adverse reactions at different levels, namely, hematological, neural, cardiac and dermatological reactions, and at gastrointestinal tract.

## 1.3. *Sambucus nigra* L.

### 1.3.1. General Considerations

*S. nigra* or black elder (**Figure 10**) is widespread native plant from the British Isles and continental Europe. In Portugal, it can be found in the Northern Region (Tarouca, Lamego, Moimenta da Beira) (Cunha *et al.*, 2016).

*Sambucus* belongs to the *Adoxaceae* family, a major group of Angiosperms (flowering plants), however some studies refer to *Sambucus* as a Caprifoliceae family member (Charlebois *et al.*, 2010; Donoghue *et al.*, 2003). *S. nigra* is a deciduous shrub or small tree that can grow up to 10 meters. There are two important subspecies of *S. nigra*: the black elder, a common plant in Europe, and American elder, also known as elder or elderberry (Charlebois, 2007).

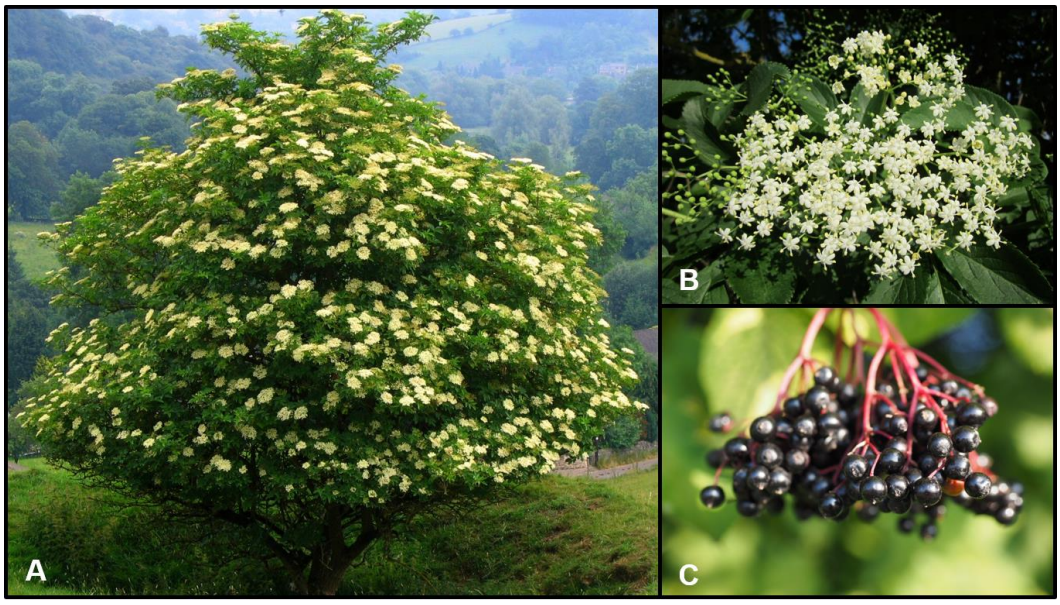


**Figure 10.** *Sambucus nigra* L. tree **(A)**, flower **(B)** and berries **(C)**.

*S. nigra* berries and flowers have a long history of usage in traditional European medicine, being used on diverse formulations that range from food products to medical formulations. Recently, *S. nigra* has been used on the development of supplements and nutraceuticals (Wildman, 2016). It has been extensively used in phytotherapy for the treatment of disorders associated with the respiratory and gastrointestinal tract, rheumatism, inflammation, diabetes, as well as viral infections and fevers and is currently one of the most-used medicinal plants worldwide (Jarić *et al.*, 2007; Sidor & Gramza-Michalowska, 2015).

The agroindustrial market pursues the consumer desire for added value-added products which are effective in disease prevention, as well as, in promoting a healthy aging (Silva, 2009). In the last decades, *S. nigra* flowers and berries secondary metabolites attracted attention due to their pharmacological activities, mainly the monoterpenic, sesquiterpenic and triterpenic compounds, sterols and phenolic compounds. The chemical composition of the berries and flowers depends on a series of factors, such as habitat/location, fertilization, maturation and harvest period.

### 1.3.2. Chemical composition and nutritional value

Elderflowers are a rich source of bioactive flavonoids and phenolic acids (Christensen *et al.*, 2008). Among flavonoids, flavonol glycosides, quercetin-3-rutinoside (rutin), kaempferol-3-rutinoside and isoharmnetin-3-rutinoside are the major flavonoids in elderflowers contributing as much as 90% of the total flavonoids content. The concentration of flavonoids is higher in elderberry flowers in comparison with berries and leaves (Dawidowicz *et al.*, 2006). The 5-caffeoylquinnic acid and 1,5-di-caffeoylquinic acid (chlorogenic acids) comprise over of 70% of the total phenolic acid content present in the *S. nigra* flowers. Opposed to the chemical composition of the elderberry fruit, which is especially rich in anthocyanins, flowers do not contain any pigments from this group.

### 1.3.3. Harmful compounds

Not all constituents of the *S. nigra* plant are safe for consumption. In fact, only *S. canadensis* and *S. nigra* flowers have been approved by the United States Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) for use as a flavoring ingredient (Food). All elderberry parts contain cyanogenic glycosides, being the most abundant sambunigrin and prunasin. Furthermore, elderberry contains *m*-hydroxysubstituted glycosides, such as zierin and holocalin (Dellagreca *et al.*, 2000). These compounds are potentially toxic and life-threatening, because they can hydrolyzed resulting in the release

of cyanide (Bromley *et al.*, 2005). However, they occur primarily in unripe berries and are degraded during heat treatment (Boon, 2010). For this reason, processed products are preferred for consumption over fresh fruit (Cejpek *et al.*, 2009).

### 1.3.4. Overview of the *S. nigra* potential health benefits

Several published evidences indicate potential benefits of *S. nigra* berries and flowers in disease prevention/management if included in diet.

#### 1.3.4.1. Antioxidant Activity

There is a high interest in plant-derived antioxidants to prevent the harmful effects of oxygen radicals and/or other reactive oxygen species (ROS) (Gülçin, 2012; Halliwell, 2013). Antioxidant activity is one of the most exploited features of *S. nigra*. These properties are often related to phenolic compounds, and especially anthocyanins and flavonols. However, it is relevant to point out possible interactions between extract components, which include non-phenolic compounds. The extraction conditions, and pre- and post-harvest conditions, might play a critical role on the result of the antioxidant capacity. **Table 1** summarizes the main findings regarding the antioxidant activity of elderflowers and elderberries.

Dietary containing phenolic molecules may help to maintain the oxidative stress homeostasis of oxidative stress once consumed. However, during digestion (ingestion, absorption, assimilation) their stability and bioavailability might be affected (Zhou *et al.*, 2016). It is described that there is a loss of elderberry bioactive composites occurs due to the digestion process (*in vitro* assay), that in the case of anthocyanins it can reach a 44% loss. Even so, the colon-digested aqueous extract decreases the excessive intracellular ROS production (22%) and oxidative damage (46%) in human colon cells (Olejnik *et al.*, 2016).

**Table 1.** Relevant finding using *in vitro* and *in vivo* assays regarding to antioxidant activity using elderflower and elderberries extracts.

| **Elderflower (*in vitro* assays)** | | | |
|---|---|---|---|
| **Extract** | **Main result** | **Bioactive compound** | **Reference** |
| Aqueous extract | Inhibition of the pro-inflammatory activity of periodontal pathogens (inhibition of oxidative burst in neutrophils) | Flavonols | (Dawidowicz *et al.*, 2006) |
| Aqueous extract | Prolonged extraction in water results in higher antioxidant activity | Phenolic compounds | (Mikulic-Petkovsek *et al.*, 2015) |
| Extracts (water and ethanol) | Hot water guarantees higher antioxidant activity when compared to ethanol | – | (Buřičová & Reblova, 2008) |
| Extract (solvent not designated) | Greater antioxidant activity when compared to standards (rutin, quercetin, etc.) | Rutin and other phenolic compounds | (Stoilova *et al.*, 2007) |
| **Elderberry (*in vitro* assays)** | | | |
| **Extract** | **Main result** | **Bioactive compound** | **Reference** |
| Extract (solvent not designated) | Anthocyanins ensure significant protective effects in endothelial cells | Anthocyanins | (Youdim *et al.*, 2000) |
| Concentrated and isolated anthocyanins | Extracellular antioxidant activity | Anthocyanins | (Pool-Zobel *et al.*, 1999) |
| ***In vivo* assays** | | | |
| **Extract** | **Main result** | **Bioactive compound** | **Reference** |
| Ethanolic extract (component not designated) | Inferior response to oxidative stress and lower levels of primary metabolites of lipoperoxidation | Flavonoids | (Bobek *et al.*, 2001) |
| Elderberry concentrates | Anthocyanins act synergistically with vitamin C and the antioxidant defense system in sparing vitamin E | Anthocyanins | (Frank *et al.*, 2002) |
| Ethanol extract of elderberries | Antioxidant activity of polyphenolic extract from *S. nigra.* Systolic and diastolic arterial pressure in rats with drug-induced hypertension were reduced | Phenolic Compounds | (Ciocoiu *et al.*, 2016) |

#### 1.3.4.2. Anti-inflammatory Activity

Inflammation is associated to several human diseases, including, asthma, diabetes, allergy, multiple sclerosis, cardiovascular diseases, neurodegenerative disorders, and some types of cancer (Debnath *et al.*, 2013; Mena *et al.*, 2014). An important mechanism of organism self-protection involves inflammation, aiming to eliminate harmful stimuli such as damaged cells, irritants or pathogens (Ho *et al.*, 2017). Usually, the inflammation process starts with the activation of monocytes and/or macrophages, which participate in the regulation of the inflammation by the release of several cytokines, such as tumor necrosis factor (TNF-α), interleukins (IL), and inflammatory mediators which include reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin E2, which are produced by inducible nitric oxide synthase and cyclogenease-2 (COX2) (Ho *et al.*, 2017).

Cytokines as TNF-α and IL(s) act as multipotential mediators on the cellular system, having an extensive diversity of biological activities. They can promote favorable or unfavorable effects on the host during immune response, depending on their local concentration, where the equilibrium between the inflammatory and anti-inflammatory cytokines will manage the outcome and the duration of the immune response (Barak *et al.*, 2002).

It is described that elderberry aqueous extract downregulated the expression of mediators such as IL-1, IL-1β, IL-6 and TNF-α (Gorchakova *et al.*, 2007; Olejnik *et al.*, 2015). The elderflowers aqueous extract inhibits the macrophage production of pro-inflammatory cytokines and block the neutrophils activation. This may be due to the inhibition of activation of NF-κB and phosphatidylinositol-3-kinase (PI3K), an enzyme essential in the regulation of immunity and inflammation process. The bioactive composites responsible for the anti-inflammatory effects of elderflower aqueous extract are unidentified. However, the ability of the aqueous extract to inhibit PI3K seems to be mediated through quercetin (Yeşilada *et al.*, 1997).

Modulation of NO production by macrophages and dendritic cells also have significative importance in inflammatory diseases. Ethanolic elderflowers extracts displayed an inhibitory activity on NO production in RAW cells and dendritic cells (Ho *et al.*, 2017). *In vivo* experiments also validated the potential of elderflower anti-inflammatory activity, when an 80% ethanol extract showed to have moderate anti-inflammatory activity in rats (Mascolo *et al.*, 1987). Studies involving elderflower and elderberries extract and their role in the inflammation process are described in **Table 2**.

**Table 2.** Inflammation process involving the activation of monocytes and/or macrophages caused by pathogens or harmful stimulus when exposed to elderflower or elderberries formulations. ↓ - downregulation; ↑ - activation/stimulation (Harokopakis *et al.*, 2006; Ho *et al.*, 2017; Olejnik *et al.*, 2015).

| | Elderflower formulation | Elderberries formulation |
|---|---|---|
| **TNF-α** | ↓ | ↓ |
| **IL-1α** | ↓ | – |
| **IL-1β** | ↓ | ↓ |
| **IL-6** | – | ↓ |
| **IL-10** | – | ↑ |
| **NO** | ↓ | – |
| **$H_2O_2$** | ↓ | – |
| **$O_2^-$** | ↓ | – |
| **NF-κB** | ↓ | – |

The evidence of *S. nigra* berries and flowers influence on the anti-inflammatory and immunological pathways on humans is still scarce.

#### 1.3.4.3. Anti-infective activity

Aqueous elderberry extracts (phenolic-type extracts) demonstrated antimicrobial activity, (fungi, bacteria) against human pathogenic microorganisms. The antimicrobial studies demonstrated that these extracts had antimicrobial activity against *Staphylococcus aureus* (methicillin-resistant and methicillin-sensitive), *Streptococcus mutans*, *Streptococcus pyogenes*, *Haemophilus influenza, Haemophilus parainfluenzae*, *Branhamella catarrhalis*, and *Helicobacter pylori* in the conditions tested (Chatterjee *et al.*, 2004; Izzo *et al.*, 1995; Krawitz *et al.*, 2011).

These experiments showed the ability of *S. nigra* to inhibit pathogens *in vitro.* However, it is essential to perform more studies, in order to understand which bacterial and fungal pathogens are vulnerable to *S. nigra*, and also their mechanisms of action.

One of the most interesting applications of *S. nigra* berries is associated with their ability to inhibit the influenza virus. Influenza virus A or B are responsible for an acute, febrile illness that happens in outbreaks varying severity every winter. Standardized elderberry extracts decreased hemagglutination and inhibited replication of numerous human and animal influenza viruses A and B *in vitro* (Zakay-Rones *et al.*, 2004; Zakay-Rones *et al.*, 1995).

Antiviral properties were also described against human immunodeficiency virus (HIV-1) on HIV-infected peripheral lymphocytes and herpes simplex virus (Vlachojannis *et al.*, 2010). Flavonoids, including quercetin, cyanidin and petunidin, and proanthocyanins, present in european elderberry extract, bind to HIV-1 virions, blocking their mechanisms, preventing to host cells infection (Fink *et al.*, 2009).

*In vivo* experiments performed on chimpanzees reinforced the *in vitro* conclusions regarding elderberry aqueous extract and its activity against influenza virus (Burge *et al.*, 1999). The suggested action mechanism stimulates the immune system; inhibit hemagglutination of the influenza virus avoiding the adhesion of the virus to cell receptor; and present anti-inflammatory effect. Furthermore, the absence of side effects of this *S. nigra* formulations offers an alternative way for a safe treatment for influenza (Zakay-Rones *et al.*, 2004).

#### 1.3.4.4. Activity on colorectal cancer modulation

According to the literature, an aqueous elderberry polar extract can block the growth of a human colorectal adenocarcinoma cell line (HT29), presenting a $IC_{50}$ of 130.3 μg of cyanidin-3-glucoside eq $mL^{-1}$ (Jing *et al.*, 2008). An aqueous acetone extracts, also with similar properties, was reported on Hepa 1c1c7 cells. This extract potentiates the induction of quinone reductase (QR) and inhibition COX2, which is indicative of anti-initiation and antipromotion properties, respectively (Thole *et al.*, 2006).

The *in vitro* experiments reported the importance of including *S. nigra* preparations in diet to positively modulate colorectal cancer. It regulates inflammatory factors, anti-initiating and anti-promoting of tumoral factors, and oxidation processes. For this reason, tests to unveil the underlying anticancer mechanisms are needed (Thole *et al.*, 2006).

#### 1.3.4.5. Diabetes mellitus

Diabetes is mainly caused by a combination of insulin resistance and β-cell failure (pancreatic cells). It can be treated with insulin-sensitizing drugs that target the nuclear receptor peroxisome proliferator-activated receptor (PPARγ) (Christensen *et al.*, 2010).

Several elderflower extracts (hexane, dichloromethane, methanol, ethyl acetate and water) demonstrated *in vitro,* an activation effect of PPAR (α, δ or γ), between 2.5 and 250 μg $mL^{-1}$, without stimulate adipocyte differentiation (Christensen *et al.*, 2009; Christensen *et al.*, 2010). The extracts had a positive effect on insulin-stimulated glucose uptake indicating that elderflowers have molecules with bioactivities comparable to those of partial PPARγ agonists (Christensen *et al.*, 2009).

The exposure to elderflower lipophilic extract (dichloromethane extract 20 mg $L^{-1}$) led to an increase on glucose uptake by primary porcine myotubes and by mouse abdominal muscle in the absence of insulin. It was also observed a decrease on fat accumulation in *Caenorhabditis elegans* model (Bhattacharya *et al.*, 2013), and a decrease on insulin secretion by clonal pancreatic β-cells (Gray *et al.*, 2000).

Antidiabetic properties of elderberry extracts were also assessed *in vivo.* STZ-induced diabetic rats were supplemented with acidified 0.5% HCl-methanol polar extracts (phenolic-type extract), with doses ranging from 28 to 350 mg $kg^{-1}$ body weight, and dichloromethane extracts (lipophilic type extracts) with doses of 190 mg $kg^{-1}$ body weight, for 4 to 16 weeks (Badescu *et al.*, 2012; Badescu *et al.*, 2015; Ciocoiu *et al.*, 2009; Ciocoiu *et al.*, 2003; Groza *et al.*, 2010; Groza *et al.*, 2011; Salvador *et al.*, 2016). The addition of these extracts to the rats dietary induced a decrease of glycemic serum levels and pro-inflammatory interleukins levels (namely, IL-6 and IL-1β) (Badescu *et al.*, 2012; Ciocoiu *et al.*, 2012; Ciocoiu *et al.*, 2003; Groza *et al.*, 2010; Salvador *et al.*, 2016).

## 1.4. Solid-liquid extraction

Solid-liquid extraction is one of the oldest extraction techniques. Its principle results on the combination of a solid sample with a solvent in which the solute is soluble (Gertenbach, 2002). To perform a specific solid-liquid extraction several parameters must be taken into account, namely solvent nature, temperature, sample granulometry, partition coefficient and liquid-to-solid ratio (Gertenbach, 2002; Petronilho *et al.*, 2014).

The use of solid-liquid extraction, also named maceration, involves the contact of the plant material (often powdered) with the solvent for a specific time that could range from few minutes to several days. This technique can be performed at room temperature or at higher temperatures to enhance extraction efficiency. This process is one of the most used on phenolic compounds extraction, including for the extraction of these compounds from *S. nigra* in which solvents like acidified methanol (with hydrochloric acid, acetic acid or formic acid) are used at room temperature (Lee & Finn, 2007; Veberic *et al.*, 2007).

## 1.5. Chromatographic-based technologies

The wide spectrum of metabolites present on a given plant implies chemical characterization studies combining multiple chromatographic based platforms to increase metabolites coverage (Jorge *et*

*al.*, 2016). Furthermore, the association between chromatographic devices and mass spectrometry (MS) brings together the high separation efficiency, selectivity and sensitivity (chromatography) with a high identification power of the spectrometric data (mass spectrometry) (Roessner-Tunali, 2007). Most representative techniques of this type include (ultra-)high pressure liquid chromatography-mass spectrometry ((U)HPLC-MS) (Grata *et al.*, 2008; Toffali *et al.*, 2011), gas chromatography-mass spectrometry (GC-MS) (Lisec *et al.*, 2006) and comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×MS-ToF) (Almstetter *et al.*, 2012). Other techniques such as nuclear magnetic resonance, are also very powerful tools in metabolites structural elucidation (Kim *et al.*, 2010).

### 1.5.1. One dimensional gas chromatography

GC-MS technology has long been used and improved for analysis of metabolites in plant species (Roessner-Tunali, 2007). The coupling of GC with electron impact ionization (EI) MS is perhaps the oldest hyphenated technique, being frequently denoted as the "gold standard", as it is one of the most advanced, robust, and very sensitive technique for metabolite studies (Dettmer *et al.*, 2007; Lisec *et al.*, 2006).

The high reproducibility attained with GC-MS analysis is in part the result of the electron impact ionization (EI) method typically employed in GC-MS, where molecules interact with kinetically activated electrons with an acknowledged average standard energy of 70 eV (Jorge *et al.*, 2016).

The operative principle of GC-MS system comprises the volatilization of the sample in a heated inlet port (injector), a chromatographic column responsible for the separation of the components in the sample, and detection of each constituent by the MS detector, being thus restricted to volatile and thermally stable composites (Kitson *et al.*, 1996).

This method has already been used on the characterization of *S. nigra* plant, namely elderflowers (Kaack *et al.*, 2006; Toulemonde & Richard, 1983) and elderberries (Kaack *et al.*, 2005).

A schematic illustration of gas chromatograph coupled with a mass spectrometer utilized along this project is present in **Figure 11**.

Depending on the target compounds, derivatization reactions are occasionally necessary to convert analytes into volatile derivatives appropriate to be eluted from GC column without thermal decomposition (Orata, 2012). Derivatization procedure can as well improve detector response, peak resolution and peak symmetry (Orata, 2012).

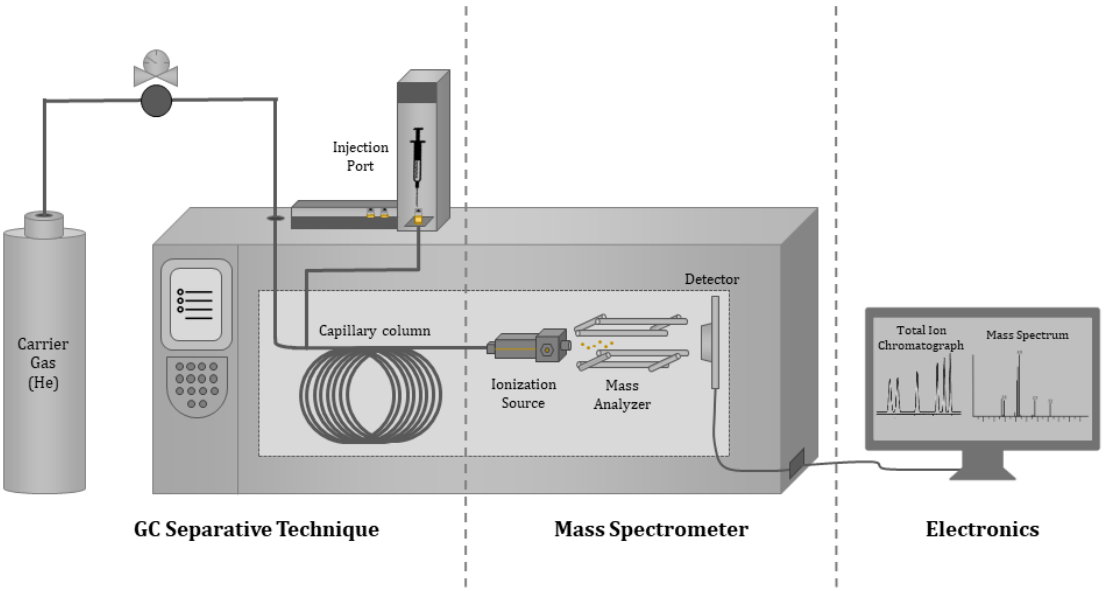


**Figure 11.** One-dimensional gas chromatographic system.

Although one-dimensional chromatography (1D-GC) is extensively used in the qualitative and quantitative analysis of an extensive range of samples, providing high quality analytical data, occasionally the complexity of the samples surpasses the separation capacity of a single chromatographic column (Dettmer *et al.*, 2013). When it occurs, peaks co-elution may happen, which complicate the identification and quantification of compounds. To overcome this problem, comprehensive two-dimensional gas chromatography (GC×GC) arises as a powerful solution, which guarantees an increase power resolution (Peter, 2016).

GC-based techniques also have some limitations. GC can only be utilized for low molecular weight (<1000 Da) molecules, which are either volatile at relative low temperatures, or that can be transformed into volatile derivatives (Roessner-Tunali, 2007).

### 1.5.2. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography-mass spectrometry displays a major advantage over GC-MS techniques the potential to study thermolabile, polar metabolites (non-volatile), and high-molecular weight composites without previous derivatization (Jorge *et al.*, 2016). The sample is initially dissolved in an appropriate solvent, and after being injected on HPLC system, the sample is then carried along a

chromatographic column by a liquid mobile phase. The choice of column, stationary phase and mobile phase are the key variables for the analytical results. Separation occurs as consequence of different interactions as liquid-solid adsorption, liquid-liquid partioning, ion exchange and size exclusion, and by solute/mobile-phase interactions (Harvey, 2000). In reverse phase chromatography, the more usually encountered system of HPLC, the stationary phase is non-polar and the mobile phase is polar. The separation of phenolic compounds by HPLC, a target chemical family in this project, is normally performed in an octadecyl bounded silica column ($C_{18}$). The mobile phase employed for phenolic compounds analysis, through reverse-phase HPLC, is normally composed by water and polar organic solvents (e.g. acetonitrile or methanol), and acetic, formic or phosphoric acids which are frequently added. Consequently, less polar composites develop a strong interaction with the stationary phase, and are more retained on the column when compared to the polar analytes (Jorge *et al.*, 2016). A schematic illustration of a possible configuration of an HPLC instrument is shown in **Figure 12**.

One detector or more are located at the end of the column, being single-wavelength ultra-violet-visible spectrophotometers (UV-Vis) and multi-wavelength diode array sensors (DAD) the most common, as well as mass spectrometers **Figure 12**.

Electrospray ionization (ESI) is the most common ionization technique used on HPLC-MS systems, as it allows an effective transfer and ionization of analytes to the gas phase. Although, ESI is a soft-ionization method which, directly offers little structural data. This information can be attained by tandem mass spectrometry techniques (tandem-$MS^n$) in which analytes ions are posteriorly fragmented. The most common tandem-in-time instruments are ion-trap mass spectrometers (Jorge *et al.*, 2016).

HPLC systems have been widely applied on the analysis of phenolic compounds from different plant extracts (Dai & Mumper, 2010), including elderflowers (Mikulic-Petkovsek *et al.*, 2015) and elderberries (Christensen *et al.*, 2008).

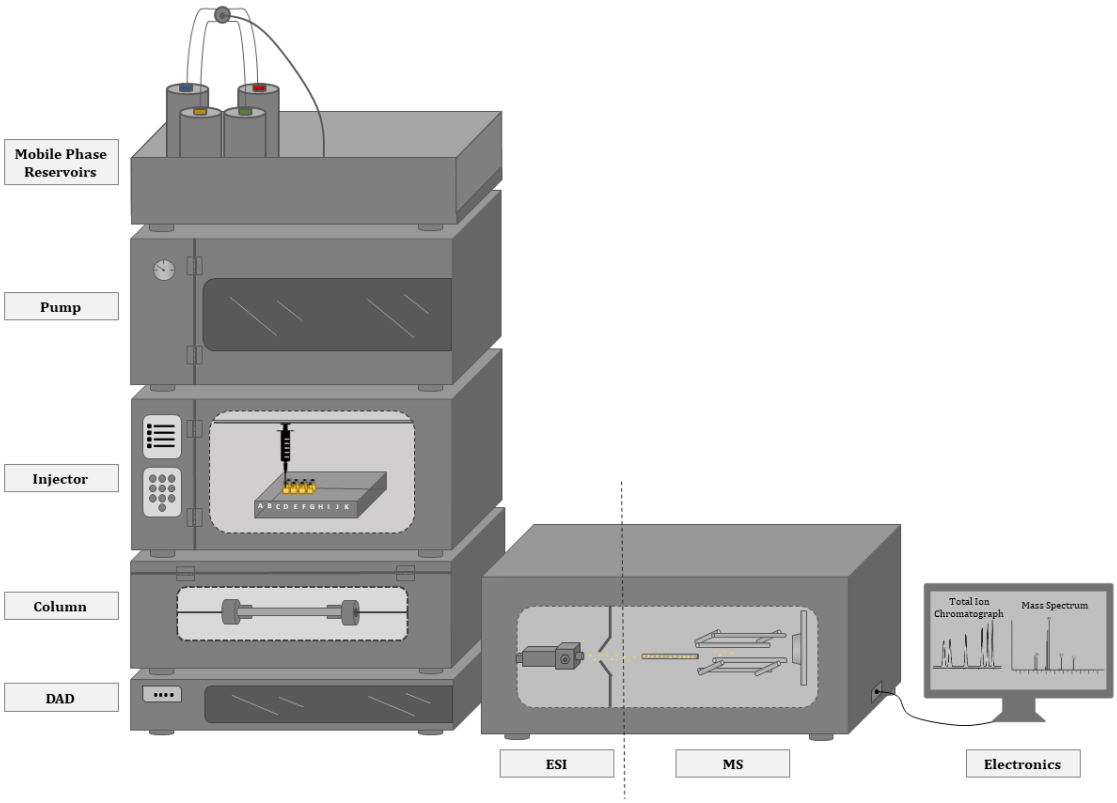


**Figure 12.** High-performance liquid chromatography system.

### 1.5.3. Data processing and interpretation

Data processing and analysis is applied in order to capture relevant information required to formulate a scientific hypothesis. The chemical complexity of natural formulation, the absence of reference mass spectra for all the compounds and the intrinsic variability in each sample associated to the uniqueness of each organism, reinforces the data analysis importance. Data analysis comprises different strategies, which include: data pre-processing, pre-treatment, identification, quantification and processing to data post-processing, validation and interpretation of the data (Goodacre *et al.*, 2007) (**Table 3**).

Data pre-processing and pre-treatment goal is to identify and delete extraneous variability features (human error, artifacts, instrument variation, etc.) from the inherent variations of the samples, displaying an essential role in data analysis (Goodacre *et al.*, 2007). These include, data deconvolution, alignment, base-line correction, normalization, transformation and scaling (Goodacre *et al.*, 2007).

**Table 3.** Data pre-processing, pre-treatment, identification and quantification, processing, post-processing, validation and interpretation (Goodacre *et al.*, 2007).

| Term | Objective | Examples |
|---|---|---|
| **Pre-processing** | Raw instrumental data to clean data | Base-line correction, deconvolution, alignment |
| **Pre-treatment** | Converting the clean data to make them ready for data processing (scaling, centering, etc.) | Normalization, transforming, scaling |
| **Identification and quantification** | Transforming of raw data into biological context | Instrument response, internal standard, MS fragmentation, standards |
| **Processing** | The actual data analysis | – |
| **Post-processing** | Transforming the outcomes from the processing for interpretation and visualization | – |
| **Validation** | Assure the quality of the conclusions collected by data analysis | Cross validation |
| **Interpretation** | Hypothesis generated, pathways affected, or visualization of the data | – |

The identification method of a chemical compound is an important function that transforms raw data into biological context. Although, the basis for what establish a valid compound identification is still now discussed, and four levels of identification can be distinguished based on Summer et al. (Sumner *et al.*, 2007):

1. Identified compounds (using chemical reference standards);
2. Putatively identified compounds (without chemical reference standards, based on physicochemical and/or spectral data);
3. Putative characterized compound classes (based on characteristics physicochemical properties of a chemical class of molecules, or by spectral similarity to recognized compounds of a chemical class);
4. Unknown compounds (beside unidentified or unclassified these metabolites can still be distinguished and measured based upon spectral data);

The identification process of a compound must be done based on more than one information source to increase confidence. Generally, this method is based on MS and retention results in

combination with databases and standards. Although, the use of reference standards in co-injection is sometimes restricted, once they are often commercially unavailable or economically unaffordable. Moreover, sample process flow (extraction, derivatization, injection mode, chromatographic column, etc.) can offer valuable data to complement identification.

Depending on the scope of the study, semi- and absolute-quantification approaches may be utilized (Sumner *et al.*, 2007). Semi-quantification reports the instrument response from analytes abundances, which could be relative to an internal standard or other metabolites. The use of an internal standard relies on the addition of a known amount of a substance to the standards and to the sample to serve as a reference for the peak area, so that small variations (ex. in injection technique and volume) are compensated by the fact that the internal standard peak and the analytes peaks are similarly affected by these variations (Kenkel, 2010; Oliveira *et al.*, 2010). Concerning absolute quantification, the analytes absolute concentrations are measured by the correlation among the response of the used instrument and known concentrations series of the same analytes, using single or several calibration curves which can be attained by an internal or external calibration (Harvey, 2000; Oliveira *et al.*, 2010).

## 1.6. Context and objectives of the thesis

Currently there is crescent interest on the exploitation of natural products as sources of bioactive compounds with potential health benefits for humans, and especially in the consumption of herbal products that are able to prevent or ameliorate chronic diseases, and specifically of those with growing incidence in the 21st century, such as cardiovascular diseases, cancer, diabetes and mental disorders (WHO, 2013).

*Sambucus nigra* flowers and berries have been extensively used on folk medicine for several applications that include antimicrobial, anti-viral, antioxidant, anti-inflammatory, anti-cancer, antidiabetic, among others (Sidor & Gramza-Michalowska, 2015). The detailed information of the chemical composition of *S. nigra* is tremendously important to comprehend its biological effects but also to improve its value and applicability. *S. nigra* flowers received increased attention due to the presence of phytochemicals with many reported health benefits, comprising phenolic compounds, monoterpenic, sesquiterpenic and sterols, although more studies are needed to in-depth establish their profiles.

The main objective of this MSc thesis was to chemical characterize an aqueous extract of *S. nigra* flowers, and evaluate its biological potential in view of their valorization. In this context, different specific objectives were drawn:

- **Production of *S. nigra* aqueous extract**

Evaluate how the temperature of extraction affects the antiradical scavenging activity and phenolic content of *S. nigra* aqueous extract.

- **Chemical characterization of *S. nigra* aqueous extract**

To establish the lipophilic metabolites (dichloromethane extractives) profile by employing one-dimensional gas chromatograph-mass spectrometry and to establish the hydrophilic metabolites (water extractives) profile by employing high pressure liquid chromatography-mass spectrometry.

- ***In vitro* studies of bioactive properties of *S. nigra* aqueous extract**

Evaluation of the antiproliferative activity, epigenetic activity of elderflower extract on demethylation of *MLH1*, antigenotoxicity activity and antimicrobial activity of *S. nigra* aqueous extract.

# Material and Methods

# 2. Material and Methods

## 2.1. Extract isolation and characterization

### 2.1.1. Plant material and extraction

*S. nigra* flower was purchased dry in a local supermarket. The aqueous extraction was performed using 5 g of dry flower mixed in 150 mL distilled water (33.33 g $L^{-1}$) (Supplementary Data VII) on erlenmeyer flasks. The erlenmeyer was placed in a thermostatic bath with controlled temperature and 130 rpm agitation for 30 min. Extraction temperatures of 50 °C, 70 °C and 90 °C were tested. After extraction, the solid was separated from the liquid extracts by centrifugation at 9,000 rpm, for 10 min at 5°C. The supernatant was and filtered with 0.22 µm filter paper. Samples were freeze-drying and stored at 4°C in the dark. This experiment was performed in triplicate.

### 2.1.2. DPPH scavenging activity and trolox equivalent antioxidant capacity (TEAC)

Antioxidant activity of elderflower aqueous extract was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The DPPH scavenging assay is frequently used to assess the free radical scavenging activity of antioxidant compounds, being acknowledge as one of the easiest colorimetric assays to evaluate the antioxidant potential (Mishra *et al.*, 2012). Trolox prepared in 100% methanol was used as standard. Mixture composed by 200 µL of sample and 100 µL DPPH solution was incubated in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 515 nm. Antioxidant activity or the ability of the sample to scavenge DPPH free radical was expressed as inhibition percentage (% of reduction of DPPH) and trolox equivalent antioxidant capacity (TEAC) as millimole of trolox equivalents per gram of dry *S. nigra* flower (mmol TE $g^{-1}$). Analyses were performed in triplicate.

### 2.1.3. Phenolic content of elderflower aqueous extract

Total phenolic content was determined according to the Folin-Ciocalteu method using caffeic acid as standard. The reaction mixture was composed by 5 µL of sample, 60 µL of sodium carbonate 15% (w/v) and 20 µL Folin-Ciocalteu reagent. Finally, 200 µL distilled water was added to the mixture. Microtiter plate was incubated at 60 °C for 10 min. The absorbance was measured at 700 nm after

cooling at room temperature. Total phenolic content was expressed as milligrams of caffeic acid equivalents per gram of dry *S. nigra* flower (mg CAE $g^{-1}$). Analyses were performed in triplicate.

### 2.1.4. GC-MS spectrometry analysis

The lipophilic fraction of *S. nigra* was obtained by mixing 30 mL liquid extract (from extraction at 90 °C) with 90 mL dichloromethane for 30 min. The solvent was evaporated in a rotatory evaporator and the solid extracts weighted (Salvador et al., 2015). The lipophilic fraction obtained was used and converted into trimethylsilyl (TMS) derivatives according to methodology of Domingues *et al.* (2011). GC–MS analyses were performed using a GC-MS QP2010 Ultra equipped with a Thermo Scientific DSQII mass spectrometer using helium as carrier gas (35 cm $s^{-1}$) equipped with a DB-1 MS capillary column (30m × 0.32mm × 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature 80 °C for 5 min, temperature rate of 4 °C $min^{-1}$ up to 260 °C, and 2 °C $min^{-1}$ until the final temperature 285 °C, then maintained at 285 °C for 13 min, injector temperature of 250 °C; transfer-line temperature 290 °C, split ratio: 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scan $s^{-1}$ over a range of m/z 33–700. The ion source was maintained at 250 °C. Compounds were identified as TMS derivatives by comparing their mass spectra with the GC–MS spectral library (Wiley-NIST Mass Spectral Library 1999) and with literature MS fragmentation (Domingues *et al.*, 2014; Freire *et al.*, 2002; Razboršek *et al.*, 2008; Vilela *et al.*, 2013). For quantitative analysis, a calibration with ferulic acid was performed to evaluate the phenolic acids compounds and palmitic acid for fatty acids. Tetracosane ($C_{24}H_{50}$) was used as internal standard as described in detail in previous studies (Vilela *et al.*, 2013).

The response factors needed to obtain a correct quantification of the peak areas were calculated based on three standards concentrations as an average of three GC–MS runs of each concentration using GCMSsolution Sotware. Three independent aliquots were derivatized and submitted to GC–MS analysis. Each aliquot was injected in duplicate. The presented results are the average of the concordant values obtained for each sample (n=3). Compound content was expressed as micrograms of respective compound per gram of dry *S. nigra* flower (μg $g^{-1}$).

### 2.1.5. Characterization of HPLC-MS

To carried out the qualitative analysis of *S. nigra* extract a solution of 10 mg $mL^{-1}$ was prepared, using water as solvent, being subsequently filtered with a 0.45 μm PTFE syringe filter. Polyphenols were

analyzed on a Thermo Finnigan Surveyor HPLC system with a diode array detector at 320 nm (flavanols, hydroxycinnamic acid derivatives, flavanone) and 360 nm (flavonols). Spectra of the compounds were recorded between 200 and 600 nm. The column was a Gemini C18 (150 mm ×4.6 mm×3 μm) operated at 25 °C. The elution solvents were 0.1% formic acid in acetonitrile (ACN) (A), 1% ACN and 0.1% of formic acid in 99% of distillated water (B), and ACN (C). Samples were eluted according to a linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20% to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30% to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions. The injection amount was 20 μL and flow rate 0.6 mL $min^{-1}$. All phenolic compounds were identified using a mass spectrometer with electrospray ionization (ESI) operating in negative ion mode. The analyses were carried out using full scan data-dependent MSn scanning from m/z 115 to 1500. The injection volume was 1 μL and the flow rate maintained at 0.6 mL $min^{-1}$. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 60 and 15 units, respectively; the source voltage was 3 kV and normalized collision energy was between 20–35%. Spectral data were elaborated using the Excalibur software.

Contents of phenolic compounds and flavonols were calculated from peak areas of the sample and using similar compounds as standards (chlorogenic acid and rutin, respectively), expressed in μg $g^{-1}$ of *S. nigra* flower.

Limits of detection (LOD) and quantification (LOQ) were also estimated using the S/N approach (n = 3). Individual compound quantification was accomplished with calibration data for the most similar standards in terms of maximum wavelength absorption, when no pure reference compounds were available. The concentration of each compound was expressed as the mean value (n=3).

## 2.2. *In vitro* studies with cell lines

### 2.2.1. Cell lines and culture conditions

For the present study 5 cell lines were used: three cell lines derived from human colon carcinoma: RKO, HCT116 and Caco-2 cells; a keratinocyte cell line, NCTC 2544; and a murine macrophage-like cell line RAW 264.7.

Firstly, a vial of frozen cells from each cell line, stored in liquid nitrogen, was thawed and the content, cells mixed with freezing mixture (DMSO and fetal bovine serum (FBS), 1:4 (v/v), respectively), was carefully resuspended in 9 mL of complete medium in a 15 mL falcon tube. The cell suspension was

then centrifuged at 1,200 rpm for 5 min and the pellet resuspended in 5 mL of fresh medium, which was transferred into a sterile culture flask.

The RKO cell line was cultured in ATCC-formulated Eagle's Minimum Essential Medium (MEM) in the presence of 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin solution at 37 °C in 5% $CO_2$ (RKO complete medium). HCT116 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution at 37 °C in 5% $CO_2$. Caco-2 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 1% (v/v) Non-essential Amino Acids (NEAA) and 1% (v/v) penicillin/streptomycin, under an atmosphere of 5% $CO_2$ at 37 °C. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% (v/v) FBS at 37 °C under a humidified atmosphere containing 5% $CO_2$.

### 2.2.2. Antiproliferative assay in human cells

For the assay, cells were plated in 48-well plate (300 µl/well) at a density of 120,000 cells $mL^{-1}$ for NCTC and RAW 264.7 cell lines, 50,000 cells $mL^{-1}$ for RKO and HCT116 cell lines, and 30,000 cells $mL^{-1}$ for Caco-2 cell line. Lyophilized *S. nigra* flower was dissolved in ultra-pure water and filtered with 0.22 µm filter. The filtrated extract was dissolved in culture medium in order to obtain concentrations ranging from 0–2,500 µg $mL^{-1}$. After 24h of cell seeding, the elderflower aqueous extract was added to the cells and incubated 48 h in a humidified medium containing 5% $CO_2$ at 37 °C. Control cells grown with complete medium were considered as 100% viability controls. At the end of the incubation, the MTT reduction assay was performed to a final concentration of MTT of 0.5 mg $mL^{-1}$ (Kupcsik, 2011). Each experiment was performed in triplicate. The results were transformed to percentage of controls, and the $IC_{50}$ values were obtained using Graphpad Prism 7 model *[Inhibitor] vs. normalized response – Variable slope*.

### 2.2.3. Epigenetic activity of elderflower extract on *MLH1* demethylation

On this study, RKO cells were seeded in 48-multiwell culture plate at density of 50,000 cells $mL^{-1}$ and incubated overnight at 37°C with 5% $CO_2$. On plate one RKO complete medium supplemented with 5-azaC (2 µM) was added (Pedro, 2015) to the cells and incubated for 48 hours at 37°C with 5% $CO_2$. On a second plate, RKO complete medium supplemented with elderflower extract (10 and 20 µg $mL^{-1}$) was added for 48 hours at 37°C with 5% $CO_2$. At this point, the medium was removed, and RKO complete

medium supplemented with 10 μM of 5-FU was added to all the plates. Cell viability was assessed by MTT as described in 2.2.2.

### 2.2.4. Effect of *S. nigra* extract on prevention of oxidative DNA damage

To evaluate protection against oxidative damage, RKO cells were pre-incubated for 24h and 48h hours at 37 °C and 5% $CO_2$, with lyophilized extract of *S. nigra* flower (extraction performed at 90° C) dissolved in ultra-pure water, added to culture medium. The final concentration of *S. nigra* extract was 200 μg $mL^{-1}$ and 400 μg $mL^{-1}$ in each assay. Luteolin-7-glucoside (L-7-G) was used as positive control at 20 μM (Ramos *et al.*, 2010). Cells were washed with PBS 1× and treated with $H_2O_2$ (75 μM) during 5 min on ice to induce strand breaks (SBs). DNA damage was evaluated by the comet assay (described in 2.2.5).

### 2.2.5. Comet Assay

The alkaline version of the single cells gel electrophoresis was used to assess DNA damage using RKO cells. Briefly, adhered RKO cells at ≈ 80% confluence were washed with PBS 1× and trypsinized. Trypsin activity was inhibited by the addition of fresh medium MEM. Cells were resuspended, and 10 μL of cell suspension was mixed with 40 μL low melting point agarose. About 5,000 cells $mL^{-1}$ were place on a microscope slide pre-coated with normal melting point agarose. Slides were then placed on ice for 10 min followed by exposure to a lysis solution (2.5M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris, pH 10) plus 1% Triton X-100 for 1h at 4°C. After lysis, slides were placed in an electrophoresis chamber with electrophoresis solution (300mM NaOH, 1mM $Na_2EDTA$, pH 13) for 40 min at 4°C for the DNA to unwind before electrophoresis was run for 20 min at 25V and 300mA. Later, for neutralization, washing and fixation, slides were removed from electrophoresis chamber and washed with a neutralizing buffer (PBS) 5min 1× and washed in distillated water 2×. Finally, fixation was performed placing slides 10 min in 70% ethanol plus 10 min in absolute ethanol, and slides were left to dry at room temperature.

For the analysis of comet images, slides were stained with SYBR Gold solution for 30 min at 4 °C, followed by analysis in a fluorescence microscope. Visual score analysis was used to calculate de parameter % tail intensity.

### 2.2.6. Cellular Repair Assay

In order to evaluate *S. nigra* ability to repair DNA from oxidative stress, RKO cells were incubated with RKO complete medium supplemented with 200 and 400 μg $mL^{-1}$ of lyophilized elderflower extract for 24 and 48 hours at 37 °C and 5% $CO_2$. L-7-G was used as positive control. After the period of incubation cells were washed with PBS and exposed to $H_2O_2$ (75 μM) for 5 min on ice to induce SBs. $H_2O_2$ was removed and cells were washed with PBS and then incubated with fresh culture medium for 5 and 10 min at 37 °C. DNA damage was evaluated by the comet assay (described on 2.2.5). Percentage of DNA repair was calculated by the following equation:

$$\textbf{\% of repair DNA damage} = \frac{T_0 - T_R}{T_0} \times 100 \quad \textbf{(Equation 2)}$$

$T_o$ – represents the DNA damage before recovery period

$T_R$ – Represent DNA damage after 5/10 min of recovery time

### 2.2.7. Susceptibility Testing

The antimicrobial activity of the elderflower extract was established determining the minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) by microdilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2010). MBC was tested against *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* ATCC 25293, *Staphylococcus epidermidis* ATCC 12228, *Klebsiella oxytoca* ATCC 13182 and *Klebsiella pneumoniae* ATCC 11296 and MFC was carried out against *Candida albicans* SC 5314. All microorganisms were preserved in cryovials at - 80 (± 2) °C to minimize putative adaptation to the laboratory environment. Prior to each experiment, bacterial and fungi cells were grown on Trypic Soy Agar (TSA) and on Sabouraud Dextrose Agar (SDA) plates, respectively, overnight at 37 °C.

Before MBC and MFC determination, bacteria and fungi were grown overnight in Trypic Soy Broth (TSB) or Sabouraud Dextrose Broth (SDB) at 37 °C, 120 rpm, respectively. Then, these bacterial and fungi suspensions were centrifuged at 9000 *g* during 5 min and washed twice with PBS 1×. MBC and MFC were assayed using a 96-well plate with rounded bottom with different concentrations of plant extract ranging from 0.064 to 33 mg $L^{-1}$ prepared in Mueller Hinton Broth (MHB) or RPMI, if bacteria or fungi were tested, respectively. At the end, bacteria and fungi were added to the wells to obtain a final concentration of 500,000 CFU $mL^{-1}$ and 150,000 cell $mL^{-1}$ for bacterial and fungi cells, respectively. Microbial suspensions were incubated at 37 °C, 120 rpm for 18-21 h in air conditions. After exposure to the elderflower extract, cultures were plated onto Mueller Hinton Agar (MHA) (for bacterial species) and SDB (for fungi species). MBC and MFC were determined by the minimal concentration of extract required

to eradicate the bacteria and fungi, respectively. All tests were at least 3 times (independent biological assays) with 3 technical replicates.

## 2.3. Statistical Analysis

The analysis of data was performed using GraphPad Prism 7 software (GraphPad Software Inc., USA). Results are represented as mean ± standard deviation (SD), and statistical comparisons were calculated with a 95 % confidence interval. Parametric tests were applied since al data sets present Gaussian distributions**.** One-way analysis of variance (ANOVA) was used for comparison of more than two means. Two-way ANOVA was used to examine the influence of two independent variables on one continuous variable. Of each test results a *p*-value indicates the significance value of each tested sample. This significance is indicated in the figures with $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$(***).

# RESULTS

# 3. Results

## 3.1. Antioxidant activity and Phenolic Content

The antioxidant potential is a crucial parameter for establishing the health benefits of a product.

As mentioned previously, the aqueous extraction *S. nigra* flower natural compounds was performed at different temperatures 50 °C, 70 °C and 90 °C, respectively. As shown in **Figure 13,** the extraction temperature clearly affects the antioxidant capacity of the extract. At the highest temperature (90 °C), the DPPH reduction was 72.9 ± 0.8 %, while for the extraction performed at 50 °C was only 62.5 ± 3.6 %. (**Figure 13 (A)**). Similarly, to the DPPH assay, TEAC demonstrated that at the highest temperature trolox equivalents of 0.157 ± 0.001 mmol TE $g^{-1}$, and for 50 °C extraction of Trolox equivalents of 0.135 ± 0.009 mmol TE $g^{-1}$ (**Figure 13 (B)**).

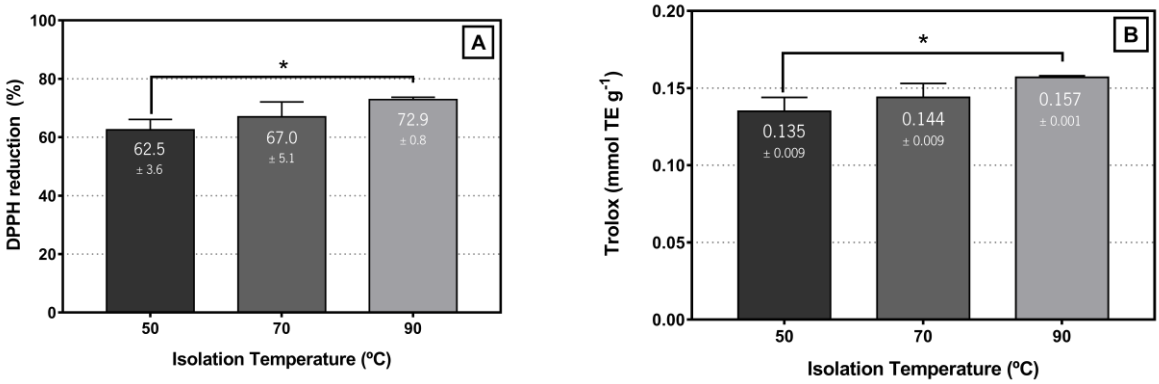


**Figure 13. Radical scavenging activity of *S. nigra* flower extracts at different isolation temperatures. (A)** Radical scavenging activity determined by DPPH assay. **(B)** Trolox equivalent antioxidant capacity (TEAC) in millimole of trolox equivalents per gram of flower (mmol TE $g^{-1}$). Values are the mean of three independent extraction flasks ± SD. Asterisks denote significant differences (*$p < 0.05$).

Phenolic content of the extract performed at different temperatures was also evaluated. This analysis revealed that 90°C extraction was responsible for ensure the highest phenolic content (45.32 ± 2.20 mg CAE $g^{-1}$), when compared to 50 and 70 °C extractions (**Figure 14**).

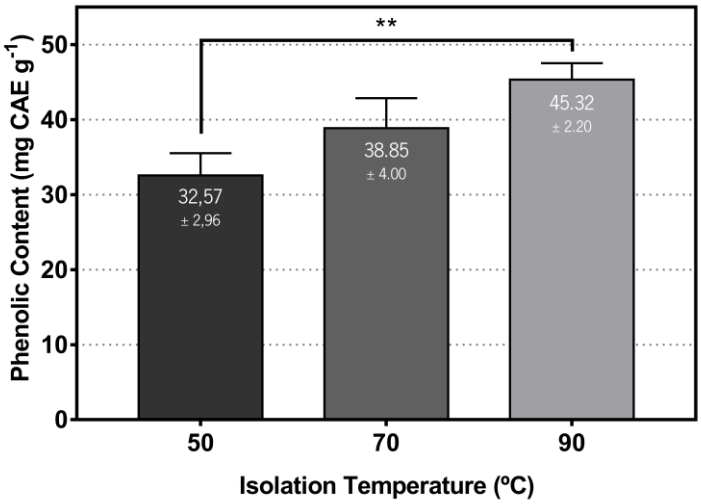


**Figure 14. Phenolic content of *S. nigra* flower extract at different isolation temperatures expressed in caffeic acid equivalents (CAE).** Values are the mean of three independent extraction flasks ± SD. Asterisks denote significant (** $p < 0.01$).

Based on these three parameters (DPPH antiradical activity, TEAC and phenolic content), it is possible to conclude that higher the extraction temperature the higher is the antioxidant activity and phenolic content. Therefore, for the following experiments, the extract with higher antioxidant properties was chemical and biological characterized.

## 3.2. Identification and characterization of the compounds on the elderflower extract

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation is a big challenge, as well as its identification and characterization.

The aqueous extract performed at 90 °C was fully characterized using GC-MS and HPLC-MS. The use of GC-MS and HPLC-MS allows a qualitative and quantitative analysis of the sample. In this case, it is possible to characterize the tested object in terms of volatile, medium-volatile, low-volatile and non-volatile substances present in it.

### 3.2.1. GC-MS Analysis

The current gold standard in the determination and monitorization of volatile organic compounds is GC-MS. It has been the method of choice for characterizing individual organic compounds, due to its high sensitivity and resolving power. To characterize the metabolite profiling of lipophilic solvent present in *S. nigra* extract an extraction with dichloromethane-water was performed. The solvent was completely evaporated and transformed into TMS derivates. The TMS derivates are more volatile than the initial molecules so their GC analysis is possible.

From this analysis it was possible to conclude that the *S. nigra* lipophilic extract yield was 0.42% (g $g^{-1}$ of flower) dry weight. The lipophilic composition of the aqueous *S. nigra* extract was studied in detail in GC-MS. The identification and quantification data (expressed as mg of compound per kg of dry elderflower weight) is summarized in **Table 4** and **Table 5**, respectively.

The GC-MS analysis revealed the presence of up to 27 compounds distributed over seven chemical families. Flavonoids, accounted for 27.45% of all identified compounds, followed by miscellaneous, which account for 22.16% of the identified compounds, phenolic acids (16.39%), fatty acid derivatives (15.45%) ($C_6$ – $C_{24}$), sugars (9.38%), monoterpenes (6.33%) and amino acids derivatives (2.84%). Regarding the fatty acids category, medium chain fatty acids (MCFAs) and long chain fatty acids (LCFAs) were detected.

The only flavonoid identified was naringenin and it corresponds to the compound in highest concentration of lipophilic fraction (27.16%), accounting of a total of 122.19 μg $g^{-1}$ of flower. The fatty acids were the second most abundant family, accounting for 45.68 μg $g^{-1}$ of the lipophilic extract, where palmitic acid was the most abundant component, representing 40.62% of the identified fatty acids.

Miscellaneous compounds accounted for 98.62 μg $g^{-1}$ of flower lipophilic extract, being vinclozolin the most significant compound detected, accounting for 67.47 μg $g^{-1}$. Sugar, monoterpenes and amino acid derivates are present, but in lower amount, 41.73, 28.17 and 12.65 μg $g^{-1}$, respectively.

**Table 4.** The compounds identified in elderflower dichloromethane extract by GC/MS analysis, and their identified biological activities in literature.

| Peak Number | RT[A] | Compound | Formula | Molecular mass (g mol$^{-1}$) | [M-H]$^-$ (m/z) | Bioactivity | Reference |
|---|---|---|---|---|---|---|---|
| 1 | 5.58 | 2-3-Butanediol | $C_4H_{10}O_2$ | 90.12 | 45, 73, 117, 147 | _ | _ |
| 2 | 6.12 | Hexanoic Acid | $C_6H_{12}O_2$ | 116.16 | 45, 75, 117, 173 | Antitumor activity | (Wang *et al.*, 2008) |
| 3 | 10.12 | Methyl leucinate | $C_7H_{15}NO_2$ | 145.20 | 45, 73, 100, 160 | _ | _ |
| 4 | 11.77 | Benzoic Acid | $C_7H_6O_2$ | 122.12 | 51, 77, 105, 135, 179 | Antifungal agent and food preservative | (Berk, 2013; Krebs *et al.*, 1983) |
| 5 | 14.21 | Glycerol | $C_3H_8O_3$ | 92.09 | 73, 117, 147, 186, 205 | _ | _ |
| 6 | 16.27 | Nonanoic acid | $C_9H_{18}O_2$ | 158.24 | 215, 117 | Antifungal agent | (Jang *et al.*, 2012) |
| 7 | 19.58 | Vinclozolin M2, trimethylsilyl ether | $C_{14}H_{19}Cl_2NO_2Si$ | 332.30 | 73, 143, 217 | Fungicide | (Müller *et al.*, 2011) |
| 8 | 20.01 | Rhamnose | $C_6H_{12}O_5$ | 164.16 | 73, 133, 147, 189, 204 | _ | _ |
| 9 | 20.73 | ρ-Anisic Acid | $C_8H_8O_3$ | 152.15 | 64, 77, 107, 135, 209 | _ | _ |
| 10 | 21.52 | Cinnamic acid | $C_9H_8O_2$ | 148.16 | 205, 131, 161 | Flavoring agent | (Pramote *et al.*, 2012) |
| 11 | 21.74 | Linolool Oxide isomer | $C_{10}H_{18}O_2$ | 170.25 | 73, 131, 157, 199, 227 | _ | _ |
| 12 | 21.93 | Vinclozolin M2, trimethylsilyl ether | $C_{14}H_{19}Cl_2NO_2Si$ | 332.30 | 73, 103, 143 | Fungicide | (Müller *et al.*, 2011) |
| 13 | 23.04 | Tyrosol | $C_8H_{10}O_2$ | 138.16 | 73, 103, 147, 267 | Antioxidant; anti-arrhythmia agent | (Covas *et al.*, 2003; |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | Maĭmeskulova & Maslov, 1998) |
| 14 | 26.45 | Linolool Oxide isomer | $C_{10}H_{18}O_2$ | 170.25 | 73, 131, 143, 183 | Anti-inflammatory; anticancer; antimicrobial; among others. | (Pereira *et al.*, 2018) |
| 15 | 27.35 | Vinclozolin M2, trimethylsilyl ether | $C_{14}H_{19}Cl_2NO_2Si$ | 332.30 | 73, 103, 143, 170 | Fungicide | (Müller *et al.*, 2011) |
| 16 | 32.88 | 4-Coumaric Acid | $C_9H_8O_3$ | 164.15 | 73, 117, 147, 179, 219, 249, 293, 308 | Antioxidant | (Zang *et al.*, 2000) |
| 17 | 35.8 | Palmitic Acid | $C_{16}H_{32}O_2$ | 256.40 | 73, 117, 145, 313 | _ | _ |
| 18 | 36.29 | Methyl 4-O-benzyl-2,5-dideoxypentonate, (3R,4S) | $C_7H_{14}O_2$ | 130.18 | 91, 115, 147 | _ | _ |
| 19 | 36.53 | Ferulic Acid | $C_{10}H_{10}O_4$ | 194.19 | 73, 117, 147, 191, 219, 249, 293, 323, 338 | , Anti-inflammatory agent, anti-diabetic, antioxidant | (Srinivasan *et al.*, 2007) |
| 20 | 39.62 | Oleic Acid | $C_{18}H_{34}O_2$ | 282.47 | 339, 117, 129, 145 | _ | _ |
| 21 | 40.32 | Stearic Acid | $CH_3(CH_2)_{16}COOH$ | 284.48 | 73, 117, 145, 201 | _ | _ |
| 22[B] | 43.72 | Tetracosane (Internal Standard) | $C_{24}H_{50}$ | 338.65 | 57, 71, 113, 141, 169 | | |
| 23 | 46.93 | n-(+)-cellobiose | $C_{12}H_{22}O_{11}$ | 342.30 | 73, 129, 147, 189, 204 | _ | _ |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 24 | 48.33 | Behenic Acid | $C_{22}H_{44}O_2$ | 340.58 | 73, 117, 145, 201 | _ | _ |
| 25 | 50.81 | (+/-)-Naringenin | $C_{15}H_{12}O_5$ | 272.26 | 73, 117, 151, 179, 224 | Antioxidant; anti-inflammatory; anticancer | (Patel *et al.*, 2018) |
| 26 | 51. 28 | glycerol monostearate | $C_{21}H_{42}O_4$ | 358.57 | 73, 129, 147, 203, 267 | _ | _ |
| 27 | 52.04 | Lignoceric Acid | $C_{24}H_{48}O_2$ | 368.63 | 73, 117, 145, 201 | _ | _ |
| 28 | 52.28 | Naringenin | $C_{15}H_{12}O_5$ | 272.26 | 73, 133, 147, 179, 229, 268 | Antioxidant; anti-inflammatory; anticancer | (Patel *et al.*, 2018) |

[A]Rt: retention time (as min); [B]Internal standard

**Table 5.** Composition of elderflower dichloromethane extract (μg $g^{-1}$ of flower) with the respective relative standard deviation (RSD).

| RT[A] | Compound | μg $g^{-1}$ flower[B] | RSD (%) |
|---|---|---|---|
| | ***Miscellaneous*** | | |
| 5.58 | 2-3-Butanediol[E] | 1.98 | 15.25 |
| 19.58 | Vinclozolin M2 Isomer[E] | 55.21 | 8.77 |
| 21.93 | Vinclozolin M2 Isomer[E] | 5.81 | 8.79 |
| 27.35 | Vinclozolin M2 Isomer[E] | 6.45 | 6.85 |
| 36.29 | Methyl 4-O-benzyl-2,5-dideoxypentonate[E] | 29.17 | 6.53 |
| | ***Fatty acid derivatives and Glycerol*** | | |
| 6.12 | Hexanoic Acid[C] | 3.12 | 12.57 |
| 16.27 | Nonanoic Acid[C] | 4.86 | 34.67 |
| 35.8 | Palmitic Acid[C] | 18.55 | 12.32 |
| 39.62 | Oleic Acid[C] | 5.95 | 11.51 |
| 40.32 | Stearic Acid[C] | 12.01 | 15.78 |
| 48.33 | Behenic Acid[C] | 5.53 | 3.64 |
| 52.04 | Lignoceric Acid[C] | 0.52 | 21.44 |
| 51.28 | Glycerol monostearate | 20.07 | 13.45 |
| 14.21 | Glycerol | 3.02 | 10.61 |
| | ***Aminoacid derivatives*** | | |
| 10.12 | Methyl leucinate[E] | 12.65 | 9.66 |
| | ***Phenolic Acids and Tyrosol*** | | |
| 11.77 | Benzoic Acid[D] | 8.83 | 8.22 |
| 20.73 | ρ-Anisic Acid[D] | 23.91 | 8.96 |
| 21.52 | Cinnamic acid[D] | 4.97 | 8.25 |
| 32.88 | 4-Coumaric Acid[D] | 8.17 | 5.28 |
| 36.53 | Ferulic Acid[D] | 22.79 | 8.10 |
| 23.04 | Tyrosol[E] | 4.30 | 7.26 |
| | ***Flavonoid*** | | |
| 50.81 | Naringenin Isomer[E] | 30.37 | 10.63 |
| 52.28 | Naringenin Isomer[E] | 91.82 | 12.19 |
| | ***Monoterpene*** | | |
| 21.74 | Linolool Oxide Isomer[E] | 15.83 | 8.52 |
| 26.45 | Linolool Oxide Isomer[E] | 12.34 | 8.67 |
| | ***Sugar*** | | |
| 20.01 | Rhamnose[E] | 10.09 | 8.57 |
| 46.93 | n-(+)-cellobiose[E] | 31.63 | 7.36 |

[A]Retention time (as minutes); [B]Content values are expressed as mean ± RSD, n=3; Calibration was performed using [C]palmintic acid, [D]ferulic acid (Supplementary Data I), or [E]tetraconase.

### 3.2.2. HPLC-MS Analysis

The HPLC method allows the separation and quantitative analysis of phytochemicals present in different extracts. HPLC along with electrospray ionization tandem mass spectrometry is the best advanced analytical platform for identification of compounds present in very low concentrations.

The hydrophilic composition of the elderflower extract was studied by high-pressure liquid chromatography -tandem mass spectrometry (HPLC-$MS^n$). For the chromatographic analysis of the polyphenolic compounds presented in *S. nigra* infusion, it was used a reverse-phase column of silica C18. The separation of the elderflower infusion under optimized gradient conditions is presented on **Figure 15**.

Phenolic compounds were identified by comparing retention times with standards and by using tandem mass spectrometric detection. The phenolic compounds identified, as well as their retention time, the maximum UV wavelengths absorption, the corresponding $[M-H]^-$ ions and the key $MS^n$ fragmentation production ions relevant for their identification are summarized in **Table 6**. The quantification was performed by HPLC-UV using calibration curves of reference compounds representative of each chemical family (**Table 7**).

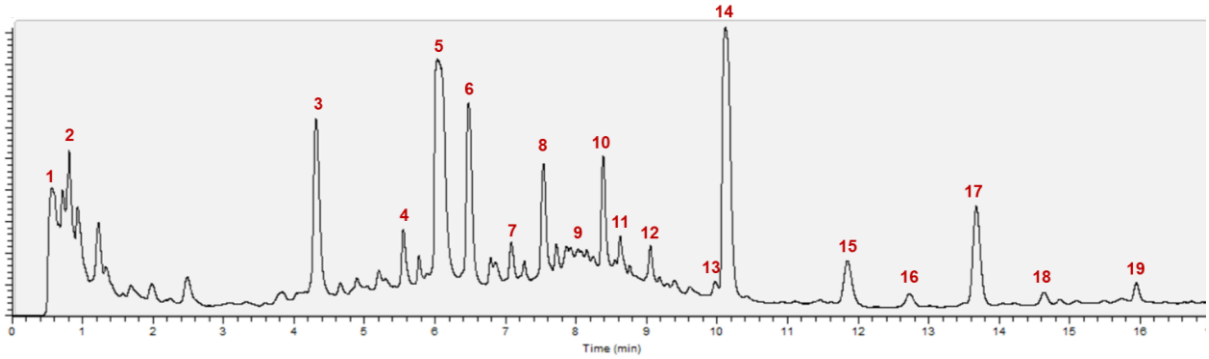


**Figure 15.** HPLC-PDA profile chromatogram of aqueous extract of *S. nigra* flowers. Time of retention is expressed in minutes. Compounds are signed by numbers as stated in **Table 6** and **Table 7**.

Nineteen phenolic compounds, were identified, representing a total of 16,32 mg $g^{-1}$ (1.63 % g $g^{-1}$ of flower) of elderflower extract. Compounds were identified by comparison of their characteristic, retention time and fragmentation patterns obtained under the same experimental conditions, by co-injection of standards or by comparing their MS fragmentation patterns with the publish data (**Table 6** and **Table 7**).

**Table 6.** Phenolic compounds identified in *S. nigra* flower hydrophilic extract and corresponding $MS^n$ fragmentation profiles, and their identified biological activities in literature.

| Peak | RT[A] | $\lambda_{máx}$[B] (nm) | Compound | [M-H]- (m/z)[C] | $MS^2$ (m/z)[C] | $MS^3$ (m/z)[C] | Biological activity | Reference |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.60 | 238, 255 | Quinic Acid | 191 | [191][D]: 85 (37), 87 (22), 109 (23), 111 (100), 127 (57), 171 (29), 173 (41) | – | – | – |
| 2 | 0.71 | 249, 259 | *p*-Coumaroyl-caffeoylquinic Acid | *499* | [499]: 191 (100), 173 (57), 481 (18) | [191]: 85 (91), 171 (100) | Antioxidant | (Sato *et al.*, 2011) |
| 3 | 4.34 | 246, 292, 322 | Caffeoylquinic Acid | 353 | [353][D]: 179 (34), 191 (100), [179]: 135 (100) | – | Antioxidant; Metalloproteinase-9 inhibitor | (Sato *et al.*, 2011) |
| 4 | 5.48 | 234, 288 | Dicaffeoylquinic Acid | 515 | [515]: 191 (100), 352 (52), 379 (28) | [353]: 191 (100) | Antioxidant; HIV integrase inhibitor | (Kim & Lee, 2005; Robinson *et al.*, 1996) |
| 5 | 6.04 | 253, 308, 358 | Caffeoylquinic Acid | 353 | [353]: 191 (100) | [191]: 85, 93 (54), 111 (40), 127 (72), 171 (49), 173 (100); [173]: 87 (23), 93 (100), 111 (100), 155 (59) | Antioxidant | (Sato *et al.*, 2011) |
| 6 | 6.48 | 241, 293, 324 | Caffeoylquinic Acid | 353 | [353]: 173 (100), 179 (49), 191 (20), [173]: 93 (100), 109 (36), 137 (76), 155 (73) | – | Antioxidant | (Sato *et al.*, 2011) |
| 7 | 7.08 | 233, 286, 321 | Quercetin dihexoside | 625 | [625]: 301 (51), 462 (24), 463 (100) | [301]: 151 (100), 179 (73), 231 (41) | – | – |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | 7.54 | 236, 288, 308 | Coumaroylquinic Acid | 337 | [337]: 191 (100), 173 (22) | [191]: 127 (100), 173 (85); [173]: 129 (21), 137 (29), 155 (100) | _ | _ |
| 9 | 8.03 | 233, 264 | Coumaroylquinic Acid derivative | 401 | [401]: 269 (100), 191 (37) | [269]: 113 (21), 159 (23), 161 (100) | _ | _ |
| 10 | 8.38 | 237, 312, 321 | Feruloylquinic Acid | 367 | [367]: 173 (45), 191 (100) | _ | _ | _ |
| 11 | 8.62 | 233, 254, 334 | Quercetin dihexoside | 625 | [625]: 255 (21), 271(24), 300 (100), 301 (61), 445 (53) | [301]: 151 (58), 179 (100) | _ | _ |
| 12 | 9.05 | 234, 254, 334 | Quercetin trisaccharide | 755 | [755]: 271 (25), 300 (100), 301 (33), 343 (39), 505 (31), 591 (79), 609 (45) | [301]: 151 (26), 179 (100); [609]: 283 (69), 300 (32), 343 (100) | _ | _ |
| 13 | 9.97 | 233, 254, 343 | Isorhamnetin derivative | 639 | [639]: 299 (27), 315 (100), 459 (29) | [315]: 300 (100) | Antioxidant; Antitumoral | (Hu *et al.*, 2015; Yokozawa *et al.*, 2002) |
| 14 | 10.12 | 254, 318, 374 | Quercetin-3-rutinoside | 609 | [609]: 301 (100), 300 (42) | [301]: 179 (100), 151 (69) | Antiallergic; anti-inflammatory; antiproliferative, anticarcinogenic | (Chen *et al.*, 2013; Guardia *et al.*, 2001; Yang *et al.*, 2008) |
| 15 | 11.85 | 232, 255, 350 | Quercetin-acetyl glucoside | 505 | [505]: 300 (62), 301 (100), 463 (23) | [301]: 151 (100), 179 73), 193 (24), 273 (46), 283 (23) | Antioxidant | (Lee *et al.*, 2002) |
| 16 | 12.73 | 232, 264, 343 | Kaempferol rutinoside | 593 | [593]: 285 (100) | [285]: 197 (24), 213 (25), 229 (45), 241 (21), 257 (100), 267 (54) | Antioxidant | (Verhoeyen *et al.*, 2002) |
| 17 | 13.68 | 234, 254, 343 | Isorhamnetin-rutinoside | 623 | [623]: 300 (23), 315 (100) | [300]: 255 (67), 271 (100), 272 (25) | _ | _ |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 18 | 14.64 | 233, 301, 385 | Kaempferol residue | 449 | 285 (100), 303 (23) | [285]: 141 (100), 123 (23) | Anti-inflammatory; anticancer; antioxidant; antimicrobial | (Chang *et al.*, 2006; Kampkötter *et al.*, 2007; Kataoka *et al.*, 2001; Park *et al.*, 2009) |
| 19 | 15.94 | 232, 254, 348 | Isorhamnetin acetylhexoside | 519 | 271 (30), 314 (100), 315 (72), 357 (36) | [315]: 300 (100) | Antioxidant | (Zielinska *et al.*, 2001) |

[A] Rt. – retention time;

[B] $\lambda_{máx}$ – maximum wavelength

[C] $MS^2$, $MS^3$ – second and third stage of mass spectrometry;

[D] [...] – product ions were subjected to further $MS^3$ fragmentation.

**Table 7.** Concentration of the polyphenolic compounds in elderflower aqueous extract (μg $g^{-1}$ of flower) determined by HPLC.

| Peak | RT[A] | Compound | μg $g^{-1}$ of flower[D] |
|---|---|---|---|
| 1 | 0.60 | Quinic Acid[B] | 545.0 ± 227.7 |
| 2 | 0.71 | *p*-Coumaroyl-caffeoylquinic Acid[B] | 747.7 ± 105.7 |
| 3 | 4.34 | Caffeoylquinic Acid Isomer[B] | 879.3 ± 29.6 |
| 4 | 5.48 | Dicaffeoylquinic Acid[B] | 145.7 ± 41.8(<LOD) |
| 5 | 6.04 | Caffeoylquinic Acid Isomer[B] | 7,394.10 ± 253.6 |
| 6 | 6.48 | Caffeoylquinic Acid Isomer[B] | 873.1 ± 79.4 |
| 7 | 7.08 | Quercetin dihexoside Isomer[C] | 181.2 ± 38.3(<LOD) |
| 8 | 7.54 | Coumaroylquinic Acid[B] | 435.6 ± 37.9 |
| 9 | 8.03 | Coumaroylquinic Acid derivative[B] | 93.6 ± 15.0(<LOD) |
| 10 | 8.38 | Feruloylquinic Acid[B] | 367.5 ± 2.7 |
| 11 | 8.62 | Quercetin dihexoside Isomer[C] | 293.1 ± 243.4 |
| 12 | 9.05 | Quercetin trisaccharide[C] | 327.8 ± 233.5 |
| 13 | 9.97 | Isorhamnetin derivative[C] | 273.4 ± 231.5 |
| 14 | 10.10 | Quercetin-3-rutinoside[C] | 1,887.7 ±269.1 |
| 15 | 11.76 | Quercetin-acetyl glucoside[C] | 413.9 ± 230.4 |
| 16 | 12.73 | Kaempferol rutinoside[C] | 286.1 ±230.1 |
| 17 | 13.68 | Isorhamnetin-rutinoside[C] | 601.2 ± 227.7 |
| 18 | 14.64 | Kaempferol residue[C] | 288.8 ± 223.7 |
| 19 | 15.94 | Isorhamnetin acetylhexoside[C] | 281.4 ± 227.7 |

[A]Retention time values are expressed in minutes; Calibration curves used: [B]rutin (LOD > 197,75 μg $g^{-1}$) and [C]chlorogenic acid (LOD > 223.19 μg $g^{-1}$), are represented in Supplementary Data II and Supplementary Data III, respectively. [D]Content values expressed as mean ± SD, n = 3.

Among all the phenolic compounds reported, caffeoylquinic acid was the major phenolic acid and quercetin the major flavonol found in *S. nigra* hydrophilic extract, accounting for 9,146.5 (± 362.6) μg $g^{-1}$ and 3,103.7 (± 771.7) μg $g^{-1}$, respectively.

## 3.3. *In vitro* analysis

After a full identification and quantification of elderflower extract metabolites by GC-MS and HPLC-MS, *in vitro* assays were performed to evaluate antiproliferative (cell viability) activity, epigenetic activity of demethylation of *MLH1*, antigenotoxic activity and antimicrobial activities.

### 3.3.1. *In vitro* proliferative activity

The proliferative activity of elderflower extract was determined by the MTT assay. The *in vitro* activity of elderflower extract was tested on five different cell lines (NCTC 2544, RAW 264.7, Caco-2, RKO and HCT-116). The results are expressed as $IC_{50}$ (mg $mL^{-1}$), defined as the concentration that inhibits the cell growth by 50% (**Table 8**).

**Table 8.** $IC_{50}$ values for elderflower extract on different cell lines expressed in mg $mL^{-1}$.

| Cell lines | | | | |
|---|---|---|---|---|
| NCTC 2544 | RAW 264.7 | Caco-2 | RKO | HCT-116 |
| 1.94 (± 0.06) | 0.51 (± 0.03) | 2.52 (± 0.31) | 1.25 (± 0.06) | 3.44 (± 0.23) |

NCTC 2544: human keratinocyte cell line; RAW 264.7: murine macrophage cell line; Caco-2: human colon adenocarcinoma cell line; RKO: human colon carcinoma cell line; HCT-116: human colon cancer cell line.

Elderflower extract exhibited a dose-response relationship in all cell lines tested (Supplementary Data IV). The most pronounced antiproliferative effect of elderflower extract was observed in murine macrophage cell line (RAW 264.7), which had the lowest $IC_{50}$ value (0.51 ± 0.03 mg $mL^{-1}$), while the less susceptible cell line to *S. nigra* extract cytotoxicity was HCT-116 (3.44 ± 0.23 mg $mL^{-1}$).

### 3.3.2. Epigenetic activity of elderflower extract on *MLH1* demethylation

Acquired defects in the MMR pathway are seen in 15%–25% of sporadic cancers of the colon. In most cases, the MMR defects are a result of *MLH1* silencing, an essential factor in the MMR pathway (Herman *et al.*, 1998). In RKO cells, *MLH1* can be reactivated by 5-azaC treatment, but becomes silenced again once the agent is removed (McGarvey *et al.*, 2006). The combination of 5-FU with other drugs seems to be a new promising therapeutic approach. Thus, the study of bioactive compounds with potential antitumor activity that act alone or in combination with other chemotherapeutic agents has been

exploited as an approach to enhance therapeutic efficacy and reduce side effects due to traditional cancer therapy, like the monotherapy of 5-FU. Some phytochemicals, such as quercetin, present in the aqueous elderflower extract, are important demethylating agents, namely on RKO cells as described by Tan and his colleagues (Tan *et al.*, 2009).

To evaluate if aqueous elderflower extract can act synergistically with 5-FU by decreasing RKO cell viability, a 2 μM of 5-azaC concentration was used, because this concentration is not cytotoxic for the RKO cells, and 10 μM of 5-FU ($IC_{50}$ concentration) (**Figure 16**).

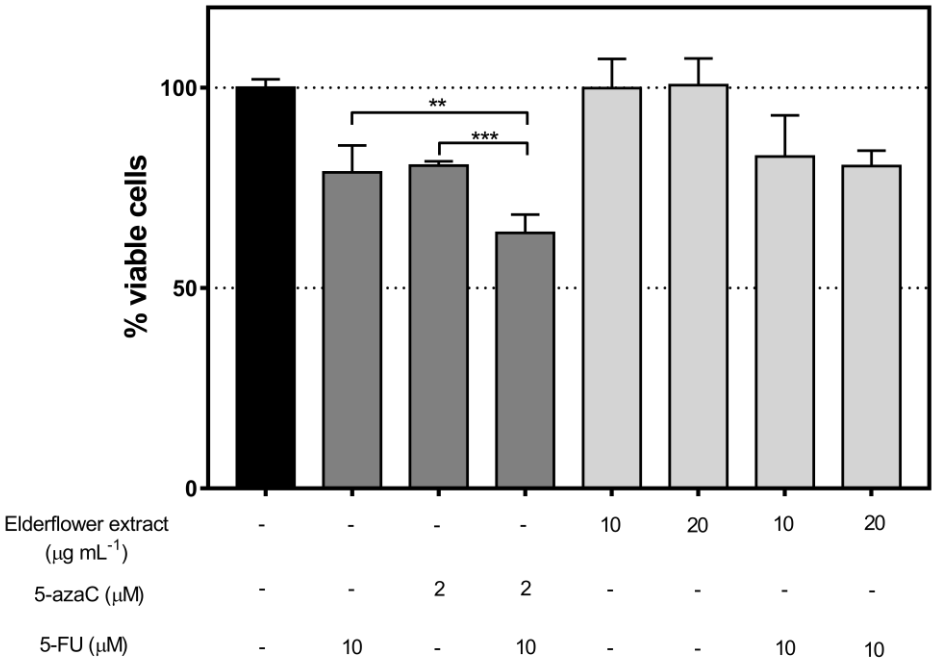


**Figure 16. Effect on cellular viability of elderflower extract, 5-azaC and 5-FU.** The cells were pre-incubated during 48h with 5-azaC (2 μM) or with aqueous elderflower extract (10 μg $mL^{-1}$ and 20 μg $mL^{-1}$) before 5-FU (10 μM) treatment for 48h. The percentage of cell viability was calculated by the absorbance relative to the value detected for the control cells (untreated cells), that was defined as 100% of cell viability. For each condition were performed three independent experiments and data are expressed as mean ± SD. Asterisks represent significant difference (** $p < 0.01$; *** $p < 0.001$).

When incubated for 48 hours with the demethylating agent, 5-azaC, followed by 48 h exposure to 5-FU, a significant decrease on viable cells (63.77 ± 4.55 %) was observed when compared to the controls (only 5-FU (78.85 ± 6.74%) or only 5-azaC (80.56 ± 1.09 %)). This might indicate a synergistic effect between these two drugs. The cells incubated with elderflower extract, followed by the exposure to 5-FU, did not present significant differences on cell viability, which indicates that the decrease on cell viability is due to 5-FU action.

### 3.3.3. Effects of elderflower extract on oxidatively induced-DNA damage

Cells must maintain the balance between the levels of free radicals and antioxidant. When the levels of free radical exceed that of antioxidants during oxidative stress, biomolecules such as lipids, proteins and DNA in particular can be damage. This damage could cause numerous chronic disorders including cancer (Halliwell, 1994). Flavonoids and their derivatives have attracted a great attention as antigenotoxic and chemopreventive agents. In this group, it is included naringenin, a flavanone detected on the GC-MS analysis, which has been associated to several genotoxic studies (Kocyigit *et al.*, 2016; Razo-Aguilera *et al.*, 2011).

To study the antigenotoxic activity of elderflower aqueous extract, RKO cells were exposed to different concentrations of $H_2O_2$ (50, 100, 150, 200, 250 μM, in order to determine the concentration that will result in a 50% of tail DNA on the Comet assay (Collins, 2004). A concentration of 75 μM $H_2O_2$ was chosen to induce a consistent level of DNA damage in RKO cells (Supplementary Data V).

As described in section 2.2.4, to study elderflower aqueous extract ability to protect against $H_2O_2$-induced DNA damage, two different concentrations of elderflower extract were evaluated (200 and 400 μg $mL^{-1}$). L-7-G with a concentration of 20 μM was used as positive control. The extracts were administrated to RKO cells for 24 and 48 hours. Subsequently, cells were exposed to oxidative stress, and for that a 75 μM $H_2O_2$ solution was added for 5 min on ice, aiming to induce DNA SBs.

As shown in **Figure 17**, elderflower aqueous extract at a concentration of 200 and 400 μg $mL^{-1}$ did not show any significant ($^*p > 0.05$) protection after 24 and 48 hours of incubation. This effect is not altered in a dose-dependent manner. On the other hand, incubation with 20 μM L-7-G, significantly ($^*p < 0.01$) decreased DNA SBs induction by $H_2O_2$ when incubated for 24h (43%) and 48h (33%).

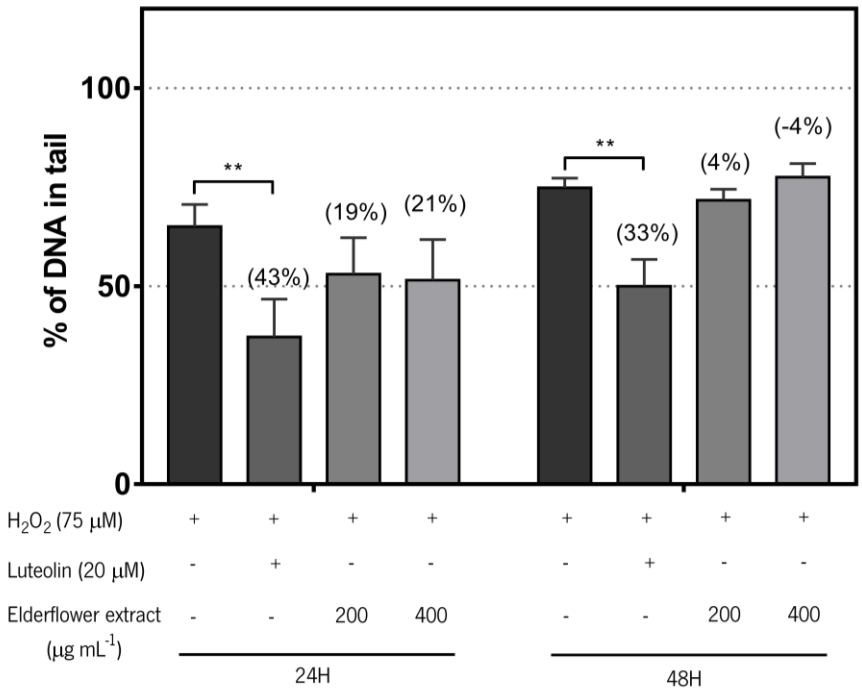


**Figure 17.** Effects of 24 and 48h of pre-treatment with luteolin (20 µM) and elderflower extract (200 and 400 µg $mL^{-1}$) on oxidative DNA damage induced by 75 µM $H_2O_2$ (5 min, on ice) in RKO cells. [+] - compound present; [-] - compound absent; () – percentage of protection regarding to the respective control. Results are expressed as mean ± SD, of at least three independent experiments. Asterisks represent significant difference (** $p < 0.01$).

### 3.3.4. Effects of elderflower extract on repair ability

DNA protection from oxidative damage can contribute to protection to reduce mutations and to maintain genomic stability. On this experiment, the repair-enhancing effects of elderflower aqueous extract and L-7-G in human colon carcinoma RKO cells when exposed to oxidative agents ($H_2O_2$) was evaluated.

Thus, to analyze the potential of elderflower aqueous extract and L-7-G on the ability of RKO cells to rejoin DNA SBs, cells were pre-treated with elderflower aqueous extract at a concentration of 200 and 400 µg $mL^{-1}$ and 20 µM L-7-G for 24 and 48 hours before $H_2O_2$ exposure. Cells were then left to recover in fresh medium for 5 or 10 min at 37 °C. Result of these experiment are shown in **Figure 18**.

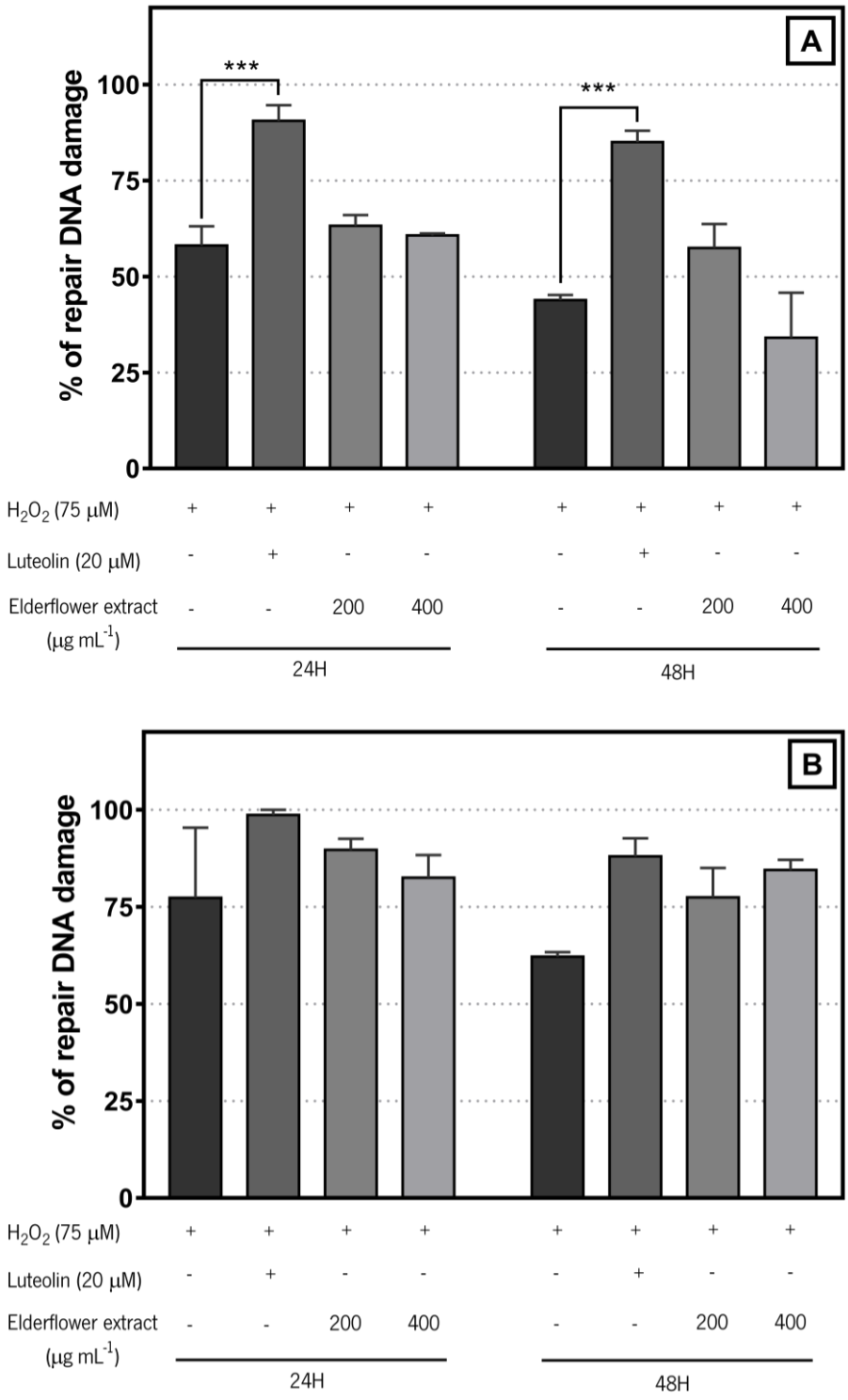


**Figure 18.** Extent of repair of 75 μM $H_2O_2$-induced damage in RKO cells after pre-incubation during 24 and 48 hours with elderflower aqueous extract (200 and 400 μg $mL^{-1}$) and 20 μM L-7-G followed by 5 minutes **(A)** and 10 minutes **(B)** of recovering time. [+] - compound present; [-] - compound absent. Results are expressed as mean ± SD, of at least three independent experiments. Asterisks represent significant difference (*** $p < 0.001$).

For recovery time, 5 and 10 minutes were selected considering the linear phase of SB repair (Supplementary Data VI). As shown in **Figure 18 (A)**, 24 and 48 hours of incubation with elderflower aqueous extract, did not induce any significant repair of DNA in comparison to control, after 5 minutes recovery time. However, after 24 and 48 hours of incubation with L-7-G there was higher % of repair of DNA when compared to the control when incubated for 24 (***$p < 0.001$) and 48 (***$p < 0.001$) hours. When 10 minutes of recovery were allowed, no significant differences were observed for all the conditions tested with elderflower extract, but once again, the positive control L-7-G, was the condition responsible for ensuring higher levels of DNA repair (**Figure 18 (B)**).

### 3.3.5. Antimicrobial activity of elderflower aqueous extract

Since the earliest times, many plants have been known to exert healing properties against human pathogens due to their secondary metabolite content. Over the past decade, much attention has been placed on the study of phytochemicals for their antibacterial/antifungal activity (Borges *et al.*, 2015). However, phytochemicals cannot be used in monotherapy, due to their high minimum inhibitory concentration (MIC) (100–5,000 μg $mL^{-1}$) compared with antibiotics (0.031–512 μg $mL^{-1}$) (Barbieri *et al.*, 2017). Some investigation showed that phytochemicals modulate or modify resistance mechanisms in bacteria, suggesting that phytochemicals can be used in combination with antibiotics to increase the activity and decrease the doses of antibiotic (Santiago *et al.*, 2015).

Determination of minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) is the most common estimation of bactericidal and fungicidal activity. MBC/MFC is defined as the lowest concentration of antimicrobial agent required to kill 99.9% of a population during 24h, under an established set of conditions (CLSI, 2010). Then, MBC can be estimated after a broth macrodilutions, by sub-culturing in non-selective agar plates to determine the number of surviving (CFU $mL^{-1}$) cells after 24 hours of incubation.

The antimicrobial activity of elderflower aqueous extract was evaluated based on MBC and MFC against several pathogen organisms and the results of this experiment are shown in **Table 9**.

**Table 9.** MBC and MFC performance of elderflower aqueous extract against pathogenic organisms. MBC and MFC values are presented in mg $mL^{-1}$. Results are expressed as mean of at least three independent experiments.

| Microbial species | Strain | MBC/MFC (mg $mL^{-1}$) |
|---|---|---|
| **Bacteria** | | |
| *Pseudomonas aeruginosa* | PA01 | > 33 |
| *Klebsiella pneumoniae* | ATCC 11296 | > 33 |
| *Klebsiella oxytoca* | ATCC 13182 | > 33 |
| *Staphylococcus aureus* | ATCC 25923 | 33-8.3 |
| | Clinical isolate | 4.1 |
| *Staphylococcus epidermidis* | ATCC 12228 | 4.1 |
| **Fungi** | | |
| *Candida albicans* | SC 5314 | > 33 |

As presented in **Table 9**, bacterial strains tested *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Staphylococcus aureus* ATCC 25923 (gram-negative bacteria) exhibited values of MBC higher than 33 mg $mL^{-1}$ (Supplementary Data VII). *Staphylococcus aureus* clinically isolated and *Staphylococcus epidermidis* (gram-positive bacteria), demonstrate higher susceptibility compared to the bacterial species previously described, both displaying MBC values of 4.1 mg $mL^{-1}$. The evaluation of MFC in fungi strain *Candida albicans* SC 5314*,* as the majority of bacterial strains tested, exhibited a susceptibility value higher than 33 mg $mL^{-1}$.

# Discussion

# 4. Discussion

Natural products such as plant-derived extracts provide numerous opportunities for new drug discoveries because of an unmatched availability of chemical diversity. In this sense, *S. nigra* has been subjected of enormous interested, and several studies involving this plant showed huge benefits of leaves, flower and fruit to human health (Sidor & Gramza-Michalowska, 2015).

## 4.1. Antioxidant activity and Phenolic Content

Based on that, the aim of this project was to obtain a natural extract from *S. nigra* flowers. Water was considered the most suitable solvent, not only for being non-toxic, but also because it is described as one of the best solvents to produce *S. nigra* extracts with high free radical scavenging activity against DPPH (Duymuş *et al.*, 2014). However, there are several factors that might affect extraction process, such as, plant matrix properties, temperature, pressure and extraction time (Hernández *et al.*, 2009). On this work, the only variable tested was the temperature of extraction, as shown in **Figure 13** and **Figure 14.** The criteria used to choose this parameter was based on the evaluation of antioxidant potential (**Figure 13**) and phenolic content (**Figure 14**) that resulted from the extraction at three different temperatures (50, 70, 90 °C). Based on the results, it was determined that the highest temperature, especially 90 °C, is responsible for the highest antiradical activity ($*p < 0.05$) and the highest phenolic content ($**p < 0.01$). These two parameters might be correlated, once polyphenols are molecules with good scavenging activity and metal chelators due to the hydroxyl groups in their structure, as represented in **Figure 4** (Nicholson *et al.*, 2012). Still, it is important to point out that the phenolic content assessed through this method does not correspond to an absolute measurement of the quantity of phenolics present, but is instead their reducing ability relatively to an equivalent reducing capacity of caffeic acid. These results are in accordance with some studies already reported, indicating that high temperatures tended to increase the polyphenol content (Dawidowicz *et al.*, 2006). This may be due to the thermal destruction of cell walls and subcellular compartments during cooking, favoring the release of these compounds (Juániz *et al.*, 2016). The analysis of DPPH assay revealed identical values with the study performed by Buricová *et al.*, demonstrating a radical scavenging activity of DPPH of elderflower aqueous extract of 60.8 %. Some studies, however, refer that phenolic antioxidants react slowly with DPPH, reaching a steady state of 1-6 hours longer than the usual, suggesting that evaluation of antioxidant activity using DPPH should be performed over time (Bondet *et al.*, 1997). For this reason, antiradical activity measure might be underestimated.

## 4.2. Identification and characterization of elderflower extract

The extraction process was followed by identification and characterization of the aqueous extract of elderflower using two instrumental analytical techniques (GC-MS and HPLC-MS) as it is described in 3.2.

The study of metabolite profiling of the lipophilic fraction of *S. nigra* extract was achieved by GC-MS as shown in **Table 4** and **Table 5**. The low yield of extraction of 0.42% (g $g^{-1}$ of flower) is explained by the extraction methodology, in particularly by the use of water as solvent. A solute will dissolve in a solvent of identical polar property, but two substances of opposing polar properties will not interact, which means that nonpolar molecules, such as fatty acids, will not dissolve in polar substance like water (Stillwell, 2013). By definition, lipid is a molecule that is soluble in solvents with low polarity and insoluble in solvents with high polarity.

The major compound detected on the GC-MS analysis was naringenin, a flavanone, which has received considerable attention regarding its pharmacodynamic activities (Patel *et al.*, 2018). These include, antioxidant activity, through the inhibition of prooxidant enzymes (Wang *et al.*, 2010); anti-inflammatory activity by the activation of transcription factor Nfr2, decreased the formation of reactive species of oxygen and inflammatory mediators (Pinho-Ribeiro *et al.*, 2016). It also has anticancer activity, namely by suppressing TGF-β signaling pathway, inducing a cytotoxic effect in many cell lines, such as colon, pancreas, leukemia, stomach, liver and breast (Kanno *et al.*, 2005; Yang *et al.*, 2011). Additionally, it has been reported anti-diabetic activity, and effects on central nervous and cardiovascular system (Capasso *et al.*, 2006; Li *et al.*, 2006; Yi *et al.*, 2010). Naringenin is freely soluble in organic solvents like ethanol, dimethylformamide and dimethyl sulfoxide, however, in aqueous buffers naringenin is sparingly soluble. It is described in literature that aqueous solubility of naringenin was found to be 475 mg $L^{-1}$ and the log *P* value observed to be 2.42. It explains the lower amounts of naringenin in our extract regarding to some studies already published, where naringenin is present at concentration of 734.15 mg $kg^{-1}$ (methanolic extract) (Mikulic-Petkovsek *et al.*, 2015).

The second family of molecules most represent in GC-MS data was a miscellaneous compounds. This family is mainly constituted by vinclozolin isomers, a dicarboximide fungicide commonly used in agricultural applications. Due to their structure, it is possible that this compound might be added during cultivation process of *S. nigra* (Sato *et al.*, 2016). It is also recognized as an androgen antagonist (antagonize the action the biosynthesis or actions of androgens) (Crews *et al.*, 2014). It has been associated to the induction of epigenetic transgenerational inheritance of increase susceptibility of disease and to induce transgenerational changes to the epigenome (Anway *et al.*, 2005).

Regarding to the fatty acids, contrary to the expected, only MCFAs and LCFAs were detected upon GC-MS analysis, instead of short chain fatty acids (SCFAs). FAs are molecules with a carboxylic group (-COOH) and a long aliphatic chain. The carboxylic group has the capability to form strong hydrogen bonds, particularly the short members of the family ($C_1$-$C_6$). As the hydrocarbon chain increases there is a loss of solubility (Stillwell, 2013), therefore it would be expected to find higher concentration SCFAs, instead of MCFAs and LCFAs. However, none of the SCFAs were detected in the GC-MS analysis. Unlike MCFAs and LCFAs, the SCFAs are molecules which exhibit a certain solubility in water, since they present a short aliphatic chain, which confers a certain polarity to these molecules (Stillwell, 2013). We suggest that during dichloromethane extraction, SCFAs were maintained in the water layer and did not got transferred to the dichloromethane layer, due to their solubility in water. For this reason, SCFAs do not appear in GC-MS analysis.

However, still remains to explain why MFCAs and LCFAs were detected in aqueous extract by GC-MS analysis, since they are insoluble in water. Amphipathic molecules must satisfy two conflicting natures simultaneously: water solubility and lipid solubility. This is achieved by forming aggregates, more precisely micelles in the case of fatty acids. As fatty acids are added to a water solution, their concentration increases up to a point that represent the limit of solubility in water, called the critical micelle concentration (CMC). As more fatty acids are added beyond this point, they form new micelles, but the concentration of free fatty acids in the water solution remains constant. CMC value will depend of the size aliphatic chain. Heavy fatty acids will have tendency to form micellar structures at lower concentrations. For example, octanoate (9:0) (CMC = 400 mM) is very soluble in water compared to palmitate (16:0) (CMC = 0.002 mM). The size of micelles will depend on the composition and concentration, but typically ranges from 2 nm to 20 nm. The size of these micelles is way lower than the pore of the filter (0.22 μm) used to separate the solid from liquid fraction. For this reason, these aggregates could easily escape to the filtration process, which might explain why they were detected upon GC-MS analysis.

The polyphenolic profile of *S. nigra* infusion (**Table 6** and **Table 7**) assessed by HPLC-MS is consistent with literature, in particular the highest content of chlorogenic acid and rutin were observed followed by the other polyphenolic compounds. The exact concentration of these compounds is, however, strongly dependent of extraneous influences (e.g. culture, place of origin, treatment of the sample). For example, the concentrations of flavonoids and their glycosides determined in the inflorescence samples of *S. nigra* present by Barros et al. are in agreement with our present results, however, the concentration levels of phenolic acids (especially isomers of chlorogenic acid) determined in our work are higher, while

the concentration levels of flavonoid rutin are lower (Barros *et al.*, 2012; Dadáková *et al.*, 2010). The composition of *S. nigra* aqueous extract demonstrates that it might be a source of valuable bioactive compounds, and particularly of phenolic acids and flavonols. Indeed, chlorogenic acid and quercetin-3-rutinoside, are both deeply studied on diverse biological activities (Ganeshpurkar & Saluja, 2017; Naveed *et al.*, 2018).

## 4.3. *In vitro* analysis

*S. nigra* extract was tested on five different cell lines as shown in **Table 8**. Several compounds detected in *S. nigra* extract by GC-MS and HPLC-MS have implication in cancer therapy as chemopreventive agents, namely quercetin, caffeoylquinic acid and kaempferol (**Table 7**). These compounds and its derivates are known to exert growth inhibitory effects in colon cancer cells by decreasing tumor growth and suppressing cell survival or proliferation rate through induction of apoptosis or autophagy (Chiang *et al.*, 2014; Kee *et al.*, 2016; Lee *et al.*, 2014). Although, analysis of the proliferation activity, revealed that *S. nigra* extract exhibited low levels of antiproliferative activity, either for non-tumoral cell lines (NCTC 2544 and RAW 264.7), or for tumoral colon cell lines (RKO, HCT-116, Caco-2), compared to other extracts (Benarba & Pandiella, 2018). These results are in accordance with some studies regarding to the anticancer properties of *S. nigra (Thole et al., 2006)*. However, some studies reported that elderberry leaves and fruits extracts moderately inhibited tumor growth in colorectal cancer cells (HT29) and leukemia, being these antitumor properties associated with the polyphenol content (Goun *et al.*, 2002; Jing *et al.*, 2008).

The analysis of antiproliferative activity was followed by the study of epigenetic activity of *S. nigra* extract on *MLH1* demethylation as an alternative to 5-azaC. The results presented in **Figure 16** showed a significant synergistic effect between 5-FU (chemotherapy drug) and 5-azaC (demethylating agent), known by its ability of revert epigenetic silencing of *MLH1* (Hinoi *et al.*, 2003). This effect could be more pronounced by adjusting times of incubation of 5-azaC to ensure that *MLH1* is demethylated when co-incubate with 5-FU. As soon as 5-azaC is metabolized, *MLH1* returns to the initially quiescent state. Furthermore, 5-azaC is unstable in aqueous solution because of the instability of 5-azacytosine nucleobase (Stresemann & Lyko, 2008). When we substituted 5-azaC by elderflower extract, no significant difference was observed, being the decrease of cell viability, mainly due to 5-FU cytotoxicity, since no significant difference was noticed between 5-FU and the group treated with 5-FU and elderflower extract. It is also observed that this effect is not altered in dose-dependent manner of *S. nigra* extract. These

results may point that elderflower extract does not possess epigenetic activity on demethylation of *MLH1* in spite of the high content of quercetins. However, some studies refered that prior to absorption, quercetin glycosides (i.e quercetin glucosides, quercetin galatactoside, quercetin arabinoside) are deglycosylated to quercetin aglycone by the lactase phlorizin hydrolase (LPH), a β-glucosidase residing in the brush border (Arts *et al.*, 2004; Németh *et al.*, 2003). Regarding to quercetin-3-rutinoside, the main source of quercetin in *S. nigra* extract, is absorbed in the colon following deglycosylation which seem to be mediated by gut microbiota-derived β-glucosidase that generates quercetin aglycone facilitating its colonic absorption (Jaganath *et al.*, 2006; Kim *et al.*, 1998) (Supplementary Data IX). The absence of this metabolization process in our assay, suggest that rutin may not have the ability to diffuse passively through the membrane, which impossibilities its action as a demethylating agent of *MLH1*.

Oxidant agents can induce severe genotoxicity and cytotoxicity as well. Regarding to hydrogen peroxide, although the chemical by itself does not have properties of a radical, it is a potential source of dangerous radical HO·, which can attack DNA at sugar residue leading to fragmentation, base loss and strand breaks with a terminal sugar residue fragment (Halliwell & Aruoma, 1991). The antioxidative potential of *S. nigra* has been demonstrated before by several studies (Dawidowicz *et al.*, 2006; Stoilova *et al.*, 2007). This property has also been suggested to be involved in DNA-damage inhibitory effect on the mutagenicity produced by agents with oxidative potential, such as naringin (or naringenin - when naringin is administrated orally, it is hydrolyzed to naringenin which is the major absorbable metabolite (Kim *et al.*, 1998)) (Jagetia *et al.*, 2007). In this sense, another bioactive property tested using *S. nigra* extract was the antigenotoxicity. With the comet assay we observed a strong DNA damaging effect of *S. nigra* extract, similar to the control, suggesting that extract (200 and 400 μg $mL^{-1}$) tested did not protect RKO cells from the radical activity of $H_2O_2$ (**Figure 17**), contrary to the results described by Olejnik *et al.* (Olejnik *et al.*, 2016), while L-7-G (positive control) exhibited a significant protection of DNA (** $p < 0.01$) for times of incubation of 24 and 48 hours. There are several variables that might had influence in this data. First, the action of chlorogenic acid, the abundant present metabolite in elderflower extract. Besides this compound being known by its antioxidant properties (Kono *et al.*, 1997), some studies refer chlorogenic acid induces genotoxic effects (Burgos-Morón *et al.*, 2012; Li & Trush, 1994). However, currently, it is unclear if chlorogenic acid can prevent or induce DNA damage. Second, it is also important to consider that the physicochemical properties of flavonoids determine their characteristics of absorption. In the absence of active transport systems, bioactive compounds must diffuse passively across biological membranes. Only molecules with appropriate lipophilicity can diffuse across membrane phospholipidic membrane. Those too hydrophilic are unavailable to the cell interior, and those to

hydrophobic unable to react in aqueous conditions. Several studies describe that rutin (log*P* = - 0.87) gets absorbed from the colon after removal of the carbohydrate moiety through bacterial enzyme action over it, as it was described before (Gupta *et al.*, 2016; Pool-Zobel *et al.*, 1999). In the case of chlorogenic acid (log*P* = -0.27), they are hydrolyzed by gastric esterease(s) most likely located in the apical membrane releasing hydroxycinnamic acids, which passively diffuses across membrane (Farrell *et al.*, 2011). L-7-G, is highly lipophilic (log*P* = 0.58), instead of glycoside and rutinoside conjugates, which had lower partition coefficient (*log P*) values. The addition of a sugar moiety give rise to hydrophilicity (Rothwell *et al.*, 2005). In this context, the protection of cells may occur through extracellular reduction of exposure to oxidative and carcinogenic factors, which indirectly, might also protect intracellular state, however, it is not observed by this assay (Olejnik *et al.*, 2016; Pool-Zobel *et al.*, 1999).

DNA damage combined with defects in repairing oxidative damage to DNA has been associated to several diseases including cancer (Loft & Møller, 2006; Mena *et al.*, 2009). Cells have multiple DNA repair pathways for specific class of lesions that mitigate the deleterious consequences of damage accumulation. Effects of natural compounds on DNA repair are still poorly understood; some reports show that polyphenols such as quercetin, present in elderflower extract, increase DNA repair activity (Ramos *et al.*, 2008). In our present study, 24 and 48 hours of pre-treatment with *S. nigra* extract did not showed increased rate of rejoining of strand breaks considering the 5 and 10 minutes of recovering time in RKO cells treated with $H_2O_2$ during 5 min (**Figure 18**). These results can be explained based on the antigenotoxicity data shown in **Figure 17**. Once again L-7-G, due to its lipophilicity, can easily diffuse through cytoplasmatic membrane, promoting an efficient repair. *S. nigra* compounds due to their hydrophilicity, cannot permeate cellular membrane, which impossibilities the enhancement of rejoining strand breaks.

The flavonoids are known for their antioxidant, anti-inflammatory, antiallergic, anticancer and antifungal properties. However, since plants synthetize flavonoids in response to microbial infection, there is a growing interest about the antibacterial properties of flavonoids and their application in the therapy for human diseases (Perumal Samy & Gopalakrishnakone, 2010). Among flavonoids, some flavonols compounds, such as quercetin (Hossion & Sasaki, 2013; Liu *et al.*, 2010) and kaempferol have shown antimicrobial activity against gram-negative and gram-positive bacteria, and so the flavanone naringenin (Górniak *et al.*). Furthermore, so phenolic acids have recently gained substantial attention due to their various practical, biological and pharmacological effect. In this group, chlorogenic acid, a majority compound of *S. nigra* infusion, has various antimicrobial effects described. Several studies indicate that chlorogenic acid has bacterial effects against *Klebsiella pneumoniae* (Bajko *et al.*, 2016), *Staphylococcus*

*epidermidis* (Fu *et al.*, 2016) and *Staphylococcus aureus* (Sousa *et al.*, 2014). On the other hand, phenolic compounds such as chlorogenic acid are not sensitive against probiotic bacteria, which makes them even more appropriate to use in food industry (Puupponen-Pimiä *et al.*, 2001). Chlorogenic acid can also exert antifungal effect against *Candida albicans* by having an impact on fungi's cell membrane (Sung & Lee, 2010). For this reason, we went to study antimicrobial properties of *S. nigra* infusion. MBC was determined to *Staphylococcus aureus* e *Staphylococcus epidermidis* (**Table 9**). These results might be justified by the action of kaempferol and quercetin, described in literature as polyphenols active against this bacterial strain (Huang *et al.*, 2015), and so by the action of chlorogenic acid. Another strains which was expected to determined MBC was *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, described by some studies as sensitive to the action of quercetin and chlorogenic acid, and naringenin, respectively (Barbieri *et al.*, 2017). The mechanism by which *S. nigra* metabolites affect namely *Staphylococcus aureus* clinically isolated and *epidermidis* is not described, however, several studies demonstrated that naringenin, quercetin and kaempferol are associated with membrane disruption, inhibition of ability to form biofilms among other effects as described in Supplementary Data VIII (Górniak *et al.*, 2018). Interestingly, would be expected a lower MFC of *Candida albicans*, since it is described that MIC of chlorogenic acid to this fungi strain is 80 μg $mL^{-1}$ (Sung & Lee, 2010) and in our extract a concentration of 33 mg $mL^{-1}$ presuppose concentration of 246 μg $mL^{-1}$ of chlorogenic acid.

# Conclusions and Future Perspectives

# 5. Conclusions and Future Perspectives

Medicinal plant extracts are a natural source of phytochemicals with health benefits, being often acknowledged by their antioxidant, anticancer and antigenotoxic features. The objective carried out during this MSc thesis was to produce a natural extract using *S. nigra* flowers followed by chemical characterization and evaluation of its biological properties, in order to valorize a product that otherwise is considered a waste. The main conclusions of this work are as follow:

- The first task of this work was to determine which temperature is responsible for ensure higher radical scavenging activity and higher phenolic content. Our result indicate that highest the extraction temperatures the highest is the radical scavenging activity and higher phenolic content as shown in **Figure 13** and **Figure 14**.

- The second task of this work was to chemically characterize *S. nigra* aqueous extract using GC-MS and HPLC-MS. The lipophilic fraction (dichloromethane extractives) of *S. nigra* aqueous extract presented a lipophilic profile, mainly composed by flavonoids, followed by miscellaneous compounds. In this context, naringenin was the most represented metabolite of lipophilic extractives. The hydrophilic fraction (water extractives) of *S. nigra* aqueous extract presented a hydrophilic profile mainly composed by phenolic acids and flavonols families, where chlorogenic acids and quercetins were the most represented of each family, respectively. This study, allowed the identification of several relevant bioactive compounds in the elderflower extract, indicating that it is a valuable source of phenolic acid, flavonol and flavonoid compounds.

- The third task of this work was to assess the bioactive properties of *S. nigra in vitro*. We observed that elderflower extract exhibited low antiproliferative activity, namely against tumoral cell lines. Regarding to epigenetic demethylation of *MLH1* was observed that *S. nigra* infusion do not possess any demethylation activity. It possibly occurs once the majority of compounds with demethylation activity (quercetins) in the studied extract are lipophobic. The antigenotoxicity of *S. nigra* extract was investigated again oxidative stress in RKO cells, where the pre-treatment with elderflower extract did not showed to prevent the $H_2O_2$-induced oxidative DNA damage. The same way, it is possible that chlorogenic acid and rutin, the major compounds of *S. nigra* extract, cannot either permeate cellular membrane, and for this reason do not exert any antigenotoxic activity. Regarding to antimicrobial activity of elderflower infusion, the only

bacterial strains susceptible to the action of it was *Staphylococcus aureus* clinically isolated and *Staphylococcus epidermidis*

Considering the future research of this project, there are considerations which may add value to this work. It is important to notice that *S. nigra* flowers are just a valuable product of *Sambucus* tree. In this particular case, *S. nigra* berries have also a rich composition in polyphenols compounds, namely anthocyanins (Vlachojannis *et al.*, 2015), recognized by its antioxidant and anti-inflammatory properties (Miguel, 2011). For this reason, formulate an extract based on *S. nigra* berries, or the combination *S. nigra* flowers and berries can lead to higher radical scavenging activities due to the presence of a wider range of compounds. Additionally, the use of different solvents and methods of extraction, may allow the recovery of compounds with different polarities, could also cause a variation in the antioxidant properties of *S. nigra* extract.

Considering the results obtained to aqueous extraction, higher temperatures (>90 °C) may be considered, since it was observed a correlation between the temperature of infusion and antioxidant activity/phenolic content, and so the adjustment of the time of extraction. Furthermore, in order to obtain a more representative fraction of lipophilic content, an enzymatic-assisted extraction should be tested (Rosenthal et al., 2001).

To evaluate if the digestive process could influence the protective properties of *S. nigra* and to infer if its components reach the colon, the human digestion of *S. nigra* should be replicated *in vitro* (gastric and small intestine digestion), to produce a digested extract of *S. nigra* (Olejnik *et al.*, 2016). After ingestion, the phytochemicals are probable to suffer structural changes during digestive process, which might alter their function once the antioxidant properties depend largely on their structure and hydroxyl groups arrangements (Loganayaki *et al.*, 2013). The absorption of flavonoids in their native form cannot occur until they are hydrolyzed by intestinal enzymes or bio-transformed by colonic microbiota (glucosidase, esterase, dihydroxylation, demethylation, and decarboxylation) (Manach *et al.*, 2004). Thus, only aglycones and some glycosides can be absorbed. Moreover, *S. nigra* extract could be exposed to faecal fermentation in order to study the interactions between the polyphenolic molecules and the colonic microbiota, since the therapeutic effects of *S. nigra* extract are expected to be induced by metabolites bio-transformed by its ecological community (Tan *et al.*, 2015).

Since *S. nigra* extract digested is expected to activate and improve the response to oxidative stress and promote DNA repair, comet assay should be complemented with an evaluation of the levels of expression of genes implicated in antioxidant defenses and in oxidative damage repair pathways (e.g. catalase, glutathione peroxidase, APE1, Ligase I and III). Thus, the mechanism of antigenotoxicity could

be studied through studies of gene expression (RT-PCR, DNA microarray) and through the measuring of the corresponding proteins by Western Blot analysis.

Besides, the modulation of NF-κB by *S. nigra* extract in response to oxidative stress could be investigated by Western Blot analysis, studying the protein levels of NF-κB and its targets (po-inflammatory proteins), as well as of its inhibitory protein, preventing the activation of NF-κB.

The acquired scientific knowledge demonstrates that elderflower aqueous extract can be potentially exploited as a source of bioactive compounds with potential health benefits, however, many questions remain unanswered whereby more research is needed.

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# SUPPLEMENTARY DATA

## Supplementary Data I – GC calibration curve

$$\frac{A_A}{[A]} = F\frac{A_S}{[S]} \qquad \textbf{(Equation 3)}$$

$A_A$ – Area of Analyte

$[A]$ – Concentration of Analyte

$A_S$ – Area of Internal Standard

$[S]$ – Concentration of Internal Standard

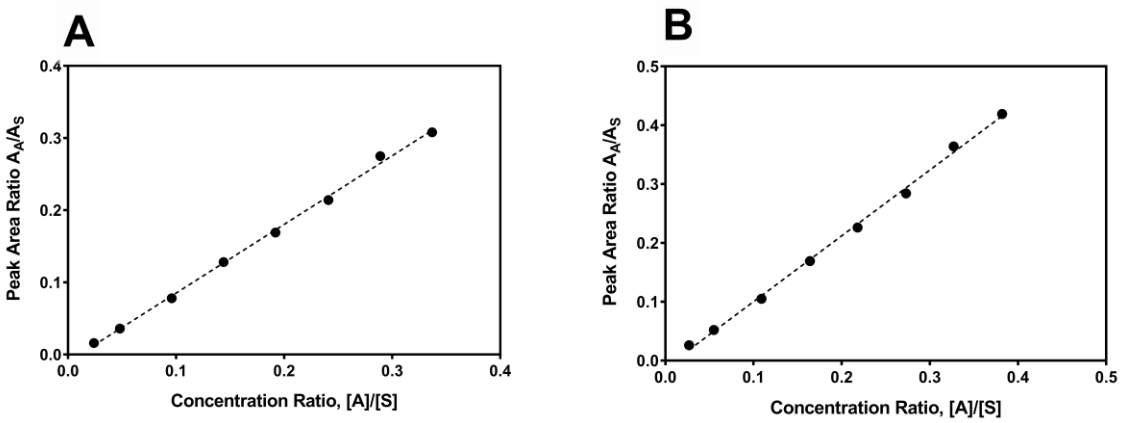


**Figure I. A.** GC-MS calibration curve of ferulic acid determined using tetracosane as an internal standard where, *Peak Area Ratio = 0.9528 • Concentration – 0.0104*. The coefficient of determination $R^2$ is ≈ 0.998. **B.** GC-MS calibration curve of palmitic acid determined using tetracosane as an internal standard, where *Peak Area Ratio = 1.1197 • Concentration – 0.012*. The coefficient of determination $R^2$ is ≈ 0.998.

## Supplementary Data II – HPLC calibration curve of rutin

| Rutin | 50 | µg.ml$^{-1}$ |
|---|---|---|

| | Rutin (360 nm) | | | |
|---|---|---|---|---|
| **Sample** | **$V_i$ (ml)** | **$V_f$ (ml)** | **C (µg.ml$^{-1}$)** | **Peak Area** |
| **G** | 0,48 | 0,5 | 48 | 314298 |
| **F** | 0,4 | 0,5 | 40 | 247292 |
| **E** | 0,3 | 0,5 | 30 | 198599 |
| **D** | 0,2 | 0,5 | 20 | 126524 |
| **C** | 0,1 | 0,5 | 10 | 69241 |
| **B** | 0,05 | 0,5 | 5 | 43379 |
| **A** | 0,01 | 0,5 | 1 | 8899 |

| | |
|---|---|
| 6867,73 | **STEYX** |
| 6284,00 | **SLOPE** |
| 3,28 | **LOD** |
| 10,93 | **LOQ** |

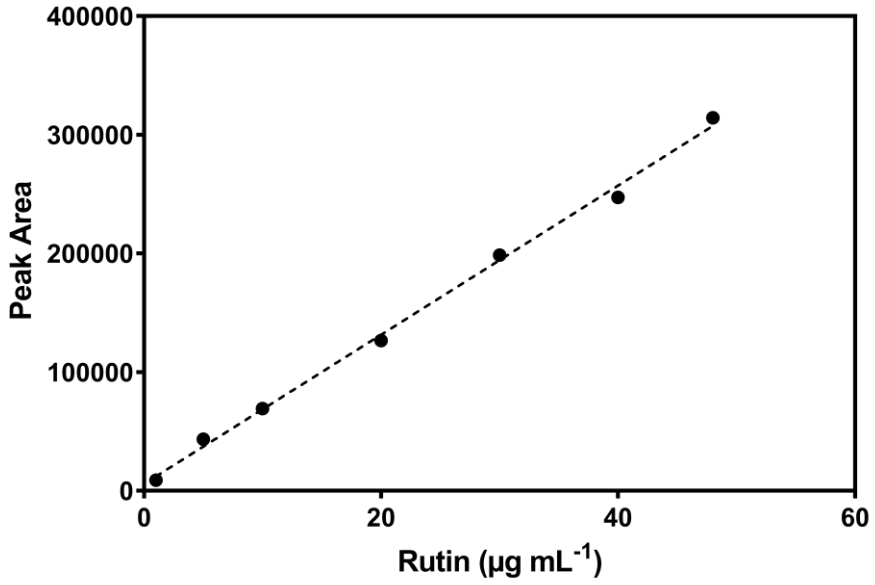


**Figure II.** HPLC-MS calibration curve of rutin based on the concentrations represented in the table above, where *Peak Area = 6284 • Concentration –5,785.1*. The coefficient of determination ($R^2$) is ≈ 0.997.

## Supplementary Data III – HPLC calibration curve of chlorogenic acid

| **Chlorogenic Acid** | 500 | **µg mL$^{-1}$** |
|---|---|---|

| | **Chlorogenic acid (320 nm)** | | | |
|---|---|---|---|---|
| **Sample** | **$V_i$ (ml)** | **$V_f$ (ml)** | **C (µg.ml$^{-1}$)** | **Peak Area** |
| **G** | 0,02 | 0,5 | 20 | 573496 |
| **F** | 0,05 | 0,5 | 50 | 1417089 |
| **E** | 0,04 | 0,5 | 40 | 1087178 |
| **D** | 0,03 | 0,5 | 30 | 832233 |
| **C** | 0,01 | 0,5 | 10 | 304730 |
| **B** | 0,005 | 0,5 | 5 | 91348 |
| **A** | 0,001 | 0,5 | 1 | 30478 |

| | |
|---|---|
| 29716,6 | **STEYX** |
| 28139,5 | **SLOPE** |
| 3,2 | **LOD** |
| 10,6 | **LOQ** |

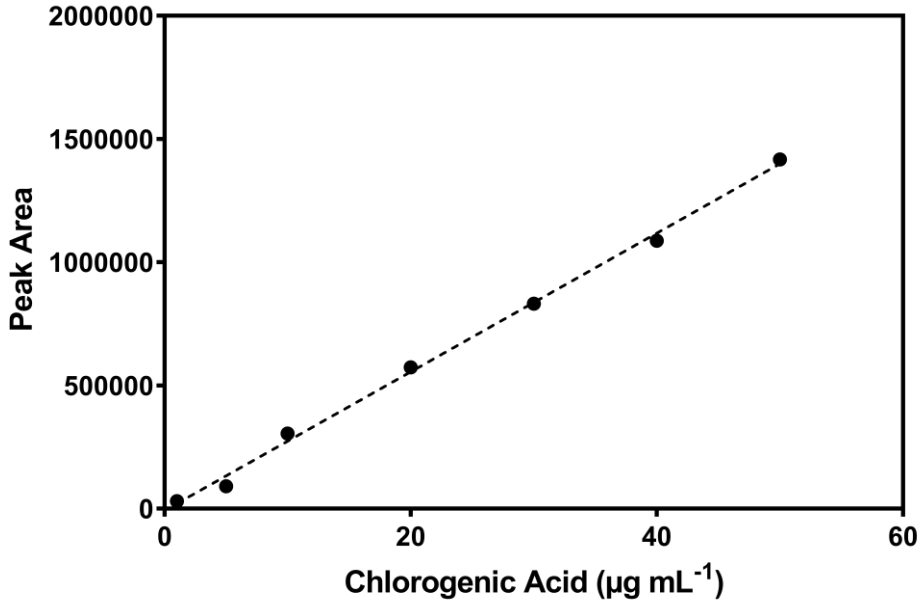


**Figure III.** HPLC-MS calibration curve of chlorogenic acid based on the concentrations represented in the table above, where *Peak Area = 28,139 • Concentration –7,600.8*. The coefficient of determination ($R^2$) is ≈ 0.997.

## Supplementary Data IV – Antiproliferative activity by MTT assay

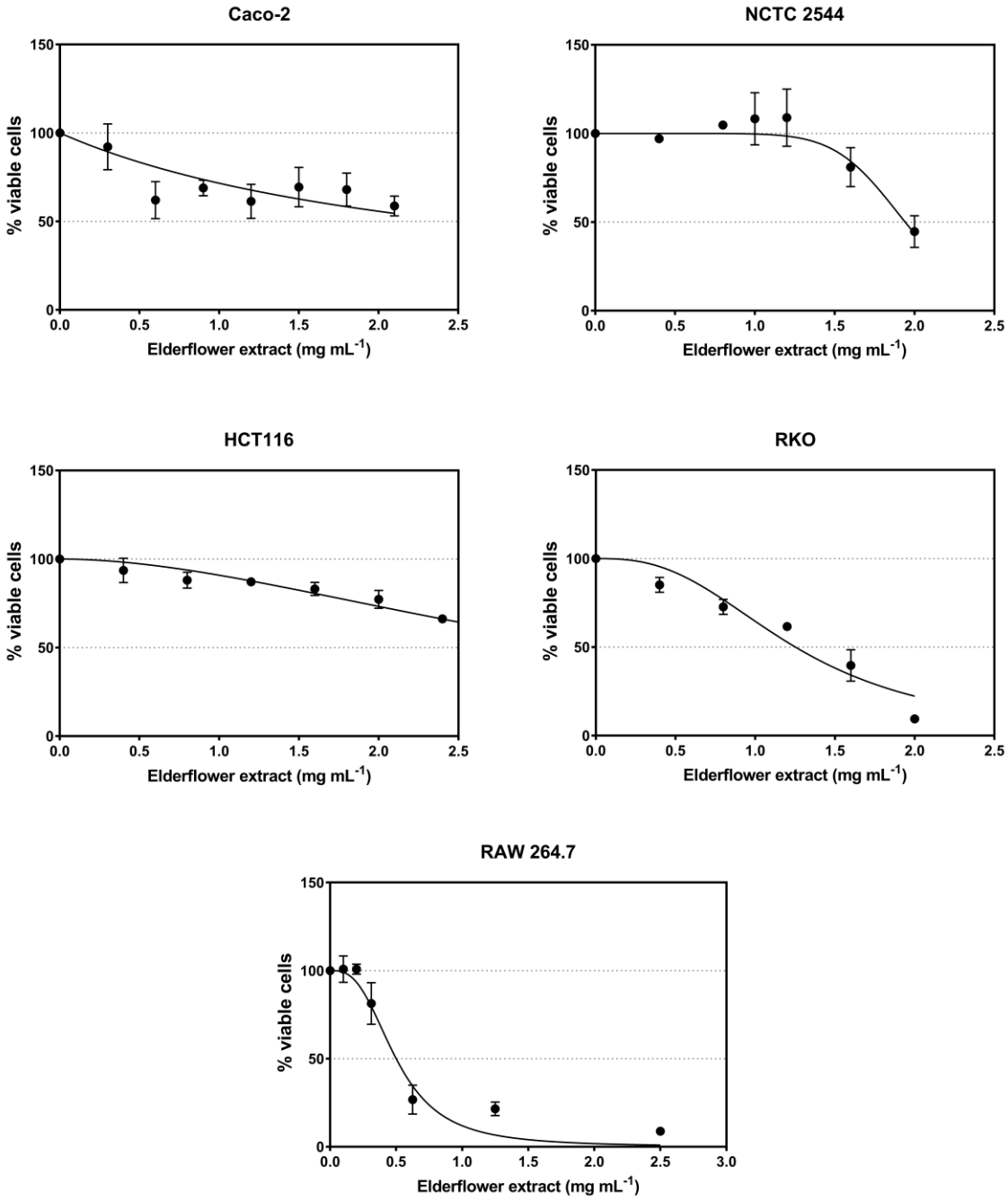


**Figure IV.** Antiproliferative activity estimated by MTT assay of aqueous elderflower extract against several cell lines. Cell lines: murine macrophage cell line RAW 264.7, human epithelial cell line Caco-2, human keratinocyte cell line NCTC 2544, human colon cancer cell line HCT116, human colon carcinoma cell line RKO. Data are expressed as mean ± S.D. (n=3).

## Supplementary Data V - Kinetics of attenuation of $H_2O_2$-induced DNA damage on RKO cells

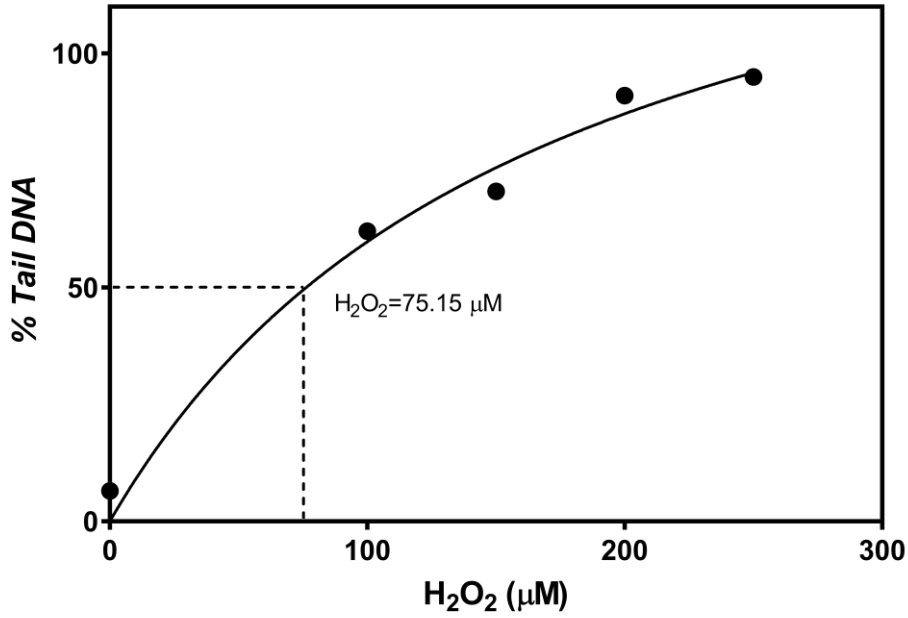


**Figure V.** Evaluation of genotoxic effects with different concentrations of $H_2O_2$ on RKO cells, and determination of 50% of tail DNA concentration by interpolation of standard curve. Value estimate of $H_2O_2$ was 75.15 μM. Results are expressed as a single value.

## Supplementary Data VI - Kinetics of SBs rejoining using $H_2O_2$

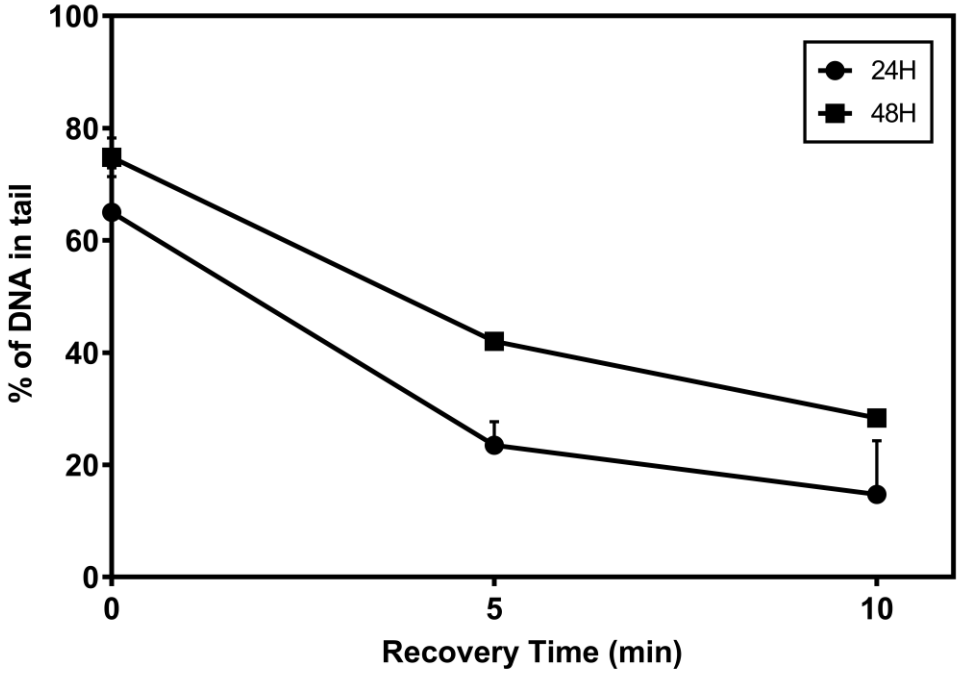


**Figure VI.** Kinects of rejoin strand breaks using $H_2O_2$ as genotoxic agent for 24 and 48 hours of incubation with fresh medium. Results are present as mean ± SD, of at least three independent experiments.

## Supplementary Data VII – Yield of elderflower aqueous extract

Extraction November of 2017

| | Sample | Massa falcon (g) | Massa final (g) | massa | mg mL$^{-1}$ | Média (g L$^{-1}$) |
|---|---|---|---|---|---|---|
| **Erlenmeyer 1** | 1.1 | 10,61 | 10,88 | 0,27 | 10,67 | 10,63 |
| | 1.2 | 10,63 | – | – | – | |
| | 1.3 | 10,88 | 11,14 | 0,26 | 10,58 | |
| | 1.4 | 10,64 | 10,91 | 0,27 | 10,64 | |
| **Erlenmeyer 2** | 2.1 | 10,95 | 11,21 | 0,26 | 10,21 | 10,39 |
| | 2.2 | 10,71 | 10,97 | 0,26 | 10,29 | |
| | 2.3 | 10,87 | 11,13 | 0,26 | 10,41 | |
| | 2.4 | 10,77 | 11,04 | 0,27 | 10,64 | |
| **Erlenmeyer 3** | 3.1 | 10,77 | 11,02 | 0,25 | 9,87 | 9,70 |
| | 3.2 | 10,55 | 10,81 | 0,25 | 10,15 | |
| | 3.4 | 10,62 | 10,87 | 0,26 | 10,24 | |
| | 3.4 | 10,95 | 11,17 | 0,21 | 8,55 | |

| Extração flor (g L$^{-1}$) |
|---|
| **5/150** |
| 33,33 |

| Média (g L$^{-1}$) |
|---|
| 10,24 |

Extraction February of 2018

| | Sample | Massa falcon (g) | Massa final (g) | massa | mg mL$^{-1}$ | Média (g L$^{-1}$) |
|---|---|---|---|---|---|---|
| **Erlenmeyer 1** | 1.1 | 10,58 | 10,83 | 0,25 | 9,97 | 9,99 |
| | 1.2 | 10,56 | 10,81 | 0,25 | 10,08 | |
| | 1.3 | 10,68 | 10,93 | 0,25 | 10,01 | |
| | 1.4 | 10,70 | – | – | – | |
| **Erlenmeyer 2** | 2.1 | 10,62 | 10,86 | 0,24 | 9,40 | 9,45 |
| | 2.2 | 10,61 | 10,84 | 0,24 | 9,49 | |
| | 2.3 | 10,69 | 10,93 | 0,24 | 9,45 | |
| | 2.4 | 10,59 | – | – | – | |
| **Erlenmeyer 3** | 3.1 | 11,00 | 11,25 | 0,25 | 10,09 | 10,13 |
| | 3.2 | 10,56 | 10,81 | 0,25 | 10,20 | |
| | 3.4 | 10,62 | 10,87 | 0,25 | 10,11 | |
| | 3.4 | 10,94 | – | – | – | |

| Extração flor (g L$^{-1}$) |
|---|
| **5/150** |
| 33,33 |

| fev/18 (g L$^{-1}$) | nov/17 (g L$^{-1}$) |
|---|---|
| 9,86 | 10,24 |

| Média final (g L$^{-1}$) |
|---|
| 10,05 |

| Rendimento extração (%) |
|---|
| 30,14 |

## Supplementary data VIII – Antimicrobial activity of flavonoids

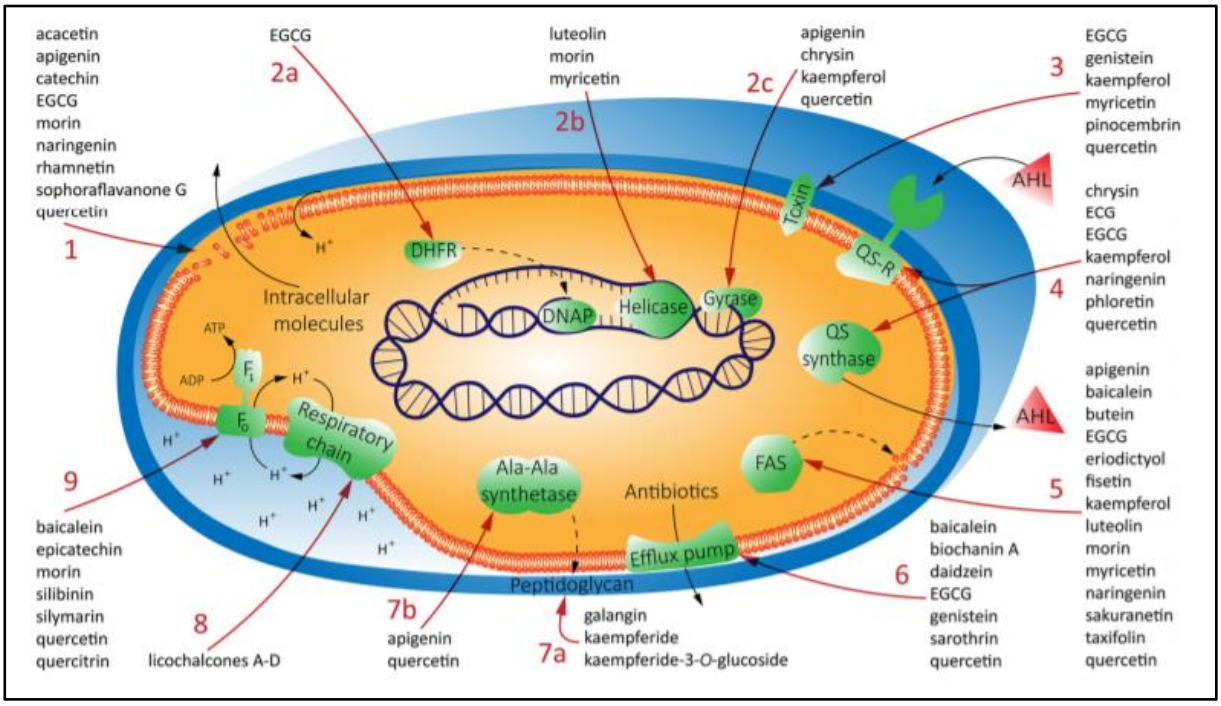


**Figure VII. Schematic representation of mechanism of action of flavonoids. (1)** membrane disruption; **(2)** inhibition of nucleic synthesis; **(3)** inhibition of bacterial virulence; **(4)** inhibition of ability to form biofilms; **(5)** inhibition of fatty acid synthesis; **(6)** inhibition of efflux pumps leading to reversing antimicrobial resistance; **(7)** inhibition of peptidoglycan synthesis; **(8)** inhibition of bacterial respiratory chain by inhibition of NADH-cytochrome c reductase activity; **(9)** and inhibition of ATP-synthase. Adapted from Górniak *et al.* (2018).

## Supplementary Data IX – Absorption of rutin and quercetin

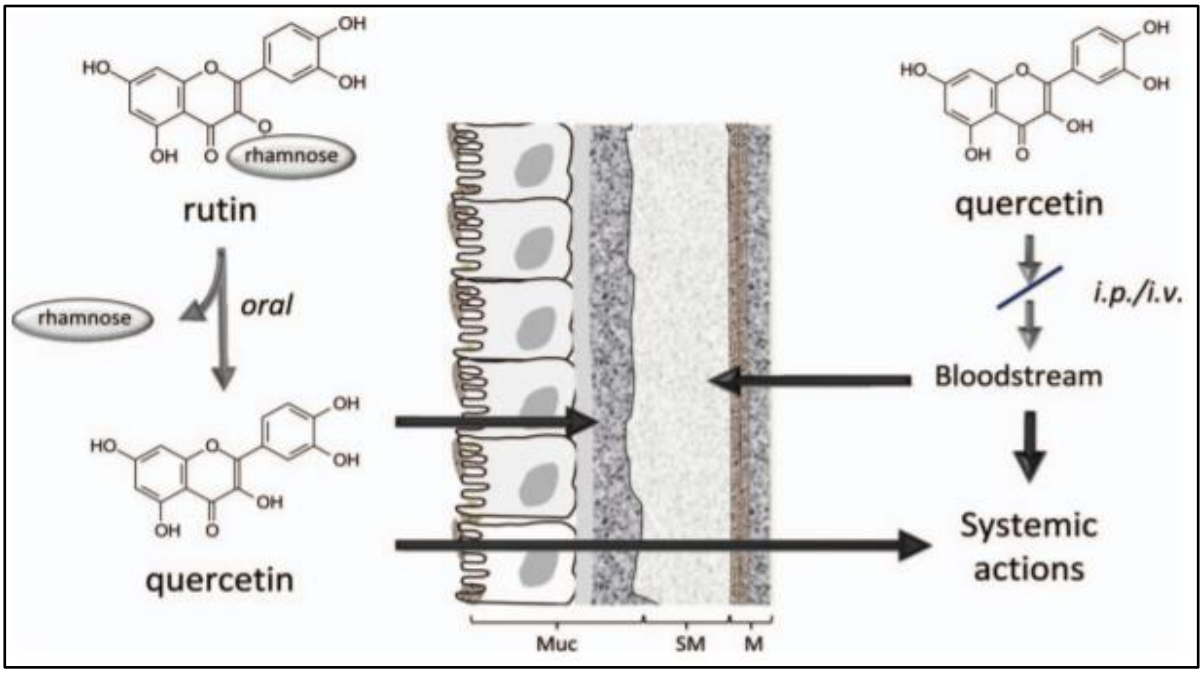


**Figure VIII.** Metabolization of rutin (glycoside hydrolysis) to quercetin to posterior absorption by gut lumen into the bloodstream. Adapted from González *et al.* (2011).

## Supplementary Data X – List of material

**Table I**. Materials, equipment and reagents used in this work.

| Product | Company | Reference |
|---|---|---|
| Acetonitrile | Sigma-Aldrich | 270717 |
| Agarose (Electrophoresis Grade) | NzyTech | MB02702 |
| Agarose, LMP, Analytical Grade | Promega | V2111 |
| Caffeic Acid | Sigma-Aldrich | C0625 |
| Cell line (Caco-2) | Sigma-Aldrich | 86010202 |
| Cell line (HCT-116) | Sigma-Aldrich | 91091005 |
| Cell line (NCTC 2544) | Institute Zooprofilattico di Brescia | – |
| Cell line (RAW 264.7) | Sigma-Aldrich | 91062702 |
| Cell line (RKO) | IPATIMUP | – |
| Dichloromethane | Sigma-Aldrich | 270997 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D8418 |
| DMEM | Sigma-Aldrich | D5648 |
| DPPH | Sigma-Aldrich | D9132 |
| EDTA-$Na_2$ | Sigma-Aldrich | E5134 |
| FBS | Sigma-Aldrich | F2442 |
| Ferulic Acid | Sigma-Aldrich | Y0001013 |
| Folin-Ciocalteu Reagent | Sigma-Aldrich | F9252 |
| GC column (DB-1) | Agilent | DB-1ms |
| GC mass spectrometer | Thermo Fisher Scientific | DSQII |
| GC software | Shimadzu | GCMSsolution Software |
| GC-MS | Shimadzu | QP2010 |
| $H_2O_2$ | Sigma-Aldrich | H1009 |
| HEPES | Sigma-Aldrich | H4034 |
| HPLC | Thermo Fisher Scientific | Finnigan Surveyor HPLC |
| HPLC column (Gemini C18) | Phenomex | 7, 563, 367 |
| HPLC mass spectrometer | Thermo Fisher Scientific | LCQ Deca XP MAX |
| HPLC software | Thermo Fisher Scientific | Excalibur™ |

| | | |
|---|---|---|
| Hypersil GOLD | Thermo Fisher Scientific | 25305-012101 |
| MEM | Sigma-Aldrich | M0643 |
| Microplate Reader | Biotek | Synergy HT |
| Microscope | Leica | DM5000B |
| $Na_2EDTA$ | Sigma-Aldrich | E5134 |
| $NaHCO_3$ | Sigma-Aldrich | S5761 |
| Non-essential Amino Acid Solution | Sigma-Aldrich | M7145 |
| Palmitic Acid | Sigma-Aldrich | P0500 |
| Penicillin/Streptomycin | Sigma-Aldrich | P4333 |
| Penicillin/Streptomycin mix | Sigma-Aldrich | A5955 |
| Pyruvic Acid | Sigma-Aldrich | P2256 |
| RPMI | Sigma-Aldrich | R6504 |
| Sabouraud Dextrose Broth (SDB) | Sigma-Aldrich | S3306 |
| Sodium Hydroxide (NaOH) | Sigma-Aldrich | S5881 |
| SYBR® Gold | Thermo Fisher Scientific | S-11494 |
| Syringe | BD | Plastipak |
| Tetracosane | Sigma-Aldrich | 87089 |
| Tetramethylsilane (TMS) | Sigma-Aldrich | 87921 |
| Thiazolyl Blue tetrazolium bromide (MTT) | Alfa Aesar | L11939 |
| Triton-X | Sigma-Aldrich | T8787 |
| Trolox | Sigma-Aldrich | 238813 |
| Trypsin | Sigma-Aldrich | T4049 |
| Tryptic Soy Broth (TSB) | Liofilchem | 620053 |