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UMinho | 2019 Ana Luísa Fernandes **Protection of plant extracts against the deleterious effects of nitric oxide**

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Protection of plant extracts against the deleterious effects of nitric oxide

Abstract

Reactive oxygen and nitrogen species are essential to the regular function of the organism. However, the imbalance on the homeostatic concentration of these species leads to oxidative stress. Oxidative stress can have a major impact in all cellular macromolecules, such as DNA, protein and lipids. Consequently, major cellular processes can be dysregulated, which can lead to disease development.

The organism has antioxidant mechanisms to deal with oxidative stress. However, often these mechanisms are not enough, and exogenous antioxidants can be helpful in dealing with this issue. Plant extracts are useful sources of antioxidant compounds and can contribute to ameliorate the effects of nitric oxide-induced oxidative stress.

In this work, extracts from *Gingko biloba and Melissa officinalis,* which already showed promising preliminary results against nitric oxide induced stress, were explored in their capacity to improve the nitric oxide effects, using yeast as model organisms. It was expected to see a protection of the extracts when cells were treated with sodium nitroprusside (SNP), the nitric oxide-donor, and extract.

According to the results obtained, each extract by itself did not present toxicity for the cells. However, regarding the concentrations used and the model organism selected, a synergistic effect seems to occur when both the extract and SNP were combined. By fluorescence microscopy, we were able to see some morphological alterations in treatments with *Gingko biloba* extract (GBE) and some abnormal chromatin structure in treatments with *Melissa officinalis* extract (MOE). However, the cell cycle progression did not seem to be affected with MOE assays and with GBE the results were inconclusive. The synergistic effect observed in both extracts could be explained by interferences with cdc2 protein kinase activity and, in order to better understand this effect, more tests should be done in the future, testing different concentrations of the compounds and using mutants for the cdc2 protein to see if the its mechanisms are affect in each situation (GBE and MOE treatments).

Key words:

Gingko biloba, *Melissa officinalis*, nitric oxide, oxidative stress, toxicity.

Proteção de extratos de plantas contra os efeitos nocivos do Óxido Nítrico

Resumo

As espécies reativas de oxigénio e nitrogénio são essenciais para o normal funcionamento do organismo. No entanto, o desequilíbrio na concentração homeostática dessas espécies leva a que ocorra stress oxidativo. O stresse oxidativo pode ter um grande impacto em todas as macromoléculas celulares, tais como o DNA, proteínas e lipídios. Consequentemente, os principais processos celulares podem ser desregulados, o que pode levar ao desenvolvimento de doenças.

O organismo possui mecanismos antioxidantes capazes de lidar com o stresse oxidativo. No entanto, muitas vezes esses mecanismos não são suficientes e a utilização de antioxidantes provenientes de fontes exógenas podem ser úteis para lidar com este problema. Extratos vegetais são fontes úteis de compostos antioxidantes e podem contribuir para melhorar os efeitos do stresse oxidativo induzido pelo óxido nítrico.

Neste trabalho, extratos de *Gingko biloba* e *Melissa officinalis*, que já mostraram resultados preliminares promissores contra o stresse induzido por óxido nítrico, foram explorados na sua capacidade de melhorar os efeitos induzidos pelo NO, utilizando leveduras como organismos modelo, esperando-se ver um efeito protetor pela utilização dos extratos em tratamentos com os mesmos e com nitroprussiato de sódio (SNP), que atua aqui como doador de óxido nítrico.

De acordo com os resultados obtidos, cada extrato por si só não apresenta toxicidade para as células. No entanto, em relação às concentrações utilizadas e ao organismo modelo selecionado, um efeito sinérgico parece ocorrer o extrato e o SNP foram combinados. Por microscopia de fluorescência, observaram-se algumas alterações morfológicas nas células em tratamentos com GBE e ainda anormalidades na forma da cromatina em tratamentos com MOE. No entanto, a progressão do ciclo celular não parece ser afetada nos tratamentos com MOE e com GBE os resultados foram inconclusivos. O efeito sinergístico observado em ambos os extratos aparenta dever-se a interferências com a normal atividade da proteína cinase cdc2., De modo a entender melhor este efeito mais testes deverão ser feitos no futuro, testando diferentes concentrações dos compostos e usando mutantes para cdc2, a fim de ver se os seus mecanismos são afetados em cada situação (tratamentos com GBE e com MOE).

Palavras-chave:

Gingko biloba, *Melissa officinalis*, óxido nítrico, stress oxidativo, toxicidade.

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Abbreviations

DDR - DNA damage response

DPPH - 2,2-diphenyl-1-picrylhydrazyl

eNOS - endothelial NOS

GBE – *Gingko biloba* extract

HX – hypoxanthine

HU - hydroxyurea

iNOS - inducible NOS

MOE – *Melissa officinalis* extract

nNOS - neuronal NOS

RNS - reactive nitrogen species

ROS - reactive oxygen species

SNP - sodium nitroprusside

VEGF2 - vascular endothelial growth factor receptor type2

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Figure 7. Chromatin observation by fluorescence microscopy in *Sch. pombe* cells. Cells from exponentially growing cultures were exposed to YES media (A), 300 mM SNP (B), 1000 μg/mL MOE (C), 100 μg/mL MOE and 300 mM SNP (D), 500 μg/mL MOE and 300 mM SNP (E) or 1000 μg/mL MOE and 300 mM SNP (F), a sample was taken at 0 (T_0), 60 (T_1), 120 (T_2) and 180 (T_3) minutes, added Hoechst fluorochrome 1 μg/mL, fixated and observed in fluorescence microscopy with a 1000x magnification. The photographs are representative of three independent experiments.

Figure 8. Fluorescence microscopy of *Sch. pombe* cells. Cells from exponentially growing cultures were exposed to YES media (A), 300 mM SNP (B), 1000 μg/mL GBE (C), 500 μg/mL GBE and 300 mM SNP (D), 700 μg/mL GBE and 300 mM SNP (E) or 1000 μg/mL GBE and 300 mM SNP (F), a sample was taken at 0 (T_0), 60 (T_1), 120 (T_2) and 180 (T_3) minutes, added Hoechst fluorochrome 1 μg/mL, fixated and observed in fluorescence microscopy with a 1000x magnification. The photographs are representative of three independent experiments.

Figure 9. Cell cycle progression analysis of *Sch. pombe* cells by flow cytometry. Cells were treated with HU to synchronize their cell cycle in S phase (C^{+4}) and compared with an asynchronous culture (C^{-4}). After washing HU, cells were exposed to YES medium (C^{-}), 300 mM SNP (C_{SNP}), 1000 μg/mL MOE(C_{MOE}), 100 μg/mL MOE and 300 mM SNP, 500 μg/mL MOE and 300 mM SNP or 1000 μg/mL MOE and 300 mM SNP. Every 30 min, a sample of each treatment was taken and processed for analysis. During the treatments, the cells were incubated at 30 °C. The images are representative of three independent experiments.

Figure 10. Cell cycle progression analysis of *Sch. pombe* cells treated with GBE by flow cytometry. Cells were treated with hydroxyurea to synchronize their cell cycle in S phase (C^{+4}) and compared with an asynchronous culture (C^{-4}). After washing HU, cells were exposed to YES media (C^{-}), 300 mM SNP (C_{SNP}), 1000 μg/mL GBE(C_{MOE}), 500 μg/mL GBE and 300 mM SNP, 700 μg/mL GBE and 300 mM SNP or 1000 μg/mL GBE and 300 mM SNP. Every 30 min, a sample of each treatment was taken and processed for analysis. During the treatments, the cells were incubated at 30 °C. The images are representative of three independent experiments

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1. Introduction

1.1. DNA damage

Cellular homeostasis is fundamental for the regular function of organisms. However, cells are constantly exposed to threats and agents that disturb their regular function. DNA damage has one of the most serious effects on cellular homeostasis and can lead to genomic instability, mutations, senescence and cell death. DNA damage is defined as modifications in the DNA coding properties or structure and can be caused by exogenous agents, such as chemical agents, or endogenous agents produced in the regular metabolism of the cells, as oxygen and nitrogen reactive species (Martin, 2008). Modifications at the DNA level can lead to deleterious effects to the cell and if not contained, through repair mechanisms or cell elimination, can be replicated and increase the risk of disease development, such as cancer (Torgovnick & Schumacher, 2015). DNA damage activates the DNA damage response (DDR) (Figure 1.), which leads to activation of repair mechanisms and to the activation of cell death pathways in case of failure to repair DNA damages. Malfunction of these mechanisms can cause genomic instability, potentially resulting in higher mutation rates, which is a propeller of carcinogenesis (Jeggo, Pearl, & Carr, 2016).

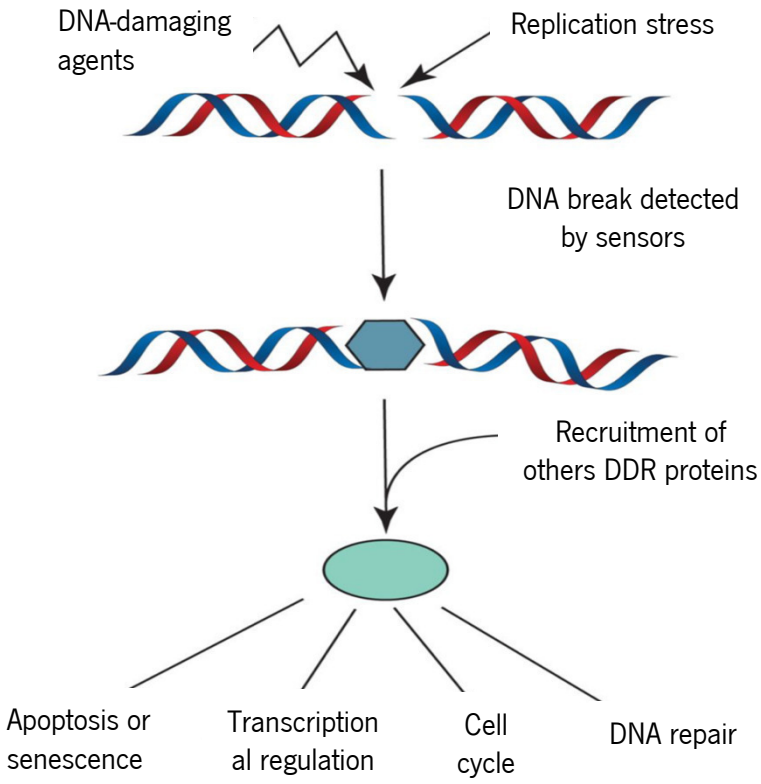


Figure 1. DNA damage response outcome when cells suffer DNA damages (adapted from (Jackson, 2009)

Reactive species such as the reactive oxygen species (ROS) and reactive nitrogen species (RNS) showed in Table 1, are oxidative species that are associated with DNA damage (Domej et al., 2014). Although associated with damage, these species also have a role in the regulation of major cell processes and maintenance of cellular homeostasis (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008). Under regular cell function, they are produced at concentrations that confer an important control of molecular mechanisms such as intracellular signal transduction cascades, modulation of the immune system, apoptosis, and differentiation (Ray, Huang, & Tsuji, 2012). For example, ROS as the superoxide anion and hydrogen peroxide, mediate biological responses such as angiogenesis induction, through the vascular endothelial growth factor receptor type2 (VEGF2) (Domej et al., 2014). Therefore, these species play a double-edge role in the cellular biology and health. ROS and RNS are generated as by-products of metabolism or exposure to natural and synthetic agents. Cells have mechanisms to maintain a healthy balance of ROS and RNS. However, this balance can be dysregulated due to overproduction and/or failure of antioxidant systems, which leads to the oxidative stress.

Table 1. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) involved in DNA damage and some of their characteristics (Domej et al., 2014).

Type	*Name*	*Formula*	*Characteristics*
ROS	Hyperoxide/superoxide	$\cdot O_2^-$	Highly unstable, signaling function, synaptic plasticity
	Hydrogen peroxide	H_2O_2	Cell toxicity, signaling function, generation of others ROS
	Hydroxyl radical	·OH	Free radical, highly unstable, very reactive agent
	Alkoxyl radical	RO·	Free radical, reaction product of lipids
	Peroxyl radical	ROO·	Free radical, reaction product of lipids
	Hypochlorite anion	OCl^-	Reactive oxygen species, reactive chloride species, enzymatically generated by myeloperoxidase
	Ozone	O_3	Environmental toxin
RNS	Nitric oxide	·NO	Environmental toxin, endogenous signal molecule
	Peroxynitrite	$ONOO^-$	Highly reactive reaction intermediate of
	Nitrogen dioxide	$\cdot NO_2$	Highly reactive radical, environmental toxin
	Nitrogen oxides	NO_X	Environmental toxins including NO and

The oxidative stress is caused by dysregulation of oxygen and nitrogen reactive species that can react and thereby damage lipids, proteins, carbohydrates and cause changes in the DNA structure and function (Ray et al., 2012). This can impact inflammation, neurodegenerative and cardiovascular diseases, cancer, as well as being associated with aging (Ray et al., 2012).

ROS and RNS dysregulation can be induced by exogenous stress such as radiation, pollutants, smoking, food, alcohol consumption and drugs (Ray et al., 2012). Ionizing radiation can penetrate the human body and lead to ·OH production, which can cause cerebrovascular, gastrointestinal and hematopoietic damage (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Cancer chemotherapy has toxic side effects, in which ROS production is predominant (Bhattacharyya et al., 2014). Therefore, both radiation and chemotherapy, through oxidative stress, have a large impact in cancer treatment toxicity. Tobacco is associated with multiple diseases, and has been shown to impact gastrointestinal diseases and multiple carcinogenesis (Bhattacharyya et al., 2014). The active chemicals of tobacco include ·NO, and compounds that react to generate oxygen radicals such as ·OH and H_2O_2 (Witschi, 2005). Food is also a source of oxidative stress, dietary iron and copper can contribute to ROS generation through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet$ + / $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$) (Bhattacharyya et al., 2014). Foods with trans fatty acids can also promote ROS, through the presence of compounds such as acrylamide (Bergmark, Calleman, He, & Costa, 1993). Furthermore, lipids when heated in microwave ovens generate free radicals (Bhattacharyya et al., 2014). Alcohol intake is associated with damage to the gastrointestinal tract, as well as various cancers due to ethanol-generated reactive species, such as alcohol induced NO synthesis (Tang et al., 2009). Moreover, many drugs and xenobiotics lead to the formation of free radicals in the organism, such as anticancer drugs. Furthermore, commonly used anti-inflammatory and antipyretic drugs, such aspirin, and non-steroidal anti-inflammatory drugs such as ibuprofen can generate ROS (Diaz de Barboza, Guizzardi, Moine, & Tolosa de Talamoni, 2017).

ROS and RNS are also generated endogenously through cells regular metabolism. One of the main organelle contributing to this is the mitochondrion (Incalza et al., 2017). The oxidative species are formed in mitochondria when electrons, to produce ATP, move through the respiratory chain, involving in this process five different complexes, two shuttles and redox reactions (Incalza et al., 2017). When the electrons flow in these complexes, protons are released into to the mitochondrial intermembrane space producing an electrochemical gradient. The oxygen involved in this process, as the last electron acceptor, is reduced to water. However, part of the total O_2 is partially reduced to superoxide anion, as electrons leak from the complexes before completing the process (Incalza et al., 2017). Moreover, these species

are also produced in phagocytic cells, vascular walls and in tissues by several enzymes, namely cyclo-oxygenases, lipo-oxygenases, myeloperoxidase, NAD(P)H oxidase, xanthine oxidase, and nitric oxide synthase (NOS) (Incalza et al., 2017). Phagocytic cells consume large amounts of oxygen during phagocytosis, mainly through activation of NADPH oxidase and release of $\cdot O_2^-$. Xanthine oxidase is expressed mainly in the liver and small intestinal mucosa. This enzyme catalyzes the oxidation of hypoxanthine (HX) to xanthine and then, to uric acid during purine catabolism, H_2O_2 and $\cdot O_2^-$ are generated during this oxidation. This pathway is one of the most important sources of oxidative stress during an injury (Incalza et al., 2017). NOS is a heme-containing monooxygenase that generates nitric oxide (NO).

1.2. Nitric oxide

NO is a weak oxidant, however the reaction with $\cdot O_2^-$ can generate $ONOO^-$ a potent reactive species. Furthermore, these molecules, ·NO and $ONOO^-$, generate very stable nitrite (NO_2^-) and nitrate (NO_3^-) ions which accumulate in cells, and lead to the formation of highly reactive intermediates, such as $NO_2^{\cdot}$ or N_2O_3· (Forstermann & Sessa, 2012; Kaneko et al., 2008).

NOS generates NO using L-arginine as a substrate and O_2 and NADPH as co-substrates. At the heme site of the enzyme the O_2 is reduced and L-arginine is oxidized to produce L-citrulline and NO (Forstermann & Sessa, 2012). NOS has three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Forstermann & Sessa, 2012).

NO is an endogenously synthesized free radical that plays an important role contributing to key functions in the organism. NO acts as a gasotransmitter, which modulates cardiovascular homeostasis, immunological and neurological functions, muscles relaxation of the vascular tissue and affects gene transcription and mRNA translation (Forstermann & Sessa, 2012). This transmitter has the capacity to easily permeate cellular membranes and act on intracellular targets due to its small size, instability, and high lipophilicity (Asiimwe, Yeo, Kim, Jung, & Jeong, 2016).

NO can modulate the nervous system and impact functions such as learning, memory and neurogenesis (Forstermann & Sessa, 2012). In the periphery, smooth muscle tissues are innervated with nerves that release NO as a neurotransmitter decreasing the contraction of various types of smooth muscle, including blood vessels. In pathologies as hypertension, stroke, and neurodegenerative diseases NO plays a significant role (Forstermann & Sessa, 2012). However, abnormal NO signaling by excessive NO production can impact these neuropathologies (Forstermann & Sessa, 2012). For example, neuronal death has been shown to be associated with energy depletion due to high levels of NO inhibiting

mitochondrial respiration and glycolysis (Brown, 2010), through interaction with oxygen and ROS (Brown & Borutaite, 2006).

NOS inducible (iNOS) isoform is not actively expressed in cells. However, the expression of iNOS can be induced by bacterial liposaccharide, cytokines, and more agents (Forstermann & Sessa, 2012). The enzyme can be stimulated in almost any cell. iNOS is particularly important in macrophages producing a large amount of NO, which is fundamental in the immunological function of these phagocytic cells, providing them a highly cytotoxic environment (Wink et al., 2011). NO can interact with enzymes involved in the mitochondrial electron transport and interfere directly with the DNA, leading to double strand DNA breaks of target cells (Forstermann & Sessa, 2012). These effects are important for the cytostatic and cytotoxic activity of macrophages in the elimination of microorganisms or even tumor cells. Despite NO crucial role in controlling the immune response, high NO levels might be harmful to healthy cells. Most of autoimmune and inflammatory diseases are characterized by an abundance of activated macrophages and high NO production (Wink et al., 2011). This elevated NO production can lead to death of cells by inhibition of mitochondria and DNA synthesis (Li, Kilbourn, Adams, & Fidler, 1991).

NO is associated with gestational diabetes mellitus, a glucose intolerance that occurs during pregnancy (Visiedo et al., 2017). Moreover, inflammation is a common feature during this stage, which leads to a higher NO production. Both factors coupled with the high-energy demand of the pregnancy stage, which is characterized by high utilization of oxygen leads to increased oxidative stress. Oxidative stress coupled with high NO production can lead to imbalances between cell proliferation and apoptosis.

NOS, particularly eNOS, is also important for the cardiovascular system in functions as blood vessel dilatation, blood pressure control and has vasoprotective and anti-atherosclerotic effects (Forstermann & Sessa, 2012). Cardiovascular risk factors, as hypertension, diabetes and smoking, are associated with increased production of ROS. The interaction of NO and ROS can be therefore a risk, aving an impact in cardiovascular diseases.

NO impact is in part due to oxidative stress caused through the interaction with free radicals, as superoxide, to form highly reactive nitrogen species, such as peroxynitrite ($ONOO^-$) and peroxynitrous acid (HNO_3) (Forstermann & Sessa, 2012). These nitrogen reactive species cause nitration and nitrosation of important biological macromolecules such as DNA, RNA, proteins, and lipids, thereby disrupting their function. For example, reaction of these species with DNA and RNA can damage the nucleotides, through generation of 8-nitroguanine, which is a potent mutagen and pro-oxidant (Kaneko et al., 2008). Furthermore, these species can cause oxidative damage in lipids. Lipids are the main form of energy storage, the structural components of cell membranes and subcellular organelles, and have a role in

signaling. Lipid peroxidation is a process where the reactive molecules disrupt lipids carbon-carbon double bonds, especially polyunsaturated fatty acids. The disruption of the lipids causes cellular damage (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012), particularly at membrane level. Moreover lipid peroxidation originates a chain reaction with the production of aldehydes, which might promote deleterious reactions such as protein and DNA crosslinking, and impairing of protein synthesis (Ayala, Munoz, & Arguelles, 2014). Lipid peroxidation has been associated with a variety of tissue injuries and diseases (Esterbauer, Schaur, & Zollner, 1991; Nimse & Pal, 2015). Moreover, $ONOO^-$ is suggested to cause protein aggregation through nitrosylation of membrane protein thiols in mitochondria, which could compromise the structure of this organelle and lead to the release of some apoptotic inducing factors and compromising ATP production (Asiimwe et al., 2016).

Sodium nitroprusside (SNP) is a water-soluble sodium salt with nitric oxide and five cyanide anions, as show in figure 2. This molecule is highly sensitive to the light and has a short life period (between one to two minutes). In the body, SNP acts as a prodrug and reacts with sulfhydryl groups on erythrocytes, albumin and other proteins in order to release NO. It could be toxic for several cell types on the human body namely cerebral endothelial and neural cells and hepatocytes, by the production of ROS, which leads to apoptotic cell death. (Hottinger, Beebe, Kozhimannil, Prielipp, & Belani, 2014). It was used in clinical practices for several years due to its quick and effective action as arterial and venous vasodilator lowering blood pressure and it was also used clinically in cardio, vascular and pediatric surgery, hypertension, heart failure, among others. However, it began to be associated with cyanide toxicity due to the progressive release of cyanide molecules. When cyanide aggregate with iron (Fe) atoms present in mitochondrial cytochrome oxidase, occurs an inhibition of ATP production. SNP was then replaced by other safer substances with the same physiological effects to treat some pathologies, but it's still used nowadays in some medical treatments.

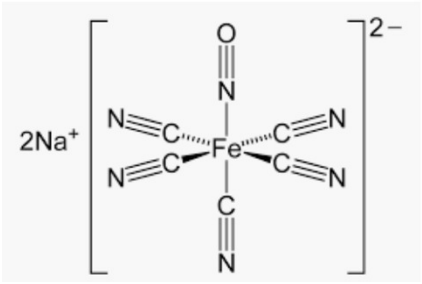


Figure 2. Chemical structure of sodium nitroprusside molecule.

1.3. Antioxidants

The organism can deal with oxidative stress with a variety of antioxidant systems. There are enzymatic and nonenzymatic molecules with the capacity to prevent the oxidative process in cells (Nimse & Pal, 2015). As with the oxidative stress, the antioxidants can also be of endogenous or exogenous origin. Enzymes as superoxide dismutase, catalase and glutathione peroxidase catalyze reactions leading to neutralization of oxidative compounds (Zhan, Sindhu, Pang, Ehdaie, & Vaziri, 2004). Nonenzymatic antioxidants act by interrupting free radical chain reactions. They can have multiple origins and a few examples are vitamin C, vitamin E, plant polyphenols, carotenoids, flavonoids, and glutathione (Birben et al., 2012).

Dietary oxidants have been identified as important molecules to prevent oxidative stress and in disease prevention. Plants are one of the major sources of antioxidant compounds which could be found in seeds, fruits, vegetables, cereals and oils among others (Shahidi & Zhong, 2010). So, it is important to identify plants that possess such compounds and identify the active compounds with antioxidant properties and their mechanism of action (Nimse & Pal, 2015).

However, it's necessary to be careful with the concentration of the antioxidant compound and the administration period. A substance with protective properties can also act as a pro-oxidant compound inducing oxidative stress by ROS formation or by antioxidants systems inhibition.

The phytochemical could be arranged by the chemical nature of their active compounds as alkaloids, terpenes and phenolic compounds. The alkaloids are a big group of compounds which contain a ring structure and a nitrogen atom and they can be found mostly in higher plants. They are good antioxidant molecules due to their capacity of free radicals scavenging, metal chelation activity and also hydrogen donation. Beta-carboline alkaloids are already reported as having ROS scavenging capacity and also antimutagenic and antigenotoxic activity (Kaur & Arora, 2015).Some of these compounds have physiological activities due to their analgesic function and anticancer properties. They are already successfully used in chemotherapeutic applications (Lu et al., 2012).

Terpenes are substances produced not only by plants but also by animals. These compounds are soluble in lipids and hydrocarbons and all of them came from the same unit, the isoprene. These compounds have an extensive range of properties described and they are used as anti-inflammatory, antimicrobial, antiviral, antifungal, antihyperglycemic, antiparasitic and in chemoprevention of cancer. (Paduch, Kandefer-Szerszen, Trytek, & Fiedurek, 2007)

The phenolic compounds are naturally produced by fruits and vegetables. There are a large variety of these compounds, but they all have a common origin which is the phenylalanine or tyrosine.

They are known for their capacity to act as antioxidant compounds due to their ability to donate hydrogen, which react with reactive oxygen species, ending the cycle of new radicals' generation. This happens because the radicals formed become chemically more stable than the initial radical. Their antioxidant properties also come from the fact that phenols can act as metal chelators. The antioxidants properties from the phenolic structures are also associated with their potential to interact with proteins due to their hydrophobic benzenoid rings and the hydrogen-bonding potential of the phenolic hydroxyl groups. Phenols compounds can also inhibit enzymes involved in radical generation, such as cytochrome P_{450} isoforms lipoxygenases, cyclooxygenases and xanthine oxidase. (Pereira, Valentão, Pereira, & Andrade, 2009)

It is important to better understand the implications of antioxidant molecules in cells. Moreover, how could they be used to improve life quality and as therapeutic compounds and to study their mechanism of action. Extracts from *Ginkgo biloba* and *Melissa officinalis* presented already good results in the literature against oxidative stress induced by nitric oxide.

1.4. *Gingko biloba*

G. biloba is a plant species dating for around 280 million years, and has been used in classical Chinese medicine for several hundred years (Ude, Schubert-Zsilavecz, & Wurglics, 2013). *G. biloba* extract contains flavonoids, terpene trilactones, and alkylphenols such as ginkgolic acids, ginkgols and bilobols that have radical-scavenging and antioxidant activities (Ude et al., 2013).

Usually, this plant is used as a supplement in the diet to prevent some disorders of the central nervous system, such as memory impairment, dementia, and Alzheimer's. Indeed, it has been shown that *G. biloba* protects against neurotoxicity which could be a key to treat diseases (Z. Y. Li et al., 2017). Terpene trilactones is a rare class of compounds in the plant kingdom. These compounds have been shown to protect mitochondria from damage, a organelle which plays a major role in Alzheimer (Shi, Liu, Wu, & Yew, 2010). Ginkgolide A, a terpenic lactone, has been shown to reduce inflammatory mediators such as NO (Y. Li, Y. Wu, et al., 2017). Furthermore, flavonoids are taken in the diet from plants as they are ubiquitous. Epidemiological data suggest that flavonoids can have a preventive effect on cardiovascular diseases, and neurodegenerative diseases (Graf, Milbury, & Blumberg, 2005; Pandey & Rizvi, 2009). These compounds appear to be potent antioxidants leading to decrease in reactive species and also inhibition of membrane peroxidation (Ude et al., 2013). Gingkgolic acids are not very stable, however they can be easily decarboxylated to form ginkgols, which seem to have an anticancer effect (Ude et al., 2013). However, these compounds can cause strong allergic reactions, in addition to cytotoxic, mutagenic, carcinogenic properties, therefore their concentration must be limited in extracts

prepared for health-related consumption (Ude et al., 2013). Some studies pointed that ginkgol C17:1 suppresses the migration and the invasion of tumor cells (Y. Li, J. Liu, et al., 2017). However, the pathways involved in this anticancer effect remain unclear.

1.5. *Melissa officinalis*

Melissa officinalis, also known as lemon balm, bee balm and honey balm is a plant cultivated all around de globe and occurs naturally in sandy and scrubby areas. It's probably one of the easiest herbs to grow and spreads really quickly. This plant has been used in tradition medicine to treat some disorders as fever, asthma, depression, colic, nausea, insomnia, indigestion among others. It acts as antimicrobial, antiviral, antioxidant, antifungal, antispasmolytic and memory-enhancer.

In the chemical composition, the extract of this plant contains flavonoids, polyphenolic compounds, monoterpenoid aldehyde, tannins, essential oils, monoterpene glycosides, triterpenes and sisterpenes. Some studies were performed with *M. officinalis* extract confirming its use in some therapeutic practices. The results show antiviral activity against herpes simplex infections and anti-inflammatory properties, supporting its use in inflammation and pain treatments and insomnia efficacy against stress-related effects.

The antioxidant activity was also studied to evaluate its pharmacological properties. The extract seems to have the ability to scavenge synthetic and also natural free radicals. Therefore, this indicates that it may have potential to prevent oxidative damage in vivo by preventing free radical-mediated oxidative stress. In an animal study, *Melissa officinalis* aqueous extract was used and the results showed a strong antioxidative and neuroprotective activity, validating its efficacy against oxidative stress in the mouse brain. (Miraj, Rafieian, & Kiani, 2017)

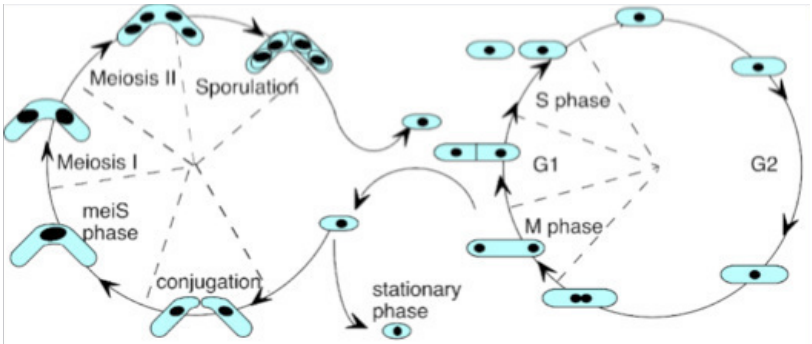
1.6. Yeast models

Eukaryotic unicellular organisms are important as model organisms as they provide tools to study cells behave when exposed to chemical compounds, physical conditions among others. This allows to work with a large number of individual organisms to identify genes involved in a biological process. Furthermore, as eukaryotes they can be used as model to study human complex processes that are highly conserved in a simpler organism. Yeast as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are good examples of such model organisms.

The yeasts have characteristics that make them useful as model organisms, namely rapid cell division under optimal laboratory conditions, they are inexpensive and easy to grow, they form colonies on plates, and they can be freeze-dried for storage. Furthermore, strains can be either haploid or diploid, in which haploid strains are useful to detect phenotypes associated with a gene and diploids to access dominant or recessive phenotypes (Hoffman, Wood, & Fantes, 2015). Yeasts can also maintain plasmids which are useful to introduce genetic material to impact the behavior of a strain and readily assess phenotypes (Hoffman et al., 2015). Moreover, introduction of exogenous DNA into the yeasts can be used to construct mutant strains as the yeast possess a highly active homologous recombination system (Hoffman et al., 2015).

Saccharomyces cerevisiae is one of the most common cellular models used with a duplication time of 90 minutes. This yeast is also used in the industry due to the fermentation process used in bakery and beverages. *S. cerevisiae* had the first fully sequenced eukaryotic genome which propelled a vast information on genome organization and evolution and cemented the use of this model organism. Therefore, the most advanced molecular tolls are available for this yeast. *S. cerevisiae* also presents a high level of protein conservation and the flexibility of genetic tools available make this organism one of the most versatile and powerful model organism (Salari & Salari, 2017).

Sch. pombe is an ascomycete yeast, which is a useful model organism. It is a fission yeast with a quite rapid cell cycle, with a duplication time between 2 and 4 hours. Strains used in laboratory studies derive mainly from a single isolate and are therefore nearly isogenic (Hoffman et al., 2015). This is particularly useful to avoid different phenotypes between studies, in wild-type strains, as occurs with models such as *S. cerevisiae*, between studies. They can follow two different reproduction pathways in laboratory as is shown in figure 3. Meiosis system has a biological advantage regarding the vegetative reproduction, allowing cells to recombine their genetic information by conjugate two organisms. However, vegetative cells are more useful when cells are been used to study some property because the mutation rate is lower. This system as a particular long phase G2, occupying about 90% of the division time.



A B

Figure 3. Cell cycle in meiotic (A) and vegetative cells (B) from *Sch. pombe.* (adapted from http://www-bcf.usc.edu/~forsburg/main4.html)

Important for molecular studies is that the genome of this yeast has a larger number of proteins involved in some major biological processes which are conserved in relation to vertebrates, when compared to *S. cerevisiae* (Hoffman et al., 2015). They share features with human chromosomes such as large and complex centromeres and replication origins and miRNA regulation. The DNA damage repair system and the cell cycle regulation are also very similar to the human cells with allows to extrapolate the results with this single cell model organism.

2. Materials and methods

2.1. Yeast strains, media and growth conditions

In this study, *Schizosacharomyces pombe* wild-type haploid strain FO656 was used. This strain was provided by Ingrun Alseth (Department of Microbioloby, Oslo University Hospital, Olso, Norway). *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was also used to test the protective effect of the pants extracts.

Strain stocks were stored at -80°C in 50% glycerol and *Sch. pombe* was cultured on solid YES medium (5 g/L yeast extract, 30 g/L glucose, 225 mg/mL adenine, 225 mg/mL histidine, 225 mg/mL leucine, 225 mg/mL uracil, 225 mg/mL lysine hydrochloride and 2% Difco Bacto Agar) at 30°C. After 3 days, the culture was stored at 4°C. Liquid YES medium (same composition as solid YES medium but without agar) was used to cultivate the *Sch. pombe* cells at 30 °C, 200 rpm. Monitoring of growth was made by optical density at 600 nm (OD_{600}).

S. cerevisiae was cultured on solid YPD media (10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone, and 2% Difco Bacto Agar) After 3 days, the culture was stored at 4°C. Liquid YPD medium (same composition as solid YPD medium but without agar) was used to cultivate the *S. cerevisiae* cells at 30 °C, 200 rpm. Monitoring of growth was made by optical density at 600 nm (OD_{600}). Each variation of media composition and growth condition are explained in the respective subsection.

2.2. Extracts preparation

In this study, samples of two different plants were used to prepare extracts. *Gingko biloba* leaves were collected from a female specimen at the end of the summer in September 2015 at the Campus de Gualtar of the University of Minho, Braga, Portugal. Leaves were completely dried at room temperature in a dark place for 7 days. To prepare the extract, 5 g of the leaves were triturated in a blender, the resulting powder was put in a cellulose extracting thimble and immersed in 100 mL of absolute ethanol in a glass laboratory bottle and left to incubate for 7 days at room temperature. At the end of this period, the cellulose thimble was removed and the remaining ethanolic solution was filtrated and evaporated using a rotavapor (RE121+461, Buchi) at 40 °C, 40 rpm and in the absence of light. The residue was dissolved in 20 mL of deionized water in order to transfer to another bottle and frozen at -80°C. After this, the sample was put in a freeze dryer (Christ Alpha 2-4, B. Braun) for 4 days in the dark. The resulting powder were used to create the stock solution of the extract with absolute ethanol to a final concentration of 50 mg/mL and stored at -20 °C. *Melissa officinalis* plants were collected in Braga at the end of the summer in September 2017. The process of extract preparation was the same as used for *Gingko biloba*.

2.3. Viability assays

Viability assays were performed by two different methods. The first one consists in cultivation on solid YES medium supplemented with extracts and/or toxicants. Cultures were growth overnight in a liquid YES medium at 30 °C and 200 rpm to an optimal OD_{600} of 0.4. Afterwards, the cell culture was serial diluted to 10^{-4}, cells were pipetted on plates with different contents in the medium, consisting in the negative control with only the medium, the positive control with 300 mM SNP and a co-incubation with 300 mM SNP and 1000 μg/mL plant extract.

The second viability assay consisted in the incubation of cells in liquid YES medium containing different treatments. Cultures were grown overnight in liquid YES medium at 30 °C and 200 rpm to an optimal OD_{600} of 0.4. Six aliquots were prepared for the different treatments: negative control, extract control with YES with 1000 μg/mL extract, positive control with 300 mM SNP or treatments with co-incubation with 300 mM SNP and extract (500 μg/mL, 700 μg/mL or 1000 μg/mL GBE or 100 μg/mL, 500 μg/mL or 1000 μg/mL MOE). Cells were incubated at 30 °C, samples were collected along time, serially diluted up to 10^{-4} and pipetted onto plates with solid YES medium. The plates were incubated at 30 °C for 3 days and the viability was assessed by spot assay.

2.4. Fluorescence microscopy

Cultures were grown overnight in YES medium to OD_{600} of 0.4 and synchronized in S phase by addition of hydroxyurea (HU)12 mM for 4h. The cell culture was then centrifuged at 4 °C, 5000 rpm for 2 minutes, cells were washed twice with sterile deionized water by centrifugation under the same conditions (to remove HU), suspended in fresh YES medium 30 °C and 200 rpm. The suspension was divided into six aliquots with treatments as described above and incubated at 30 °C. Samples were taken every hour, including time 0, up to 3 hours. Each sample was washed with PBS solution by centrifugation at 1.200 rpm for 2 minutes, suspended in the same volume of PBS and 5 μL of cells was fixated by heat in microscopy slides. Hoechst fluorochrome, a DNA marker, was added at the concentration of 1 μg/mL, slides were incubated at the dark for 15 minutes and then they were observed in a fluorescence microscope (DM5000B+CTR5000+ebq100, Leica)

2.5. Cell cycle

Cultures were grown overnight on YES liquid medium to DO_{600} of 0,4. A 500 mL sample was taken from the culture, centrifuged at 14,100 rpm, suspended in 500 mL of ice-cold 70% ethanol for fixation of cells and stored at 4 °C until further processing. All samples taken from the cell cycle study were treated as above. The culture of *Sch. pombe* was synchronized in S phase by addition of HU12 mM for 4h, 30 °C and 200 rpm. At the end of the incubation, another 500 μL sample was collected to be used as a synchronized culture control. The cell culture was then centrifuged at 4 °C, 5000 rpm for 2 minutes, cells were washed twice with sterile deionized water by centrifugation under the same conditions (to remove HU), suspended in fresh YES medium and another 500 mL sample was collected to serve as hydroxyurea-free culture control sample. After synchronization, cells were divided in six aliquots the treatments were applied as described above and each suspension was incubated at 30 °C and 200 rpm. From each suspension, 500 μL samples were collected every 30 min, including time 0 min, up to 180 min. Each sample was washed twice by centrifugation at 2,600 rpm, 4 min, with 50 mM sodium citrate pH 7.5 and suspended in the same buffer. Samples remained in sodium citrate and 0.1 mg/mL of RNAse A was added and left to act overnight at 30 °C. Cells were sonicated twice for 10 s and propidium iodide was added to a final concentration of 24 μg/mL. Cell cycle analysis was performed in two cytometers (Sony Biotechnology, EC800 BR for GBE analysis and C02946, CytoFLEX V2-B2-R2, Beckman for MOE analysis) 20.000 events and the results were processed using FlowJo Software.

2.6. Chemical analysis

Chemical analysis was performed by ESI-MS technic using 40 ppm of *Gingko biloba* and *Melissa officinalis* extracts dissolve in 50 % methanol. The extract was injected in an ion trap mass spectrometer (Finnigan LXQ) at a flow rate of 5μL/min and vacuum. The analysis was performed for positive and negative selective ions.

3. Results

3.1. Viability assays

The viability assays performed consisted in a spot test used to see the growth rate of the yeast cells on the solid media. The first viability assay was performed using both *S. cerevisiae* and *Sch. pombe* and *G. biloba* extract in order to see if there is some protective effect when cells are exposed to an antioxidant agent SNP and the results are shown in Figure 4.

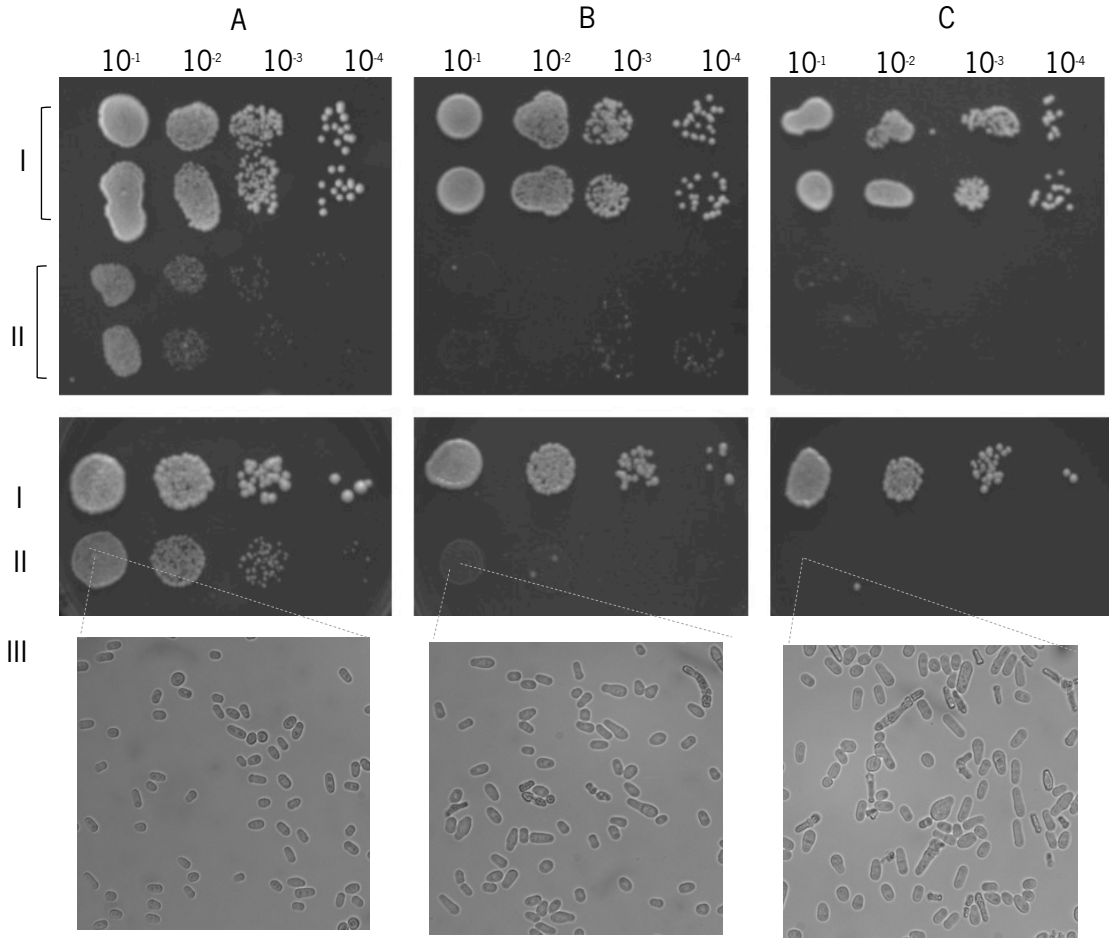


Figure 4. Spot test used in viability assay performed with *S. cerevisiae* (I) and *Sch. pombe* (II) in the presence of SNP and GBE. Cells from exponentially growing cultures were serially diluted to 10^{-4} and 5 µL of each dilution was placed onto to solid medium (A), solid medium with 300mM SNP (B) or solid media with 300 mM SNP and 50 µg/mL GBE (C). Plates were incubated at 30 °C, 48 h and plates were photographed. YES medium was used for *Sch. pombe* and YPD for *S. cerevisiae*. *Sch. pombe* cell from each treatment were observed in optical microscopy with 500x magnification (III). The photographs are representative of three independent experiments.

Regarding the first assay, *S. cerevisiae* does not seem to be affected by any treatment. The number of formed colonies does not have any visible reduction when cells are exposed to the SNP agent. This result indicates that *S. cerevisiae* is rather resistant to SNP, therefore the concentrations of SNP would have to be considerably high for the assays. As *Sch. pombe* displayed higher sensitivity to SNP (Fig. 4 and 5), we have decided to use this yeast as experimental model for the subsequent experiments. Despite *Sch. pombe* being sensitive to SNP, as the viability drops with this treatment, the extract does not seem to have a protective effect, regarding the reduced number of formed colonies and also the morphological alterations observed in optical microscopy that seems to be aggravated in treatments with the extract and SNP (Fig. 4C).

The second viability assay was performed using only *Sch. pombe* and *Gingko biloba* extract. The results are presented in Figure 5.

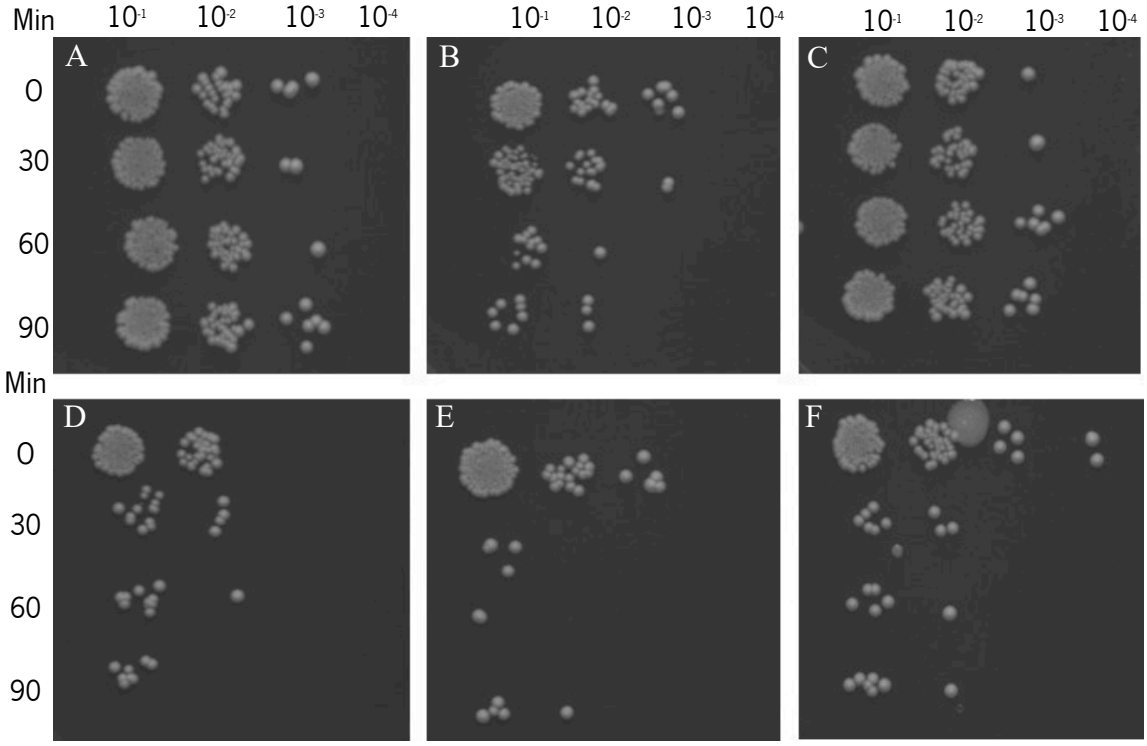


Figure 5. Spot test used in viability assay performed with *Sch. pombe* in the presence of SNP and GBE. Cells from exponentially growing cultures were exposed to YES medium (A), 300 mM SNP (B), 1000 µg/mL GBE (C), 500 µg/mL GBE and 300 mM SNP (D), 700 µg/mL and 300 mM SNP (E) or 1000 µg/mL GBE and 300 mM SNP (F), a sample was taken every 30 minutes, serially diluted to 10^{-4} and 5 µL of each dilution was placed onto YES solid medium, incubated at 30 °C, 48 h and plates were photographed. The photographs are representative of three independent experiments.

In this second assay, viability of *Sch. pombe* decreased in the presence of SNP along time and the extract did not affect viability, therefore the extract is not cytotoxic. However, the viability was more affected in the treatments of SNP and extract than those of SNP alone, suggesting that GBE is acting synergistically with NO. The last viability assay was performed with *Sch. pombe* using *Melissa officinalis* extract in order to see if this plant shows some protection against the NO provided by the SNP compound. The results of this assay are shown in Figure 6.

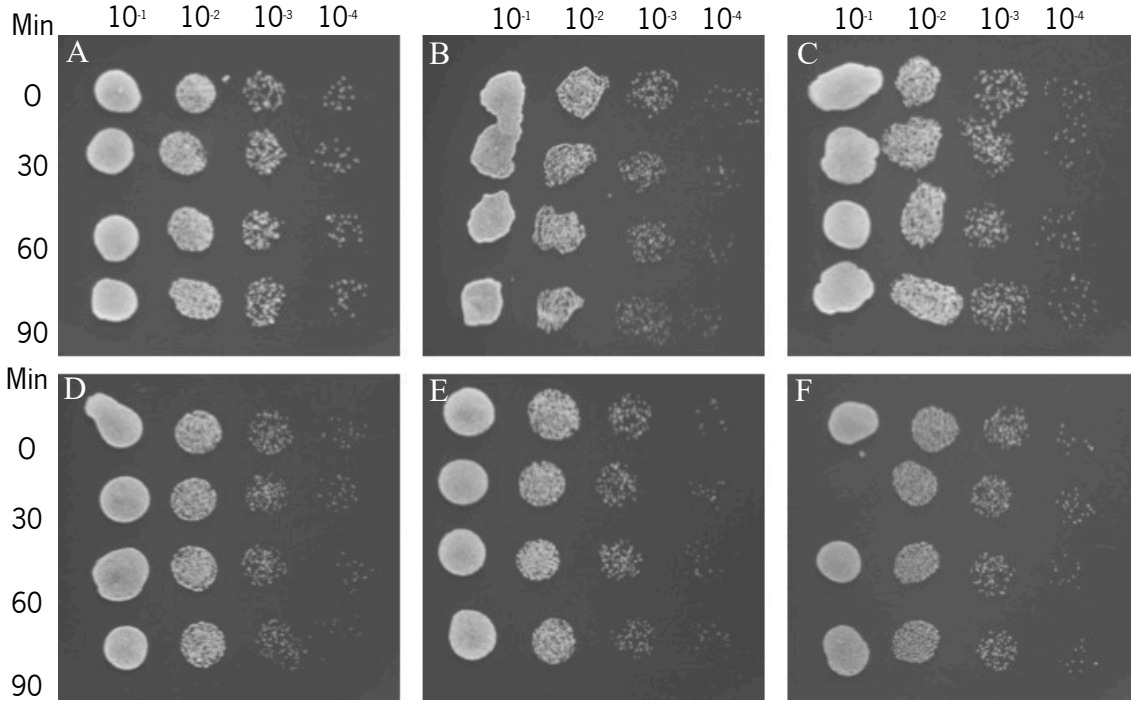


Figure 6. Spot test used in viability assay performed with *Sch. pombe* in the presence of SNP and MOE. Cells from exponentially growing cultures were exposed to YES medium (A), 300 mM SNP (B), 1000 µg/mL MOE (C), 100 µg/mL MOE and 300 mM SNP (D), 500 µg/mL MOE and 300 mM SNP (E) or 1000 µg/mL MOE and 300 mM SNP (F), a sample was taken every 30 minutes, serially diluted to 10^{-4} and 5 µL of each dilution was placed onto YES solid medium, incubated at 30 °C, 48 h and plates were photographed. The photographs are representative of three independent experiments.

In this last assay, it is not evident any activity regarding the SNP compound. The number of formed colonies was not affect regardless the incubation period which made this experience inconclusive.

3.2. Fluorescence microscopy

The cultures of *Sch. Pombe* were treated with MO extract. Hoechst fluorochrome was incorporated by the nucleus and allowed a chromatin visualization by fluorescence microscopy as is showed in Figure 7, in order to see if MOE protect cells against oxidative stress or induce structural DNA damages.

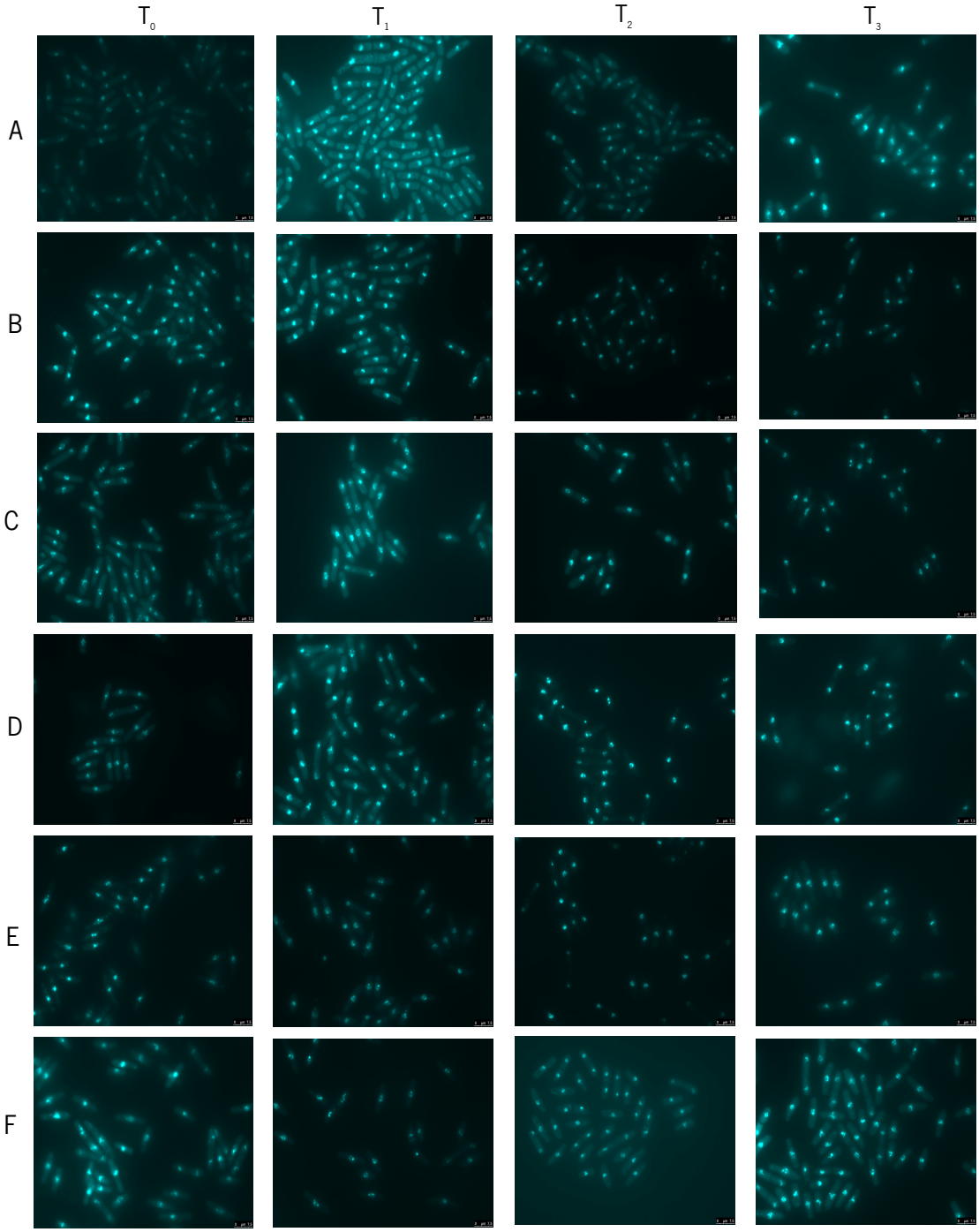


Figure 7. Chromatin observation by fluorescence microscopy in *Sch. pombe* cells. Cells from exponentially growing cultures were exposed to YES media (A), 300 mM SNP (B), 1000 μg/mL MOE (C), 100 μg/mL MOE and 300 mM SNP (D), 500 μg/mL MOE and 300 mM SNP (E) or 1000 μg/mL MOE and 300 mM SNP (F), a sample was taken at 0 (T_0), 60 (T_1), 120 (T_2) and 180 (T_3) minutes, added Hoechst fluorochrome 1 μg/mL, fixated and observed in fluorescence microscopy with a 1000x magnification. The photographs are representative of three independent experiments.

According to the fluorescence microscopy results, we are able to see some alterations in the chromatin structure when cells were treated with SNP, which indicate that the compound induce the expected outcome in cells: the production of NO molecules, originating oxidative stress in cells and hence structural changes in chromatin as result of DNA damages.

When cells were treated with *M. officinalis* extract, it did not seem to affect the cells chromatin, which indicates that, the extract by itself does not presents toxicity to the cells. Besides that, a synergistic effect seems to occur when cells are in the presence of MOE and SNP. The nuclear DNA has an unregular shape, which is more aggravated with higher concentrations of the extract. Regardless the time of the exposure, the chromatin was not properly condensed, and some fragmentation of the nucleus occurred. Not only the synergistic effect seems to occur. At 120 minutes of exposure, cells are completing their cell division cycle and un improvement seems to occur with the chromatin folding. Regardless this adjust who seems to occur during the cell cycle progression, some cells appear to stop or suffer a delay in their cell cycle division in M phase, presenting a majority of the cells with typical folding of this stage in which occur chromosomal condensation with the nuclear DNA preparing to divide.

Similarly to MOE experiment, cells of *Sch. pombe* were also treated with GB extract and the results from the fluorescence microscopy are shown in Figure 8.

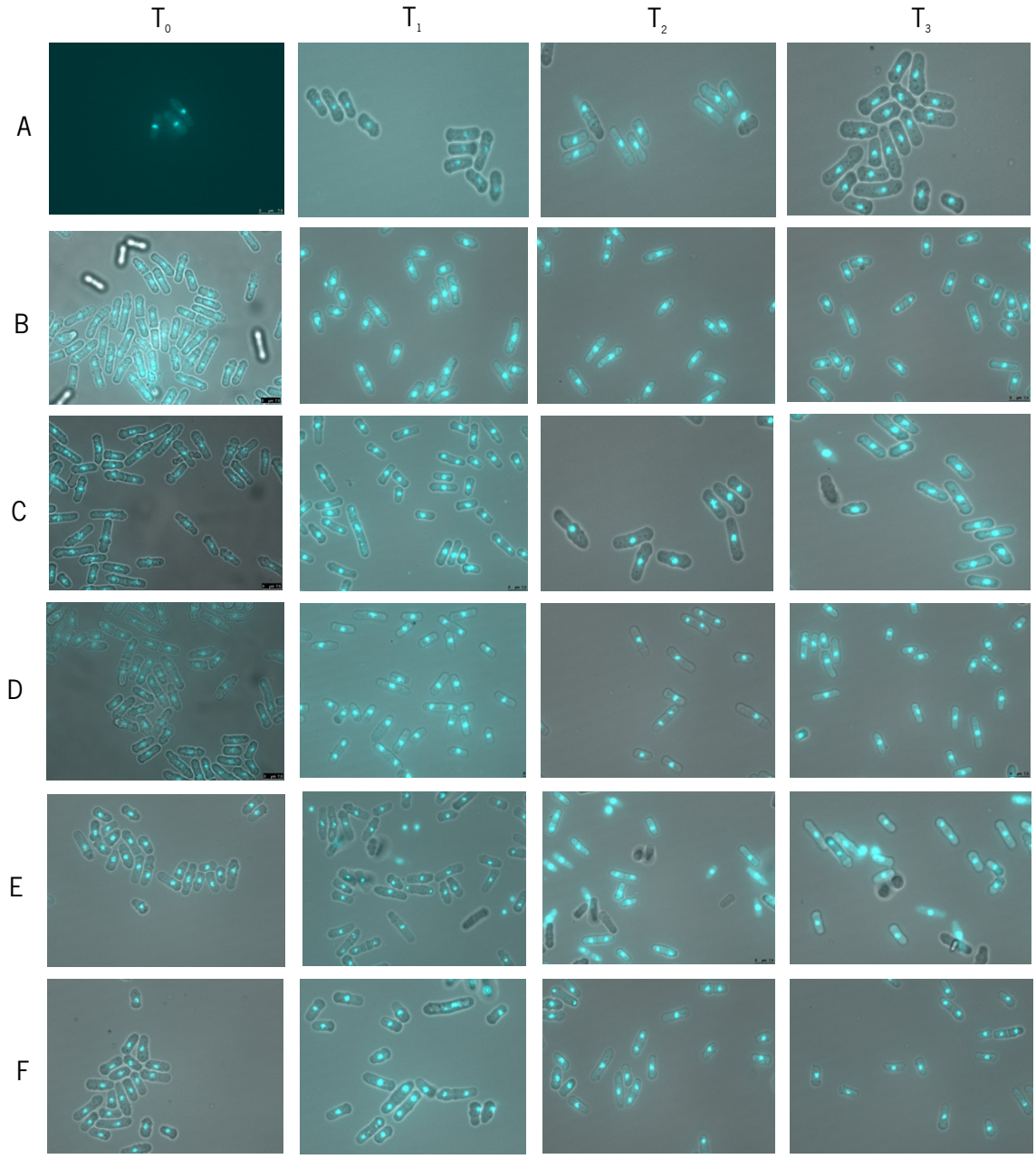


Figure 8. Fluorescence microscopy of *Sch. pombe* cells. Cells from exponentially growing cultures were exposed to YES media (A), 300 mM SNP (B), 1000 µg/mL GBE (C), 500 µg/mL GBE and 300 mM SNP (D), 700 µg/mL GBE and 300 mM SNP (E) or 1000 µg/mL GBE and 300 mM SNP (F), a sample was taken at 0 (T_0), 60 (T_1), 120 (T_2) and 180 (T_3) minutes, added Hoechst fluorochrome 1 µg/mL, fixated and observed in fluorescence microscopy with a 1000x magnification. The photographs are representative of three independent experiments.

According to the fluorescence microscopy results to GBE treatments, the SNP effect it is not evident. The cells seem to become shorter with time, but no nuclear abnormalities are clear. However, it

seems to occur an abnormal elongation in the cells morphology that contrast with very small cells in treatments with SNP and GBE.

3.3. Cell cycle

The cell cycle was performed using *Melissa officinalis* and *Gingko biloba* extracts in a *Sch. pombe* culture. The first assay was made with MOE in order to see if the chromatin alterations showed by fluorescence microscopy were somehow correlated with the normal cell cycle and the replication process. The results are shown in the Figure 9.

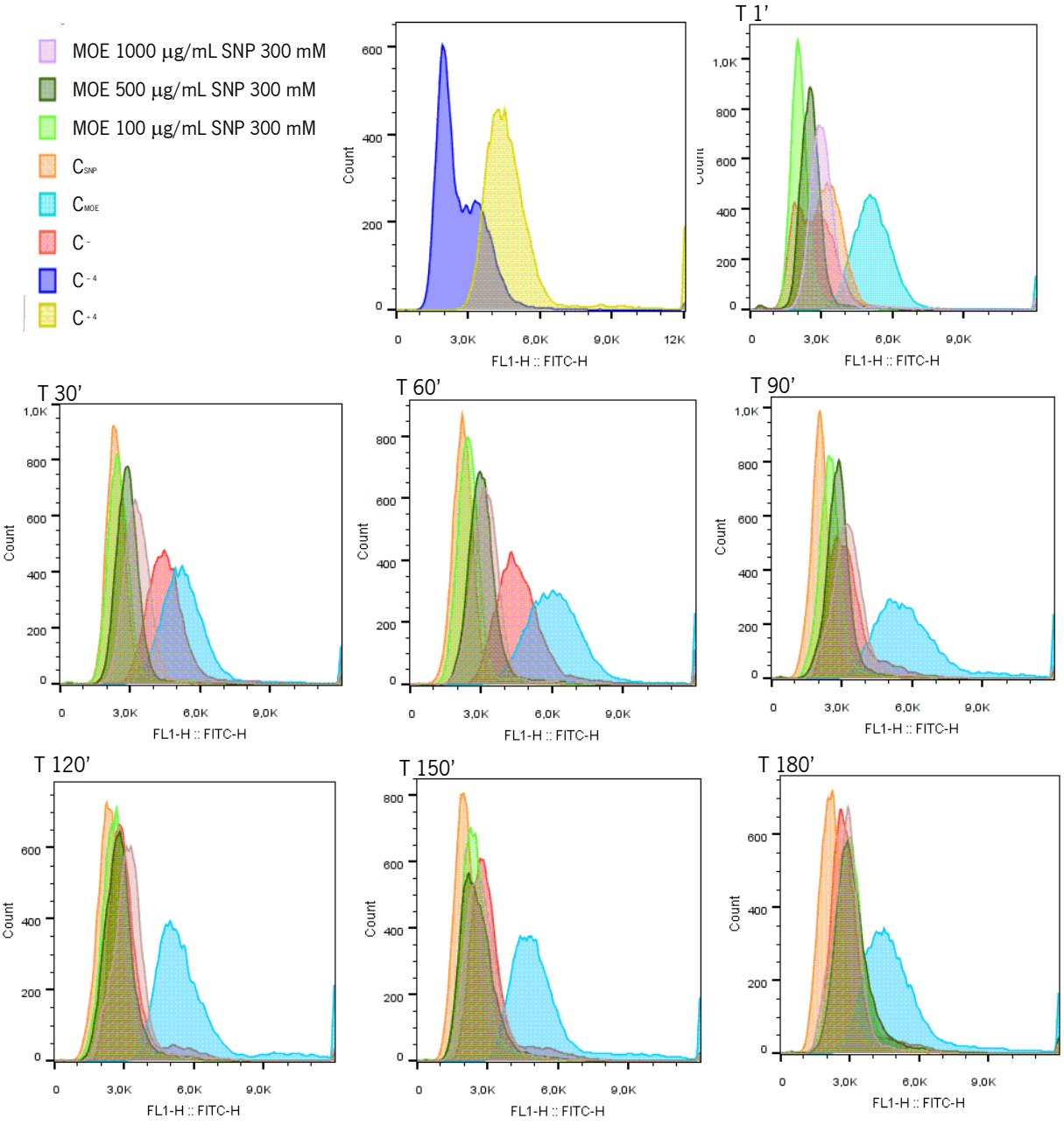
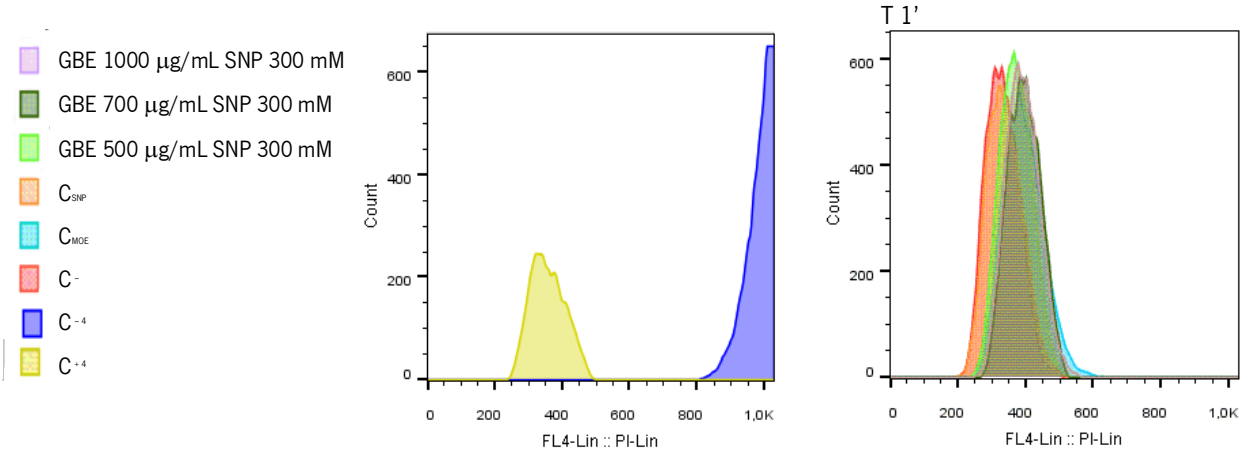


Figure 9. Cell cycle progression analysis of *Sch. pombe* cells by flow cytometry. Cells were treated with HU to synchronize their cell cycle in S phase (C^{+4}) and compared with an asynchronous culture (C^{-4}). After washing HU, cells were exposed to YES medium (C^{-}), 300 mM SNP (C_{SNP}), 1000 µg/mL MOE(C_{MOE}), 100 µg/mL MOE and 300 mM SNP, 500 µg/mL MOE and 300 mM SNP or 1000 µg/mL MOE and 300 mM SNP. Every 30 min, a sample of each treatment was taken and processed for analysis. During the treatments, the cells were incubated at 30 °C. The images are representative of three independent experiments.

According the results by flow cytometry, in the control samples before and after the treatment with HU, a synchronization of the cell cycle seemed to occur. We were able to see only one peak which indicates that all the cells have the same amount of DNA. The curve correspondent to the control with MOE have a clear separation from the other treatments regardless the time of exposure. However, the curve corresponding to the SNP control indicates that the cell cycle progression is delayed when compared to the other curves. Regarding the treatments with the extract and SNP, it seems to be advanced in the cell cycle progression when compared to the SNP control and when the extract is present in a higher concentration, more advanced seems to be. The second cell cycle analysis was made with GBE in order to see if the cells modifications observed in fluorescence microscopy interfered with the cell cycle progression and the results are showed in figure 10.



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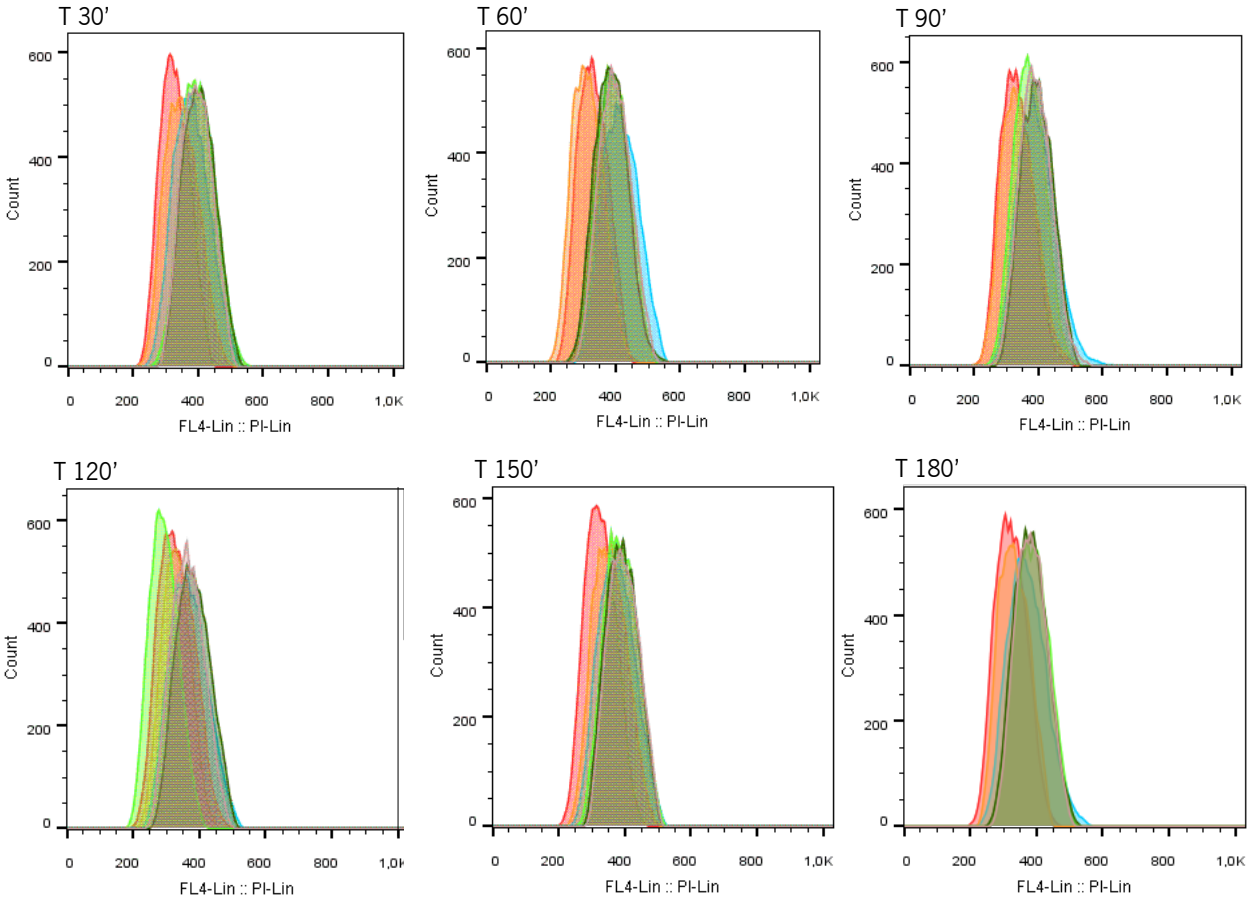

Figure 10. Cell cycle progression analysis of *Sch. pombe* cells treated with GBE by flow cytometry. Cells were treated with hydroxyurea to synchronize their cell cycle in S phase (C^{+4}) and compared with an asynchronous culture (C^{-4}). After washing HU, cells were exposed to YES media (C^{-}), 300 mM SNP (C_{SNP}), 1000 µg/mL GBE(C_{MOE}), 500 µg/mL GBE and 300 mM SNP, 700 µg/mL GBE and 300 mM SNP or 1000 µg/mL GBE and 300 mM SNP. Every 30 min, a sample of each treatment was taken and processed for analysis. During the treatments, the cells were incubated at 30 °C. The images are representative of three independent experiments

Regarding the results obtained by flow cytometry, there are no indication that the treatment worked. There are no visible alterations in the cell cycle progression and any expected effect by the treatments is seen. These results were inconclusive.

3.4. Chemical analysis

The analysis of chemical composition of the extract allows the identification of the major compounds potentially responsible for bioactivities. According the results from ESI-MS some of the compounds present in GBE are described in Table 2.

Table 2. Chemical analysis of *Gingko biloba* extract. Tentative identification of compounds according to the mass presented by positive and negative selective ions and the literature record.

	m/z	TENTATIVE OF COMPOUND IDENTIFICATION
POSITIVE	*218*	Perfluorotributylamine
	246 *274*	4-hydroxy-2-alkylquinolines
	284	Rhamnocitrin Diosmetin 7-O-glucoside Astragaloside
	302	quercetin
	330	6,7-dimethoxyquercetin-3-O-β-D-glucopyranoside Piroxicam
	568	homalicine tertraacetate
NEGATIVE	*255*	Rhamnocitrin
	319	Piptocarphin A piptocarphin B glaucolide A glaucolide B di-*O*-*p*-coumaroylquinic acid
	345	ginkgolic acid I syringetin tetrahydroxydimethoxyflavone
	373	ginkgolic acid II
	485	di-*O*-*p*-coumaroylquinic acid 3,4-di-*O*-(*E*)-*p*-coumaroylquinic acid
	609	bisrhamnosyl–glucosylquercetin quercetin-2″-glucosylrhamnoside quercetin-3-O-rhamnoside-glucoside quercetin-3-O-rutinoside rutin
	673.	oleic acid
	755.	kaempferol 3-O-2″-glucosyl-6″-rhamnosyl-glucoside kaempferol 3-O-rhamnosylhexoside-7-O-glucoside quercetin 3-O-2″, 6″-dirhamnosideglucoside quercetin 3-O-2″, 6″-dirhamnosylglucoside quercetin 3-O-2″-(6″-p-coumaroyl)glucosylrhamnoside quercetin 3-O-p-coumaroyldiglycoside quercetin p-coumaroyldiglycoside quercetin-3-O-rhamnoside-glucoside-coumaroyl

The extract chemical analysis results to MOE, according ESI-MS are described in Table 3.

Table 3. Chemical analysis of *Melissa officinalis* extract. Tentative identification of compounds according to the mass presented by positive and negative selective ions and the literature record.

	m/z	TENTATIVE OF COMPOUND IDENTIFICATION
POSITIVE	218	Perfluorotributylamine
	246 274	4-hydroxy-2-alkylquinolines
	284	Rhamnocitrin Diosmetin 7-O-glucoside Astragaloside
	302	quercetin
	330	6,7-dimethoxyquercetin-3-O-β-D-glucopyranoside Piroxicam
	353	Chlorogenic acid
	568	homalicine tertraacetate lutein
NEGATIVE	255	Rhamnocitrin
	283	dihydroxymethoxyflavone
	345	ginkgolic acid I syringetin tetrahydroxydimethoxyflavone
	373	ginkgolic acid II
	485	di-O-p-coumaroylquinic acid 3,4-di-O-(E)-p-coumaroylquinic acid
	565	ginkgetin isoginkgetin
	609	bisrhamnosyl–glucosylquercetin quercetin-2″-glucosylrhamnoside quercetin-3-O-rhamnoside-glucoside quercetin-3-O-rutinoside rutin

4. Discussion

The chemical analysis of the extract elucidates about the major compounds potentially responsible for bioactivities and was based in the literature records. Compounds as Rhamnocitrin, identified in both GBE and MOE, is a flavonoids member and is a kaempferol derivate, which is known for its antioxidant properties. Chlorogenic acid, identified in MOE, is a phenolic compound with radical-scavenging properties, which suggests an antioxidant activity. It acts in the organism reducing blood pressure and is pointed as having anti-inflammatory properties (Suzuki et al., 2006). However, some of the compounds identified in both extracts are associated with cellular damages. Ginkgolic acids I and II are known for their potential to induce cytotoxicity, mutations and genotoxic effects in cells, being restricted to a low concentration in commercial extracts (Ndjoko, Wolfender, & Hostettmann, 2000). Glaucolide B, a sesquiterpene lactone, was identified in GBE and pointed as being cytotoxic and clastogenic in studies performed *in vitro* with mammal cells, increasing the frequency of chromosomal aberrations (Burim, Canalle, Lopes, & Takahashi, 1999).

According to the results obtained for the treatments with extracts, the GBE and MOE seem to act synergistically with SNP compound. However, the effect induced in cells is different. In the GBE case, the number of *Sch. pombe* colonies is affected in the presence of the NO-donor and even more when the extract was mixed with SNP in co-incubation with the extract, suggesting higher yeast sensitivity to the treatment (Fig 5). A higher number of colonies was expected in the presence of the extract due to its already reported protection activity against the oxidative stress (Chao & Chu, 2004). However, it seems to occur a synergistic effect when the extract is mixed with the SNP once the number of colonies is smaller than when compared with the SNP treatment.

When cells treated with GBE were analyzed by optical microscopy, it was possible to observe differences in their morphology, with very elongated cells contrasting with very small ones. This suggested that the treatments were affecting their replication, disturbing their cell cycle. The effect observed could be explained by some mutations in the cdc2 protein. In fission yeasts, cdc2 is the only cyclin-dependent kinase and is involved in mitosis and S phase (Kommajosyula & Rhind, 2006). Cdc25 and Wee1 are responsible to control the progression in the cell cycle by cdc2 protein phosphorylation (Perry & Kornbluth, 2007) and this complex is responsible for cells elongation during replication, as represented in figure 11.

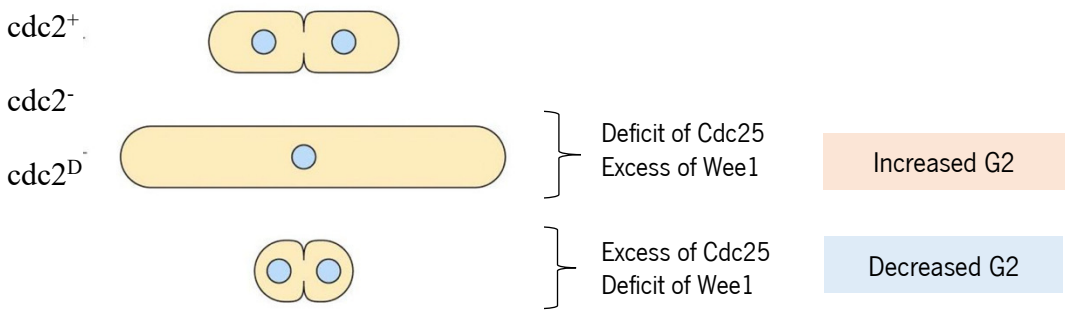

Figure 11. Diagrams of *Sch. pombe* cdc2 mutants. When occurs a deficit on Cdc25 and an excess of Wee1, cells tend to be elongated due to an increase of the G2 phase. When there is an excess of Cdc25 and a deficit of Wee1, cells became smaller and occurs a decrease of the G2 phase (adapted from Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition)

cdc2 needs to be dephosphorylated by Cdc25 to be activated and for the normal cell cycle progression. However, the results obtained suggest an imbalance between Cdc25 and Wee1 (Perry & Kornbluth, 2007). Regarding the extract composition, some of its chemical compounds could be interfering with the normal phosphorylation of cdc2. The experiences of flow cytometry to evaluate the cell cycle progression does not allow more conclusions once the treatment did not worked.

In treatments with *Melissa officinalis* extract, the synergistic effect is present differently. Cells are able to replicate in a normal rate but when their nucleus are analyzed by fluorescence microscopy, we observe that in treatments with SNP and in co-incubation, chromatin structure becomes irregular. In fact, a higher concentration of the extract seems to aggravate these modifications, suggesting the absence of extract antioxidant properties. Besides the fact of the chromatin modifications, the cell cycle does not seem to be affected. When cells were analyzed by flow cytometry, it becomes possible to observe that SNP was active once cells cycle progression was affected and in the treatment with MOE, the cell cycle appear to stop. The T'1 correspond to 1 minute after the cells were added to the treatment, allowing a quick reaction by the extract, which is the probable cause of the cell cycle arrest. The other treatments indicated that, in co-incubation, a small protective effect was observed due to the advance regarding the SNP control. In fact, with a higher concentration of MOE, more protection activity seems to occur.

Against the expected, modifications in the chromatin does not seem to compromise the cellular viability and the cells replication. However, the extract by itself appear to induce genotoxicity and could be interfering with M phase of the cell cycle due to DNA damages, affecting cdc2 protein normal function.

The chemical analysis supports the results obtained once some of the compounds mentioned before are described as inducers of damages in DNA structure.

5. Conclusion and future perspectives

The analyze of chemical composition of the extract allows the identification of the major compounds responsible for antioxidant action. In fact, both *Gingko biloba* and *Melissa officinalis* were already mentioned in the literature as having good antioxidant properties and they are usually used as food supplements as prophylactic to prevent disorders caused by oxidative stress. In the future, the extracts could be used in some therapeutic applications against diseases, as the ones already mentioned if their use proves to be unharmful for health.

In this study, a small protection activity was observed in the treatments with *Sch. pombe.* However, the extract co-incubation with SNP, a compound used to induce oxidative stress, seems to act synergistically and induce more damages in cells. The theory of cdc2 mutants induced by extracts co-incubation should be explored in the future and mutants for DNA damages repair should also be tested in order to discover the cellular mechanisms affected by the extract treatment.

Due to the extreme sensitivity of the SNP compound some of the experiments should also be repeated. The effect was not clear in all the experiments and in order to improve the results, SNP could be replaced for S-nitrosothiol, an agent also acting as NO donor and widely used in experiments (Kevil & Patel, 2010). However, the NO-donor used in future tests should be lower concentrated once the stress induced can be too strong for the extracts in use. The extract concentration should also be taken in consideration due to its potential to induce cellular toxicity when is highly concentrated or the lack of protective effect when is present in low concentrations. Therefore, in future trails, different concentrations should be tested.

Chemical analysis of the extracts should be repeated in the future by HPLC, which allows more accurate identification of the compounds present in each extract, in order to confirm the tentative of identification by ESI-MS.

Regarding MOE, the genotoxicity observed in the experiments could be explored to its potential for chemotherapies applications in which a toxic environment is required. In the future, cellular lines could

be used to test the compounds toxicity and improve the conditions for an application in treatments as cancer.

It is important to keep in mind that, the experiments were made in unicellular organisms and these results could not be extrapolated for complex organisms. Cellular response could induce protection against toxic agents when administered in low concentrations by the activation of defense mechanisms. However, physiological response could attenuate the effects observed at cellular level and have different biological responses.

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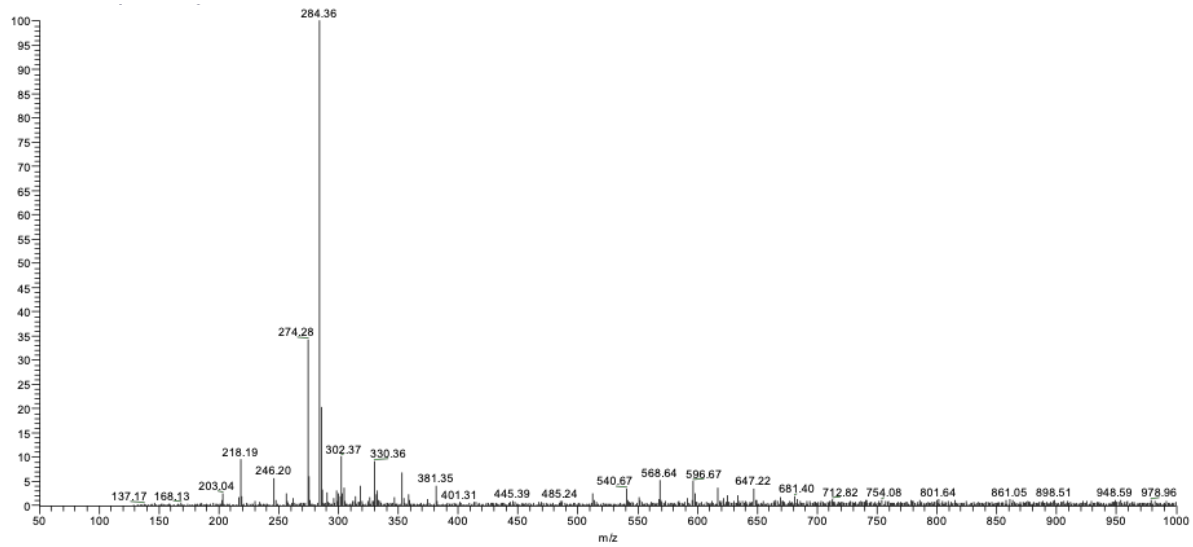
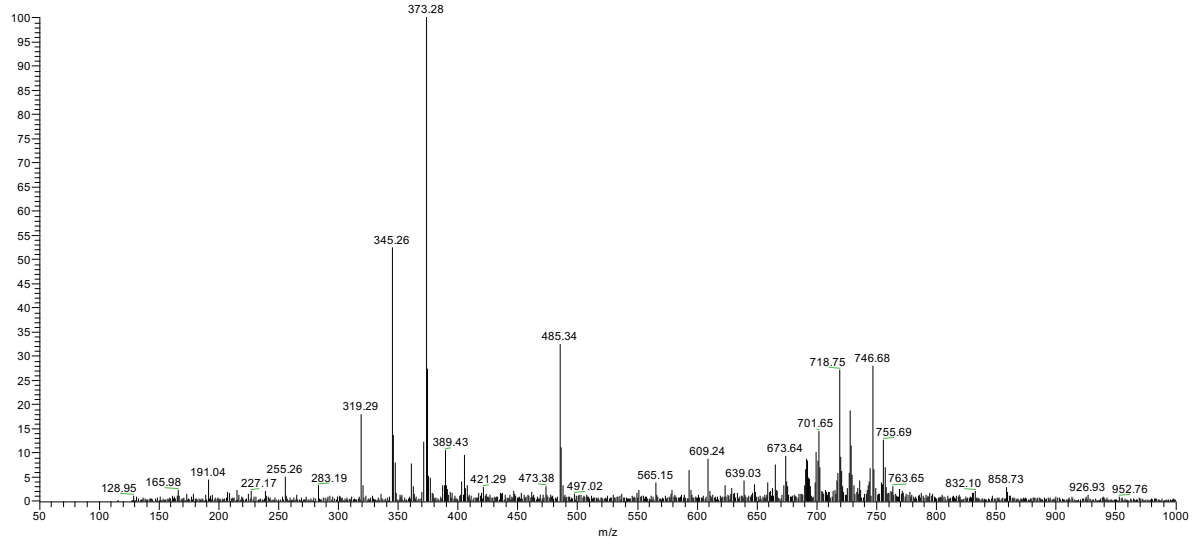
Appendix

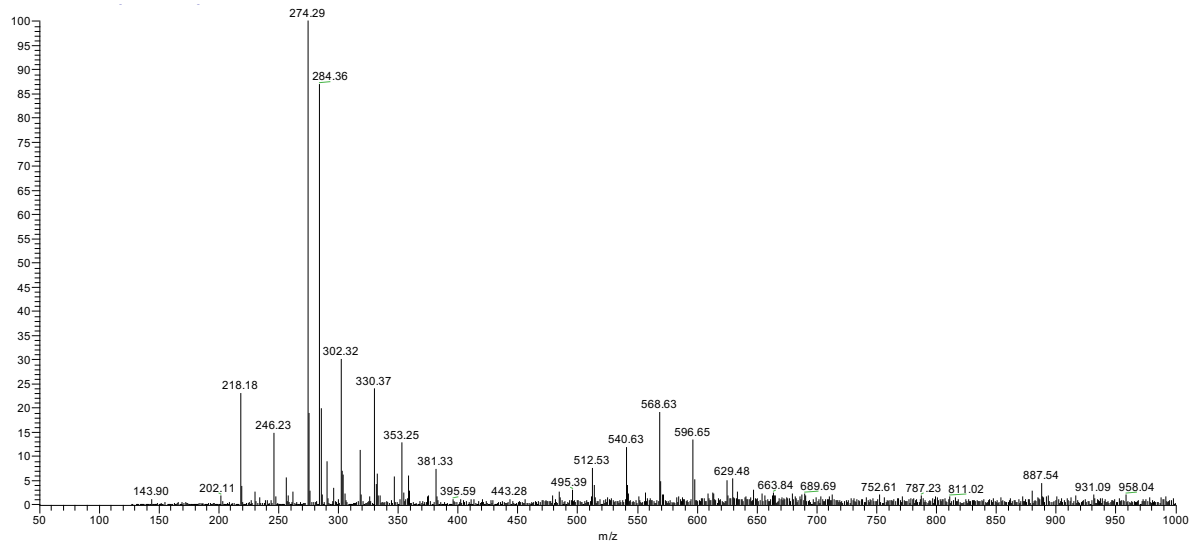
Appendix 1 – Mass spectrum of GBE in positive ionization.



Appendix 2 – Mass spectrum of GBE in negative ionization



Appendix 3 – Mass spectrum of MOE in positive ionization



Appendice 4 – Mass spectrum of MOE in negative ionization