Universidade do Minho
Escola de Ciências

Daniela Filipa Alves Neves

Assessment of antigenotoxic activity of *Dittrichia viscosa*

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Master Thesis
Master's in Molecular Genetics

Work under the supervision of
Doctor Rui Pedro Soares Oliveira

july 2021

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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

Resumo

A resistência do nosso genoma é testada diariamente não só por agentes exógenos, de natureza física, química e biológica, mas também por fatores endógenos, que podem causar genotoxicidade e citotoxicidade. Para contra-atacar estes efeitos nocivos, as células possuem vários mecanismos capazes de reparar o ADN ferido, no entanto, em algumas situações os danos estão para além da reparação e a patogénese ocorre. Os investigadores têm a oportunidade de criar novas estratégias terapêuticas com plantas que têm sido usadas na medicina tradicional desde há séculos. Esta possibilidade pode ser muito benéfica para o bem-estar dos seres humanos, por exemplo, para travar ou retardar o desenvolvimento de várias doenças associadas ao envelhecimento. Uma dessas plantas de interesse é a *Dittrichia viscosa*, típica dos países que circundam o mar Mediterrâneo e consequentemente empregada pela sua população na medicina tradicional.

O objetivo deste estudo é verificar se o extrato aquoso de *Dittrichia viscosa subsp. revoluta* tem atividades biológicas, concretamente, atividade antigenotóxica. E se assim for, compreender o seu mecanismo de ação.

Os resultados dos ensaios *in vitro*, da atividade antioxidante pela captura do radical livre DPPH e da quantificação do conteúdo fenólico total, sugerem que o extrato estudado tem atividade antioxidante devido ao seu conteúdo fenólico, em parte. Além disso, foram realizadas experiências *in vivo* com o organismo modelo, *Schizosaccharomyces pombe*, onde foram avaliados as viabilidades, a morfologia nuclear e os comprimentos das leveduras tratadas com o extrato da planta, hidroxiureia e a combinação de ambas. Os resultados obtidos desses testes indicam que o extrato da planta afetou a atividade de HU, um inibidor da replicação do ADN, após um longo período de tempo.

Em conclusão, a o extrato aquoso de *Dittrichia viscosa subsp. revoluta* exibiu atividade antigenotóxica parcialmente explicada pela sua atividade antioxidante.

Palavras chave: *Dittrichia viscosa*, levedura de fissão, hidroxiureia, ciclo celular, antigenototxicidade

Abstract

The resistance of our genome is tested on daily basis by not only exogenous agents of physical, chemical and biological nature, but also, by endogenous factors, that can cause genotoxicity and cytotoxicity. To counterattack these harmful effects, cells possess several mechanisms capable of repair injured DNA, nevertheless, in some situations the damage is beyond reparation and pathogenesis takes place. Investigators have the opportunity to create new therapeutic strategies with plants that have been used in traditional medicine from centuries ago. This possibility could be very beneficial for the wellbeing of humans, for example, to halt or retard the development of several diseases associated with ageing. One of those plants of interest is *Dittrichia viscosa*, typical from countries surrounding the Mediterranean Sea and consequently employed by their population in traditional medicine.

The objective of this study is to verify if *Dittrichia viscosa subsp. revoluta* aqueous extract has biological activities, concretely, antigenotoxic activity. And if so, comprehend its mechanism of action.

The results from *in vitro* assays, DPPH radical scavenging activity and the quantification of total phenolic content, suggest that the studied extract has antioxidant activity due to their phenolic content, in part. Moreover, *in vivo* experiments were performed with the model organism, *Schizosaccharomyces pombe,* where the viability*,* nuclear morphology and lengths of yeasts cells treated with the plant extract, hydroxyurea and the combination of both, were assessed. The obtained results from those tests point out that the plant extract affected the HU activity, an inhibitor of DNA replication, after a long period of time.

In conclusion the *Dittrichia viscosa* subsp. revoluta aqueous extract had antiogentotoxic activity partially explained by its antioxidant activity.

Key words: *Dittrichia viscosa*, fission yeast, hydroxyurea, cell cycle, antigenotoxicity

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Abbreviations List

AP endonuclease – Apurinic/apyrimidinic endonuclease

BER – Base excision repair

CAT - Catalase

CPD - Cyclobutane pyrimidine dimer

DDR – DNA Damage Response

DVAE – *Dittrichia viscosa* aqueous extract

Exo I – Exonuclease I

GPx – Glutathione peroxidase

GR – Glutathione reductase

h - hour

HR – Homologous recombination

HU – Hydroxyurea

IC50 - Concentration of drug required for 50% inhibition

IR – Ionizing radiation

MI – Mitotic index

min - Minutes

MMR – Mismatch repair

NER – Nucleotide excision repair

NHEJ – Non-homologous end joining

NOS - Nitric oxide synthase

NOX - NADPH oxidase

PCNA - Proliferating cell nuclear antigen

PPs – Polyphenols

Prx - Peroxiredoxin

RFC - Replication factor c

RNR - Ribonucleotide reductase

ROS – Reactive oxygen species

RPA – Replication protein A

ssDNA – single strand DNA

SOD – Superoxide dismutase

UV – Ultra violet

WT – Wild type

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1- Introduction

1.1 - Unraveling DNA structure

Stability and preservation of genome is crucial to the development and conservation of species since DNA encodes essential information at molecular scale that is crucial for maintenance, reproduction and development of organisms. Until the new perspective about the molecular structure of deoxyribonucleic acid, by Watson and Crick in 1953 (Watson & Crick, 1953), relevant aspects at biochemical dimension had remained unknown to the scientific community. In their revolutionary hypothesis, DNA is constituted by a double helix (Figure 1A), unlike the three intertwined helical chains (Figure 1B) theory from Pauling and Corey (L. Pauling & R. B. Corey, 1953; Linus Pauling & Robert B. Corey, 1953). This helix is linked by hydrogen bridges between one purine and one pyrimidine from each strand, in a perpendicular plane in relation to the axis of the helix, and the existing phosphates groups close to this axis (that are negatively charged) would repel each other (Watson & Crick, 1953).

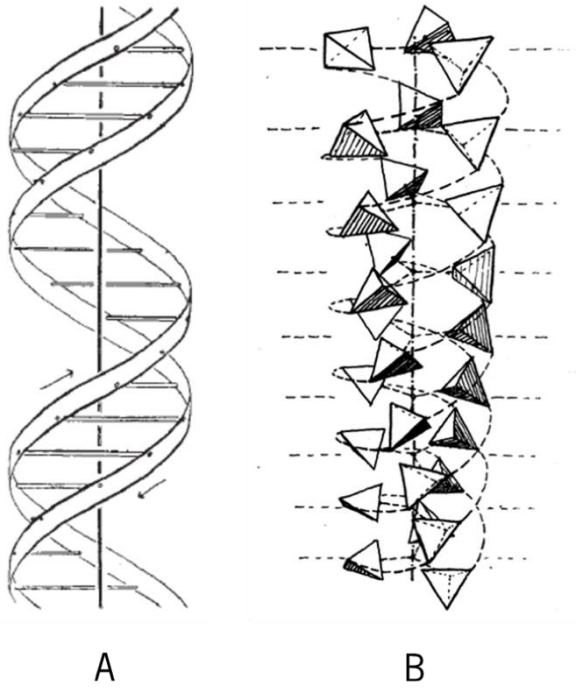


Figure 1. DNA structure models proposed in 1953: DNA double helix structure (Watson & Crick, 1953) and DNA triple helix structure (L. Pauling & R. B. Corey, 1953).

After that moment several important discoveries in the most diversified scientific fields were achieved. Until the present day, the discovery of DNA structure remains relevant to medicine, because new knowledge about some diseases was and continues to be acquired, which enables the development of

new methods of diagnosis and therapy. In forensic science, with a small amount of DNA from a crime scene or a victim, investigators are able to identify the perpetrator. In genealogy and history, for example, researchers had acquired new data on the intricate immigration patterns of humanity since antiquity. In ecology, DNA barcodes are employed to identify and discriminate species, this methodology is based in DNA sequencing. As a result, new species can be discovered, populations from one ecosystem can be monitored, invasive species can be identified and also cryptic species. These are few examples about the importance of DNA sequencing techniques in differentiated scientific fields.

1.2 - Genotoxicity

The resilience of our genetic information is constantly tested by not only exogenous sources, for instance, ultra violet (UV) radiation; xenobiotics; viruses, but also by endogenous sources, for example byproducts of cellular metabolism; reactive oxygen species (ROS); replication errors. Interactions between these genotoxic agents of chemical, biological and physical nature, and DNA can generate abasic sites, 8-oxoguanine, pyrimidine dimers, DNA adducts, intra or/and interstrand crosslinks and single or/and double DNA strand breaks, micronuclei, chromosomal aberrations, sister chromatid exchange (Fenech et al., 2011; Ghosal & Chen, 2013). Abasic sites are locations where purines or pyrimidines are absent, they possibly were produced spontaneously by eliminating damaged or improper bases due to ionizing radiation (IR), UV light, alkylating agents and base oxidation (Dianov, Sleeth, Dianova, & Allinson, 2003; Mohni et al., 2019). When an increase of ROS can't be counterbalanced with endogenous antioxidants, guanines are oxidized and new biochemical products are obtained, among them is 7,8-dihydro-8-oxoguanine also known as 8-oxoguanine (the most representative oxidation product) (Morikawa et al., 2014; Pázmándi et al., 2019). UV rays prompt the formation of pyrimidine dimers (Fig. 2) since UV radiation induces the covalent link of two adjacent pyrimidines (Yoon et al., 2019). Bulky DNA adducts are formed due to interaction of UV or pollutants with DNA (Lee, Mann, da Silva, Scalici, & Gassman, 2019). Intra or/and interstrand crosslinks are produced by both endogenous (byproducts of cellular metabolism) and exogenous alkylating agents (chemotherapeutic agents) (Guainazzi & Schärer, 2010; Legerski, 2010). Single strand breaks are generated by IR, ROS, and secondarily due to base excision repair of abasic sites or modified DNA bases (Aleksandrov et al., 2018; Caldecott, 2008). Double strand breaks (DSBs) are generated by ionizing and UV radiation, chemotherapeutic drugs, during replication to correct some anomalies (Aleksandrov et al., 2018; Ghosal & Chen, 2013).

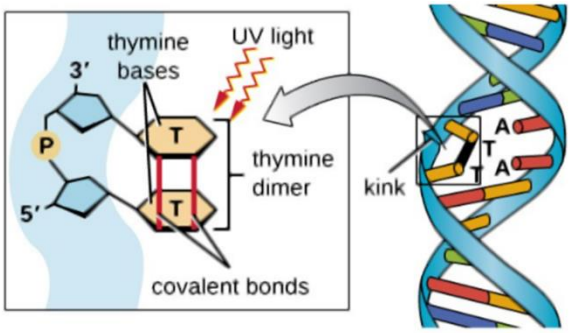
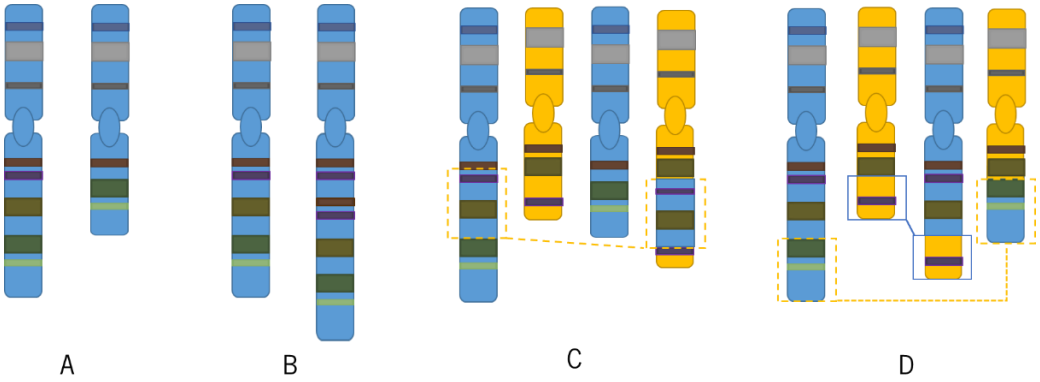


Figure 2. Thymidine dimer, example of DNA lesion (adapted from: Mutations; 2019, September 25. Retrieved January 1, 2021, from https://geo.libretexts.org/@go/page/6188).

Micronuclei are a form of nuclear anomaly, there are several explanations to micronucleus formation, although all of them take place at anaphase, such as faulty checkpoint genes, misrepaired or unrepaired DNA breaks, malsegregation of a chromosome, defects in kinetochore proteins or assembly and dysfunctional spindle (Fenech et al., 2011). Errors that weren't repaired during or after mitosis and meiosis, or interactions with chemicals (as pesticides), can originate chromosomal anomalies, which are classified as numerical, in which cells exhibit an abnormal number of chromosomes, or structural, where the typical structure of chromosomes is changed. There are more types of structural aberrations comparatively to numerical. The structural abnormalities are classified as deletions when a portion of a chromosome is deleted (Fig. 3A). Duplications, a part of a chromosome is duplicated (Fig. 3B). Insertions, a segment of a chromosome is inserted into another chromosome after being eliminated from its original locus (Fig. 3C). Translocations, a fragment of two chromosomes is changed between each other (Fig. 3D). Inversions, a segment of genetic information of a chromosome is inverted (Fig. 3E). Ring, two ends of a chromosome are joined and a ring is shaped (Fig. 3F). Isochromosome, an arm of a chromosome is deleted and the other is duplicated (like a mirror; Fig. 3G) (Ferguson-Smith, 2001).



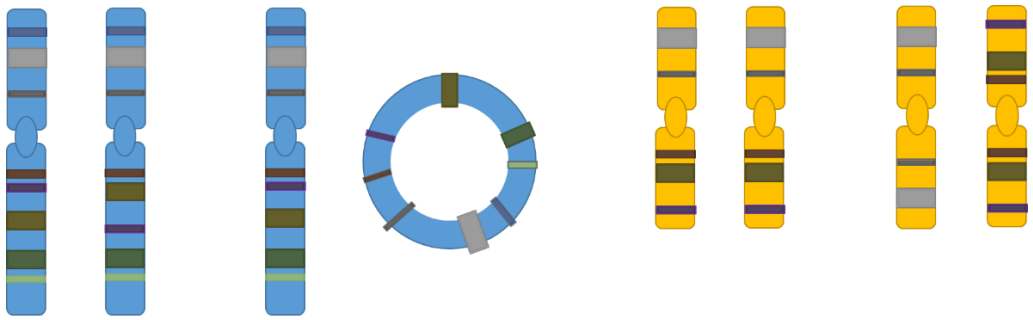


Figure 3. Structural chromosome aberrations: A-Deletion, B-Duplication, C-Insertion, D-translocation, E-Inversion, F-Ring and G-Isochromosome.

In regard to chromosomal numerical abnormalities, they can be classified as polyploidies or aneuploidies (Fig. 3). In polyploidies the normal set of chromosomes in the nucleus is augmented (Sigl-Glöckner & Brecht, 2017). Aneuploidy means that the number of chromosomes present in a cell is atypical, this type of numerical anomalies are more common (Hwang et al., 2019). They can be monosomies, when a chromosome is missing, or trisomies, when an extra chromosome is present. Thereafter, if these DNA injuries are not identified and subsequently mended the survival of the organism is compromised and in more extreme cases they can be lethal. The irregular number of chromosomes can be the basis of spontaneous abortions and development deficiencies and be connected with cancer and aging (Hwang et al., 2019). For example, due to an extra 18 chromosome, in the condition recognized as Edwards syndrome (trisomy 18 syndrome), the frequency of fetal loss is higher, prenatal deficiencies in growth occurs, characteristic craniofacial structure; major deformities; and minor abnormalities are manifested, and slow development at cognitive and psychomotor level takes place (Cereda & Carey, 2012).

1.2.1 - Oxidative Stress

Oxidative stress, in aerobic organisms, occurs when the equilibrium between reactive oxygen species (ROS) and the antioxidant defense system of cells is disrupted. ROS have oxygen atoms in their chemical structure, such as superoxide anions ($O_2^{\cdot-}$); hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), the most recognized ROS. ROS molecules can be free radicals and also non-radicals, the former being the most hazardous ones since they possess one or more unpaired electrons that react with biomolecules, causing chemical alterations (S. C. Gupta et al., 2012).

The imbalance between oxidants and antioxidants inside organisms is caused and/or favored not only by exogenous factors, for instance, radiation, pollutants, smoke, tobacco, xenobiotics and heavy/transition metals (Warraich, Hussain, & Kayani, 2020), but also by endogenous factors, for example, mitochondrial

respiratory chain and the activity of enzymes, such as, NADPH oxidase (NOXs), nitric oxide synthases (NOS), xanthine oxidase, cyclooxygenase, lipoxygenase, and cytochrome P450 (Gangwar, Bevan, Palanivel, Das, & Rajagopalan, 2020). Superoxide anions are generated after the reduction of oxygen by the electrons previously leaked to the mitochondrial matrix and the intermembrane space, during the electron transport chain and within its complexes, namely, ubiquinone binding sites of complex I and III, flavin prosthetic group of complex I, ubiquinone oxidoreductase, glycerol 3-phosphatase dehydrogenase and electron transferring flavoprotein (Brand, 2010; Figueira et al., 2013). After that, $O_2^{\cdot-}$ are converted to H_2O_2 by superoxide dismutase. Later, H_2O_2 oxidizes a transition metal, like iron (II) into iron (III), and hydroxyl radicals and hydroxide anions are formed after this reaction, known as Fenton reaction (Fig. 4).

Figure 4. Fenton Reaction: hydrogen peroxide (H_2O_2) oxidizes iron (II) (Fe^{2+}) into iron (III) (Fe^{3+}), hydroxyl radicals ($OH^{\cdot}$) and hydroxide anions (OH^-).

The outcomes of cumulative oxidative stress are lipid peroxidation, protein oxidation, DNA and RNA damage, mainly for the reason that these macromolecules interact with highly reactive hydroxyl radicals that result from Fenton reaction. After the irreversible damage of DNA, RNA, proteins and lipids and the accumulation of this type of lesions, the process of pathogenesis begins. Several studies established a connection between oxidative stress and several ailments, such as, several types of cancer (Cheung et al., 2020; Cruz-Bermúdez et al., 2019; Yang, Guo, Albers, Sehouli, & Kaufmann, 2019), neurodegenerative diseases, cardiac pathologies (Boulghobra et al., 2020; Sverdlov et al., 2016; Yao et al., 2015), diabetes (Manea et al., 2018; Zujko, Witkowska, Górska, Wilk, & Krętowski, 2014) and also the progression of aging. On the other side, when cells are in redox homeostasis, ROS are crucial in numerous and diverse biological processes, such as, cell signaling transduction, differentiation, migration, proliferation, apoptosis (e.g. tumor cells), regulation of the cell cycle, immune response, inflammation and gene transcription (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018; Warraich et al., 2020).

To control and stabilize ROS levels, organisms possess a complex antioxidant defense system machinery, constituted by enzymatic and non-enzymatic antioxidants (endogenous antioxidants). They are scavengers of ROS but they also take action at different levels. Firstly, a portion of antioxidants avert reactions between ROS and biological structures, secondly, other antioxidants are able to inactivate ROS swiftly, the rest of antioxidants repair the damages caused by ROS (Mirończuk-Chodakowska et al., 2018). The most

prominent enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD regulates superoxide radical's levels, after their generation in mitochondria, thus this enzyme converts $2O_2^{\cdot}$ into H_2O_2 and $O_2^{\cdot}$ (table 1). The concentration of H_2O_2 is managed by catalase, glutathione peroxidase, peroxiredoxins (Prx) or thioredoxin-2 (Trx), depending on the localization of H_2O_2 (Figueira et al., 2013).

Table 1 – Examples of oxidants and the corresponding antioxidants and the reactions between them

Oxidant	Antioxidant	Reaction
Superoxide	Superoxide dismutase (SOD)	$2O_2^{\cdot} + H^+ \longrightarrow O_2 + H_2O_2$
Hydrogen peroxide	Catalase (CAT), glutathione peroxidase (GTPx), peroxiredoxin (PRX)	$2H_2O_2 \longrightarrow O_2 + 2H_2O$ $H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$ $H_2O_2 + TrxS \longrightarrow Trx(SH)_2 + H_2O$

Beyond endogenous antioxidants, organisms can resort to exogenous antioxidants, through their diet, with the ingestion of fruits, vegetables, and cereals, since they are sources of, for example, vitamins, polyphenols, minerals with antioxidant activity. Furthermore, exogenous and endogenous antioxidants possibly act synergistically in the maintenance of ROS levels. One strategy to retard the progression of illnesses associated with age is a diet rich in antioxidants (Catalán, Barrubés, Valls, Solà, & Rubió, 2017).

1.2.2 - Hydroxyurea

Hydroxyurea (HU) or hydroxycarbamide was synthetized for the first time in 1869 by Dresler and Stein (Dresler & Stein, 1869) as an attempt to generate derivatives of urea. These doctors accomplish this using hydrochloric acid, potassium cyanide and hydroxylamine (Dresler & Stein, 1869). In the 60's decade, *in vitro* studies were made and the results revealed that HU counteracted leukemia cell lines and some types of tumors, later, in the same decade HU was introduced in the medical scene as antineoplastic agent (Marahatta & Ware, 2017; Stearns, Losee, & Bernstein, 1963).

HU is currently used as medication in the management of some blood related diseases and cancers, which includes sickle cell anemia, myeloid leukemia, polycythaemia vera and essential thrombocythemia (Marahatta & Ware, 2017). HU is applied in large scale in the management of sickle cell anemia (SCA) since it stimulates fetal hemoglobin production (Davies & Gilmore, 2003). HU is applied in chemotherapy because this molecule targets proliferating cells and suppresses DNA replication through the inhibition of ribonucleotide reductase (RNR) activity (Fig. 5, Fang et al., 2019).

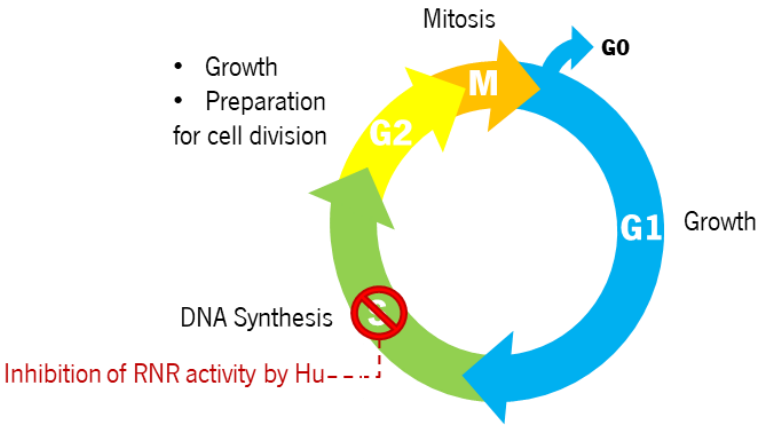


Figure 5. Inhibition of DNA synthesis by Hydroxyurea (HU).

The role of RNR in DNA synthesis is essential because it catalyzes the reduction of ribonucleoside 5'-diphosphates (NDPs) into 2'- deoxyribonucleoside 5'-diphosphates (dNDPs), which contributes to the equilibrium of DNA precursors. HU inhibits specifically the activity of Class I RNRs (Jordan and Reichard, 1998). RNR is an enzyme involved in DNA replication and also repair, as a result, the proliferation of cells can be on jeopardy when this enzyme is inactivated, since their cycle can be under arrest at G1/S phase due to unbalanced deoxyribonuclieotides pool and stalling of replication forks. As a result, the management of RNR action not only is crucial in DNA replication but is also relevant in the maintenance of genome stability (Herrick & Sclavi, 2007).

1.3 - DNA Damage Response (DDR)

Mechanisms of defense and repair of DNA were developed and selected along millions of years in all forms of life but their complexity differs from species to species. The DNA damage response (DDR) is defined as a myriad of mechanisms and procedures that are initiated to face aggressions from external and internal sources that target DNA. DDR includes cell cycle checkpoints, senescence, apoptosis, transcription and DNA repair mechanisms (Ghosal & Chen, 2013).

At cellular level, in response to DNA damage, there are checkpoints in different phases of the cell cycle (G1-S, intra-S and G2/M, and M phase) that are triggered and the normal progression of these processes is blocked or delayed in the phase where DNA lesions were verified (Fig. 6).

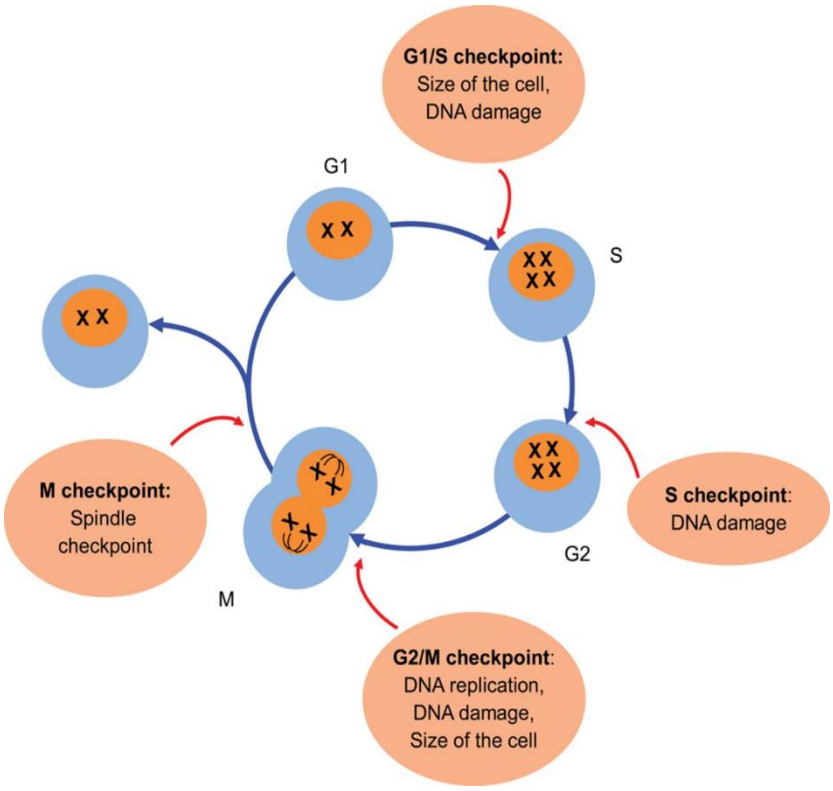


Figure 6. Cell cycle and different checkpoints (Canaud & Bonventre, 2014).

Chao and collaborators (Chao et al., 2017) showed with their study that in both checkpoints, in G1 and G2 phase, the progression of the cycle stops, however, the checkpoint in G2 is more sensitive to DNA injury. The checkpoint at S phase is more tolerant in relation to DNA damage since it only slows the time length of this phase instead of suspension. In some occasions, DNA lesions are so severe that more drastic outcomes occur such as cellular senescence (permanent cellular proliferation arrest) and apoptosis (programmed cell death).

The most relevant pathways for DNA restoration upon DNA damage are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ).

The BER mechanism is activated when lesions as deamination, alkylation and oxidation cause base damage. Base excision repair (BER) begins with the recognition of the damaged base by DNA glycosylase, afterwards, DNA glycosylase cleaves the base in question, which creates apurinic or apyrimidinic (AP) sites. Next, AP endonuclease I creates a cut resulting 3'-OH and 5'- deoxyribose phosphate termini at the abasic site of DNA backbone and then polymerase exchanges the 5'-deoxyribose into 5'-phosphate and induces the incorporation of nucleotides on the 3'-OH. At the end of BER, DNA ligase closes the gap at the backbone (Schermerhorn & Delaney, 2014).

Nucleotide excision repair (NER) pathway is induced after intrastrand crosslinks and bulky base adducts are originated by UV radiation (Evdokimov et al., 2018). In this mechanism, after the recognition of DNA injury, the affected nucleotide is removed from the DNA strand after cutting a segment with 25 or 30 nucleotides (where the nucleotide in question is localized) by exonucleases. Later, the repair synthesis (reconstruction of DNA segment from a normal opposite DNA strand) and ligation follows (Prakash & Prakash, 2000; Spivak, 2015).

Mismatch repair (MMR) occurs to fix small insertions, deletions and misincorporations of bases that arise from DNA replication errors (Blanpain, Mohrin, Sotiropoulou, & Passegué, 2011). In summary this process has three stages: licensing, degradation and resynthesis (Peña-Diaz & Jiricny, 2012). First, MutSα connects to the place where the error was detected, then Mutα forms a ternary complex with MutSα. Afterwards, this MutSα/Mutα complex search for PCNA (proliferating cell nuclear antigen), that was previously charged into the 3' nick terminus by RFC (replication factor c) (D. Gupta & Heinen, 2019; Peña-Diaz & Jiricny, 2012). In the degradation step, the MutSα/Mutα complex loads EXO I (exonuclease I) into the nick (previously generated by this complex), then the strand is degraded, starting in the nick and finishes after 150 nucleotides (Peña-Diaz & Jiricny, 2012). Ultimately, polymerase δ and DNA ligase end this process and also PCNA is also implicated in this stage.

Double strand breaks arise from correction of DNA replication errors, inadequate activity of nuclear enzymes, ionizing radiation and ROS existence (H. H. Y. Chang et al., 2017). They can be fixed by two distinct mechanisms of DNA repair that can compete with each other or function together, the non-homologous end joining (NHEJ, Fig. 7) and the homologous recombination (HR). NHEJ mechanism of DNA repair, takes action when DSBs occur during G0/G1 phase in the cell cycle (H. H. Y. Chang et al., 2017). In this repair pathway, DSBs are directly joined, this process has four key stages: DNA end recognition, association and maintenance of NHEJ machinery; joining of DNA ends; DNA ends processing; ligation of the ends and dissociation of NHEJ machinery (Davis & Chen, 2013). First the heterodimer ku70-Ku80 (Ku) identifies and attaches to DSBs sites, later, this heterodimer function as carrier of other proteins necessary to the DNA end joining process, like DNA dependent protein kinase catalytic subunit (DNA-PKcs) that combined with Ku originates the DNA-Pk complex (H. H. Y. Chang et al., 2017). NHEJ process is finalized when the complex XRCC4/DNA ligase IV reconnects DNA strands (Davis & Chen, 2013). As intact DNA is not employed as a template, nucleotide changes, deletions, insertions and translocations can happen (Blanpain et al., 2011).

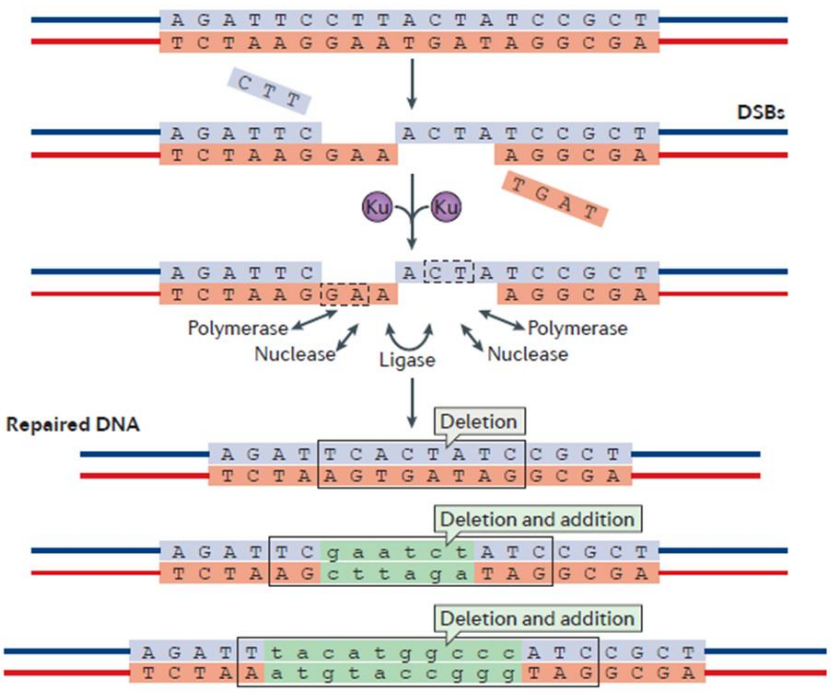


Figure 7. Non-homologous end joining (NHEJ) mechanism of DNA double strand breakage (adapted from: H. H. Y. Chang, Pannunzio, Adachi, & Lieber, 2017)

HR (Fig.8), similar to NHEJ, is initiated as a response to DNA double strand breaks, but happens during S-G2/M phases (Blanpain et al., 2011; Wright, Shah, & Heyer, 2018). HR is considered more accurate than NHEJ because in this procedure undamaged sister chromatids or homologous chromosomes are used as DNA templates (Blanpain et al., 2011; Ghosal and Chen, 2013). First, DNA 5'-ends are resected by the MRN complex (MRE11-Rad50-Nbs1) to form 3'-ends (single strand DNA, ssDNA) overhangs (Yan et al., 2019). Replication protein A (RPA) then shields the 3'-ends from nucleases and avoids DNA coiling (Mazina et al., 2020). Substitution of RPA by RAD51 is necessary for the progression of HR, because RAD51 is implicated in the search of homology and strand invasion, this happens with the help of and BRCA 2 (Jensen, Ozes, Kim, Estep, & Kowalczykowski, 2013). As a result of invasion by 3'-ends of the homologous chromosome, D-lops structures are formed. Then, the invading DNA strand elongation occurs due to the action of DNA polymerase, which uses as a template the evaded DNA strand. Later, branch migration of holiday junction occurs. Posteriorly, the holiday junctions (two) are cleaved and individual DNA molecules are produced, this step is termed resolution (Wright et al., 2018). Cross over HR repair happens when the final product is the outcome of DNA exchange between homologous chromosses, the non-cross over HR repair is the opposite.

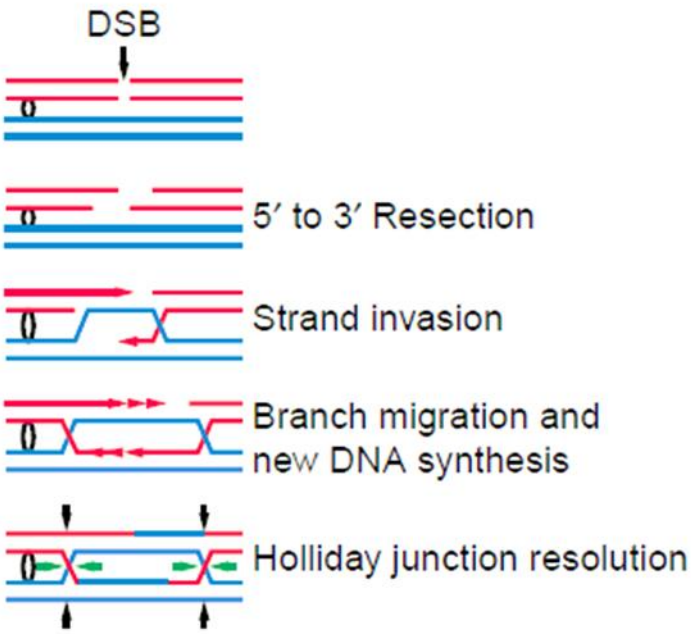


Figure 8. Simple scheme of Homologous recombination of DNA double strand breaks (adapted from: Hiom, 2001)

1.4 - Phytochemicals and their potential as nutraceuticals

From ancient times humans had employed plants in their diet, medicine, cosmetics, perfumes, and recreational time (e.g. tobacco). Currently, the interest by scientific community, that began in recent years, in phytochemicals to prevent the development of pathologies or to combat their progression increases continuously. Polyphenols and flavonoids (Fig. 9) are examples of those phytochemicals in question, since, previous studies proved their antioxidant activity (López-Cobo, Gómez-Caravaca, Švarc-Gajić, Segura-Carretero, & Fernández-Gutiérrez, 2015; Mendoza, Cuaspud, Arias, Ruiz, & Arias, 2018; Sytar, Hemmerich, Zivcak, Rauh, & Brestic, 2018; Thiruvengadam & Chung, 2015) . As previously referred, oxidative stress is implicated in the initiation and/or evolution of innumerous diseases, such as, cancer (Cheung et al., 2020; Gill, Piskounova, & Morrison, 2017; S. C. Gupta et al., 2012), cardiac (Boulghobra et al., 2020; Sverdlov et al., 2016; Yao et al., 2015) and neurodegenerative diseases (Griñán-Ferré et al., 2021; Madreiter-Sokolowski, Thomas, & Ristow, 2020; Pan, Liu, Zhao, Wu, & Liu, 2020) and diabetes (Bahadoran, Mirmiran, & Azizi, 2013).

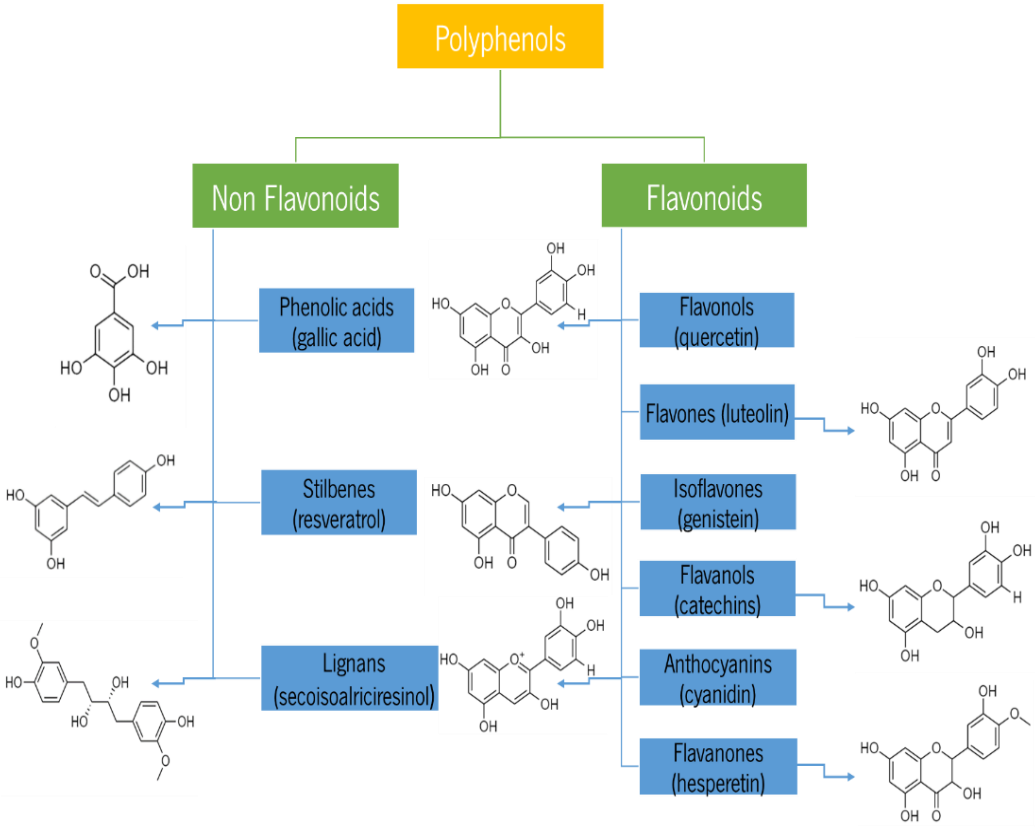


Figure 9. Classification of polyphenols and examples of polyphenols and their chemical structure

Polyphenols (PPs) are secondary metabolites of plants, they take part in defense mechanisms against pathogens and herbivores, adaptation to environmental stress factors (e.g. UV radiation) and also act as signaling molecules (Kim, Pälijärvi, Karonen, & Salminen, 2020; Mendoza et al., 2018). The general chemical structure of PPs is one aromatic ring or rings with hydroxyl groups attached to them (Fig. 9). Considering their structural differences, specifically, the number of aromatic rings, the elements that bind the rings and the functional groups connected to the aromatic rings, PPs are divided into two major classes, flavonoids and non-flavonoids, additionally, non-flavonoids PPs are further sub classified in four groups, phenolic acids (e.g. gallic acid), lignans (e.g. secoisoalriciresinol), stilbenes (e.g. resveratrol) and other polyphenols (Fig.9, Behl et al., 2020; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). The structural base of all flavonoids is composed by two aromatic rings connected by a three carbon atoms chain and a heterocyclic ring. This class of PPs can also be separated in six categories, flavonols, flavones, isoflavones, flavanols, anthocyanins, flavanones (Fig. 9) (Abotaleb et al., 2018). They exhibit biological activities of great interest, such as, antioxidant, anticancer, antiviral, anti-inflammatory and anti-ageing (Jia et al., 2020).

According to the World Health Organization (WHO), in the top ten causes of death worldwide in 2019, are heart diseases (e.g. ischemic heart disease, which is at the top), dementia (e.g. Alzheimer) and diabetes.

Since cancer, cardiovascular disorders, neurodegenerative disease, and diabetes are intimately associated with oxidative stress and ROS, one possible way to prevent and treat these illnesses is the intake of dietary polyphenols and also the use of extracts from medicinal plants (Bahadoran et al., 2013; Behl et al., 2020; Bhullar & Rupasinghe, 2013; de Carvalho et al., 2020; Khurana, Venkataraman, Hollingsworth, Piche, & Tai, 2013; Samodien et al., 2019). Fruits, vegetables, teas, coffee, cocoa, red wine and extra virgin olive oil are the main sources of nutritional PPs (Catalán et al., 2017; D'Archivio, Filesi, Varì, Scazzocchio, & Masella, 2010). However, PPs are metabolized by hepatic and intestinal enzymes and also by the intestinal microflora (Behl et al., 2020), their bioactivity can be affected, besides, their bioavailability differ from each other (Manach et al., 2004). Important knowledge is still missing, as a result, new studies about several diseases pathways and biological properties of medicinal plants are mandatory to develop new preventive and treatment strategies to avoid pathogenesis or mitigate their consequences. *Dittrichia viscosa* is a medicinal plant and has high levels of polyphenols, consequently, this plant species is a matter of interest.

1.5 - *Dittrichia viscosa*

Dittrichia viscosa (L.) Greuter (the accepted scientific terminology) also known as *Inula viscosa* (L.) Ainton (designation present in the majority of published scientific papers); *Erigeron viscosus* (L.), *Cupularia viscosa* (L.) Godr. & Gren.; *Jacocobaea viscosa* (L.) and more ordinary names like inula, aromatic inula, false yellowhead, woody fleabane and sticky fleabane, is a typical plant species from countries that surrounds de Mediterranean Sea but it can also be present in North America, United Kingdom and Belgium (Hertel et al., 2016; Seca, Grigore, Pinto, & Silva, 2014; Trimech et al., 2014). This herbaceous (possesses a flexible stem) perennial plant species belongs to the *Dittrichia* genus, Asteraceae family and Asterales order. *Dittrichia viscosa* specimens can be easily recognized since they can grow up between 1 m and 2 m in abandoned or neglected areas, and resemble a small shrub, they exhibit alternate slim leafs and yellow flowers in flowering season (Fig. 10, Danino, Gottlieb, Grossman, & Bergman, 2009; Haoui, Derriche, Madani, & Oukali, 2015; Hertel et al., 2016; Trimech et al., 2014).

Parts of this plant, decoctions, ointments, cataplasms of *D. viscosa* had been used to treat a large spectrum of pathologies and their inherent symptoms, from antiquity. The use of *D. viscosa* persists in traditional medicine, these days, in countries such as Morocco, Algeria, Spain, Italy, Israel and others, since this plant reveals anti-inflammatory, antipyretic, antiseptic, antimicrobial and antiproliferative properties (Brahmi-Chendouh et al., 2019; Seca et al., 2014).

Figure 10. The typical yellow flowers of *Dittrichia viscosa* (author: Miguel Porto).

Fifty one phytochemicals were identified in this species, from an ethanolic extract of *D. viscosa* leaves, counting 11 phenolic acids, 23 flavonoids, 1 lignan and 12 terpenoids, with liquid chromatography coupled to photodiode array detection and electrospray ionization mass spectrometry (LC-DAD-ESI-MS/MS) (Kheyar-Kraouche, da Silva, Serra, Bedjou, & Bronze, 2018). Several scientific reports had attested the antiradical and antioxidant activity of extracts from *D. viscosa*, due to the existing PPs in this plant (Brahmi-Chendouh et al., 2019; Brahmi et al., 2015; Chahmi et al., 2015; Danino et al., 2009; Kheyar-Kraouche et al., 2018; Wafa Rhimi et al., 2019; Schinella, Tournier, Prieto, Mordujovich de Buschiazzo, & Ríos, 2002; Trimech et al., 2014). It was demonstrated, with an ethanolic extract, that the most representative PPs are dicaffeoylquinic acids derivatives and quercetin derivatives, which can explain the antioxidant activity (Wafa Rhimi et al., 2019). Furthermore, 1,3-dicaffeoylquinic acid was studied in more detail and it was verified that the acid in question is a scavenger of superoxide radicals and hydroxyl radicals (Danino et al., 2009).

Sesquiterpenes lactones isolated from *D. viscosa*, such as, tomentosin and inuviscolide, revealed anticancer activities. Since they inhibited the proliferation of different human melanoma cell lines (SK-28, 624 mel, 1363 mel) depending on the dosage. The cell cycle was arrested at G2/M phase and apoptosis occurred (Rozenblat et al., 2008). The same biological events were observed in human cervical cancer cell lines (HeLa and SiHa) subjected to hexane and dichloromethane fractions from *D. viscosa* extracts. Additionally, telomere shortening and the decrease of tumor cells resistance was also observed (Merghoub et al., 2016). Furthermore, it was proved, specifically, the association of tomentosin and cytotoxicity of human cervical cancer cells (SiHa and HeLa), because this compound can shorten

telomeres, arrest cell cycle at G2 and M phases and trigger apoptosis (with diminution of mitochondrial membrane potential and caspase dependent cascade) (Merghoub et al., 2017). In addition, cytotoxic effect of *D. viscosa* soxhlet (chloroform and methanol) extracts against neuroblastoma (SH-Sy5Y), hepatoblastoma (HepG2) and colon carcinoma (HCT 116) cells was also detected due to inhibition of mitochondrial redox activity and cell viability in a dose-dependent manner (Brahmi-Chendouh et al., 2019). In opposition, the immortalized human keratinocytes (HaCaT) were not affected at that degree (Brahmi-Chendouh et al., 2019).

Different types of extracts made from this plant exert different biological activities important to humans, such as, antibacterial and antifungal (Hertel et al., 2016; Masadeh, Alkofahi, Alzoubi, Tumah, & Bani-Hani, 2013; Miguel, Faleiro, Cavaleiro, Salgueiro, & Casanova, 2008; W. Rhimi et al., 2017; Wafa Rhimi et al., 2018; Talib & Mahasneh, 2010), antiviral (Abad, Guerra, Bermejo, Alicia, & Carrasco, 2001), wound healing (Khalil, Afifi, & Al-Hussaini, 2007; Wafa Rhimi et al., 2019), anti-inflammatory (V. Hernández et al., 2001; Victoriano Hernández, Recio, Máñez, Giner, & Ríos, 2007; Máñez, Hernández, Giner, Ríos, & Recio, 2007; Wafa Rhimi et al., 2018; Schinella et al., 2002) and hypoglycemic (Orhan, Gökbulut, & Deliorman Orhan, 2017; Zeggwagh, Ouahidi, Lemhadri, & Eddouks, 2006; Zhang et al., 2010) properties. Differences between results from scientific papers about *D. viscosa* are explained by different origins of samples (which means that the abiotic and biotic conditions were not the same), diverse methods of extraction and analysis, and various procedures to assess the biological properties.

1.6 - *Schizosaccharomyces pombe* as a model organism

Schizosaccharomyces pombe is a unicellular haploid fungus that belongs to the Ascomycota phylum (Farlow et al., 2015). This yeast, also called fission yeast due to its process of cellular division (Fig. 11) where their tips extend and division happens trough medial fission, was isolated for the first time in 1890, from East African millet beer, by Paul Lidner (Forsburg, 2005; Patterson, Swaffer, & Filby, 2015). Fission yeast exhibits a rod morphology, its length ranges from 7 to 14 µm and its diameter varies between 3 and 4 µm (Pérez, Cortés, Cansado, & Ribas, 2018). Usually, new yeasts are the outcome of asexual reproduction through fission. But yeasts switch to sexual mode of reproduction, in this case sporulation, when they're in deprived environment. Then the previously formed haploid spores are united and diploid yeast cells are the end result (Egel, 2013; Farlow et al., 2015; Tusso et al., 2019). *S. pombe* is worldwide dispersed, they can be founded in fermentations of plants (Egel, 2017).

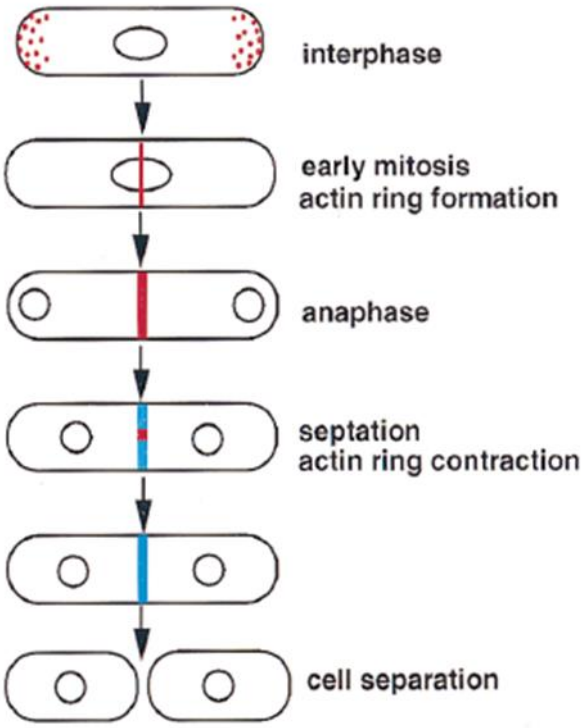


Figure 11. Process of cell division in fission yeast (adapted from: F. Chang & Nurse, 1996).

S. pombe is distant from *Saccharomyces cerevisiae* (budding yeast) in terms of evolution and taxonomy. Unlike budding yeast, the fission yeast has more similarities with higher eukaryotes (Egel, 2013; Takegawa et al., 2009). After a few decades of its discovery, specifically in the 40's, genetic studies with *S. pombe* were performed by Leupold, moreover, the majority of strains that are employed nowadays in experimental works derive from Leupold's cultures (Egel, 2017; Leupold, 1949). Later, in the 50's, fission yeast was employed in many studies to understand biological processes, like, cell cycle, cell morphology, meiosis, genome organization and preservation, gene regulation, signaling and stress responses. In 2002, an international consortium of scientific institutions published the complete genetic sequence of *S. pombe*, becoming the sixth eukaryotic genome fully sequenced and annotated (Wood et al., 2002). The total 13.8 Mb genetic information of fission yeast is organized in three chromosomes I, II and III with 5.7 Mb, 4.6 Mb and 3.5 Mb respectively, plus the 20 kb mitochondrial genome (Wood et al., 2002).

S. pombe is an excellent model system for several reasons, its life cycle is short (between 2 to 4 hours), the conditions of maintenance are well established and the costs are low. It can grow in solid and liquid medium and a collection of wild type strains and mutants are available for scientific research. Since its genome is totally sequenced and annotated, several genomic edition tools are available. In addition, fission yeast genome is more similar to higher eukaryotes then *S. cerevisiae*, for example more than 40 % of fission yeast genes possess introns (non-coding sequences) on the contrary budding yeast genes

only show 5% of introns (Forsburg, 2005). For that reason, *S. pombe* is utilized to understand the biochemical basis and course of human diseases (Harris, Lock, Bähler, Oliver, & Wood, 2013; Patterson et al., 2015). There is an increasing interest in *S. pombe* as a model organism in comparison with *S. cerevisiae*, which can be translated in the crescent number of scientific papers in the last decades. Nevertheless, an information void persists about evolution, ecology and diversity about this yeast species.

2 - Objectives

Nowadays, some current therapeutic strategies employed to treat some pathologies, like cancer, or their symptoms, show some drawbacks, such as lack of efficacy, side effects and inefficiency to combat some bacteria and fungi. To overcome these issues, it is crucial to develop alternatives, for instance, scientists can resort to compounds that are already available in nature. Plants synthetize phytochemicals that provide many human health benefits. As such, they can be an option to create new treatments or supplements (to synergistically potentiate the efficacy of therapeutic agents already available).

To illustrate the previously described situation, for example, it is known that oxidative stress is associated with several pathologies, such as, several types of cancer, neurodegenerative diseases, cardiac pathologies, diabetes and the aging process. One possible prophylactic measure to avoid or delay their development is the consumption of aliments rich in antioxidants or functional foods.

The employment of plants in the pharmaceutical industry offers several advantages, decrease of production costs, because it is not necessary to synthetize the required active principle. Since the human population is continuously increasing, it is necessary to produce medication in large scale at low cost. Pharmaceutics could resort to weeds, typically regarded as something without value and a plague, since they possess compounds with nutraceutical properties and exist in abundance.

One of those weeds, that possesses potential to be employed in the previous mentioned situation, is *Dittrichia viscosa* subsp. *revoluta*. Previous studies reported that extracts from this plant have booth antigenotoxic activity and cytotoxic activity. Consequently, the main purpose of this work is to collect new data about a subspecies of the mentioned plant present in Portugal. More specifically if the aqueous extract of this plant has antigenotoxic activity. These studies are important to understand and realize both beneficial and hazardous effects of plants to humans and the environment. Additionally, they are fundamental for the development of new medications or nutraceuticals with antioxidant and antigenotoxic activities that can be taken isolated or in combination with other medicines.

3 - Materials and Methods

3.1 - Fission yeast as model organism

In this work the fission yeast *S. pombe* was employed as model organism to evaluate cell viability with fluorescence microscopy. The strain utilized in this study was the wild type (wt) 972h-.

3.2 - Plant material collection and extraction

Young stems and leaves were collected in Gambelas (Faro, Portugal) from *Dittrichia viscosa* specimens in the wild, in June/July of 2017. After collection, the vegetal material was dried at 40 °C until stable weight (24-48 h) and grounded with a blender until a powder with particle size lesser than 2 mm was obtained. Later, boiling distilled water was added to the plant material (5 min), the infusion was putted at rest until room temperature was reached (10 min). Afterwards, the infusion was filtered, frozen and lyophilized. The resultant extraction yield was 32.67%. The steps previously described were performed in Anabela Romano's lab at the University of Algarve. The aqueous extract of *D. viscosa* (DVAE) was made by solubilizing the lyophilized sample with ultra-pure water (50 mg/ml) in a boiling water bath and then the final solution was filtered with syringe filter of 0.2 µm. Afterwards the stock solution was employed in the *in vitro* assays.

3.3 - *In vitro* assays

3.3.1 - DPPH scavenging activity

The antioxidant activity, via free radical scavenging, of the extract was assessed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. DPPH is a stable radical that exhibits a purple color, after its reduction his typical color progressively changes to yellow.

Firstly, an ethanolic stock solution of DPPH 0,04% (p/v, 10 x) was made with of absolute ethanol. Different solutions of extract (2.5; 5; 10; 15; 40 and 50 µg/ml) and gallic acid (0.375; 0.5; 0.75; 1.25 and 1.5 µg/ml) were prepared with ultra-pure water and ethanol, respectively. Later, 50 µl of ultra-pure water or ethanol and 100 µl of DPPH were employed as negative controls. One hundred microliters of ultra-pure water or ethanol and 50 µl of sample were utilized as blanks. Fifty microliters of extract or gallic acid and 100 µl of DPPH (samples) were added in a 96-well plate. After 20 min of incubation, at room temperature and at the dark, the blanks, controls and samples observances were measured at 517 nm in microplate reader. Gallic acid was used as a positive control. The anti-oxidant activity of samples was expressed in percentage (%) of DPPH inhibition according to the following equation:

$$\% \text{ Inhibition} = \frac{100 \text{ x } (\text{Abs blank} - \text{Abs sample})}{\text{Abs blank}}$$

Where $\mathrm{Abs\ blank}$ corresponds to the absorbance value of blanks and $\mathrm{Abs\ sample}$ corresponds to the absorbance value of samples.

3.3.2 - Total phenolic content (TPC)

The total phenolic content of the extract was measured with Folin-Ciocalteu method. Molybdenum and tungsten (that are present in Folin-Ciocalteu reagent) when reduced, by for example phenolic compounds, forms a blue complex. TPC was calculated using the adapted version (Granato, Katayama, & de Castro, 2011) of Folin-Ciocalteu assay (Singleton & Rossi, 1965).

Briefly, 18.18 µl of extract (25; 50; 100; 200; 400 and 500 µg/ml) or gallic acid (2, 5, 20, 30 and 40 µg/ml) were mixed with 18.18 µl of Folin-Ciocalteu reagent and 145.5 µl of ultra-pure water in 96 well-plate. After 5 min, 18.18 µl of a previously prepared sodium carbonate solution (0.16 g/ml) was added to the mixture and the plate was protected from the light at room temperature for 60 min before reading of the absorbances at 725 nm with a microplate reader. Later, the TPC was calculated with the calibration curve of gallic acid standard, results were expressed as µg of gallic acid equivalents (GAE)/ µg of extract.

3.4 – Assessment of DVAE antigenotoxicity

3.4.1 – Assessment of viability of *S. pombe* cells – Overnight and 6 h treatments

Acridine orange/propidium iodide dual staining was employed to detect apoptosis since acridine orange permeates the nucleus and when binds to dsDNA and then emits green fluorescence. On the other hand, propidium iodide only binds to DNA, by intercalating between the bases, of dead cells whose cell wall integrity is compromised, and red fluorescence is emitted. Hoechst 33342 staining was applied to examine the nuclear morphology of cells. This marker emits blue fluorescence when exposed to ultraviolet light.

Initially, cultures were grown in YES medium (glucose 30 g/L, yeast extract 5 g/L, adenine 0.02 g/L, uracil 0.02 g/L, histidine 0.02 g/L, leucine 0.02 g/L, lysine 0.02 g/L, and water) to the next day, until an $OD_{595\ nm}$ of 0.2 and 0.9 was reached. Posteriorly, cells were distributed between 4 aliquots and treated with *D. viscosa* extract (1000 µg/ml) and or hydroxyurea (12 mM) and incubated for 6 h or overnight at 30 °C with 200 rpm. Two hundred microliters of cell suspension from each aliquot, was centrifuged (30 °C, 200 rpm) and the supernatant was rejected. After, cells were resuspended with 50 µl acridine orange/propidium iodide mixture that was previously prepared (5 µl of acridine orange (1 mg/ml), 3 µl of propidium iodide (1 mg/ml) and 1 ml of PBS solution) and 20 µl of sample was observed under florescence microscope. Moreover, 200 µl of cell suspension from each aliquot was washed with PBS two times, and 10 µl of cells were fixed at the flame. Then, 4 µl of Hoechst (5 mg/ml) was placed above

the earlier fixed cells. Then cells were examined under fluorescence microscopy and photographs were taken. The photographs were taken in three days.

3.4.2 – Yeast viability

From the previously taken photographs with the fluorescence microscope, from overnight and 6 h treatments, 100 fission yeasts, randomly selected, from each group (cells from the negative control, cells from extract control, cells exposed to HU and cells exposed to HU and the extract) viable yeasts and yeasts in apoptosis were counted, from three independent experiments (100 x 4 x 3).

3.4.3 – Assessment of cellular length

Also, for yeasts length measurements, the photographs already taken with the fluorescence microscope, from overnight and 6 h treatment, were used. Yeast length of 100 cells per sample from three independent experiments was measured with Gimp, image edition program (total number of counted cells: 100 x 4 x 3). Yeasts were randomly selected and then measured.

3.4.4 – Cell cycle progression monitoring - Mitotic Index

Cultures of fission yeast were grown in YES liquid medium, overnight at 30 °C and 200 rpm. In the next day, the yeast culture was diluted to a OD of 0.3. After, to synchronize the cell cycle of yeasts, HU (12 mM) was added and the culture was incubated for 3 h (30 °C a 200 rpm). Before and after the cell cycle synchronization, from the original suspension 50 µl were collected, centrifuged and washed with cold distilled water (dH_2O). Next, they were resuspended in 50 µl of EtOH 70 % and stored at 4 °C (samples -3 and +3). The suspension was centrifuged (30 °C, 200 rpm) and washed with cold dH_2O. After, yeasts were resuspended in 2.5 ml of YES liquid medium, distributed between 4 falcons of 50 ml as follows: 500 µl of cell suspension (negative control); 450 µl of suspension and 50 µl of *D. viscosa* extract (extract control); 470 µl of suspension and 30 µl of HU; 370 µl of suspension and 50 µl of *D. viscosa* extract and 30 µl of HU, and incubated (30 °C a 200 rpm). Subsequently, from each falcon 50 µl of sample was collected from each 30 to 30 minutes for a 3h period. Later, cells were centrifuged (30 °C, 200 rpm) and washed with cold dH_2O. Then, cells were resuspended in 70 % ethanol and stored at 4 °C. From each sample 10 µl were collected and stained with 10 µl of Prolong Gold antifade reagent with DAPI for fluorescence microscope visualization of nucleus. Then a series of photographs were taken for counting purpose. One hundred cells per sample (these corresponds to yeasts from the controls, yeasts exposed to HU and yeasts exposed to HU and the extract, collected between 1 h and 3 h, with intervals of 30 min) were randomly selected and counted. Within the 100 cells, the number of dividing cells (cells with two nucleus) was also counted. The counts were made from three independent experiments (total number of counted cells: 100 x 4 x 5 x 3). Posteriorly, the mitotic index was calculated with the following equation:

$$Mitotic\ Index\ (\%) = \frac{total\ nº\ of\ dividing\ cells}{total\ number\ of\ cells\ observed}\ x\ 100$$

3.5 - Statistical analysis

Three replica were done for all the assays in this work. The results from DPPH assay is presented with medium and standard deviation (SD). Statistical analyses of yeast viability and length measurements were made with the program GraphPad Prism 7.00 using the 1-way ANOVA-multiple comparisons, Turkeys multiple comparisons test, the results are presented with medium and standard error of mean (SEM) with $p < 0.001$; $p < 0.004$; $p < 0.0374$.

4 – Results

4.1 - Antioxidant activity of the *D. viscosa* extract

Plant extracts are rich in PPs that are reported as having antioxidant activity, therefore, this study started by assessing the radical scavenging capacity and the TPC of DVAE. The DVAE exhibited antioxidant activity against the DPPH radical. The extract IC50 was 7.69 ± 0.03 µg/ml (Table 2). The IC50 of gallic acid (compound used as positive control), 0.62 ± 0.03 µg/ml, was lower in comparison with the studied extract (Table 2). Moreover, both standard and extract demonstrated antiradical activity in a dose dependent manner. The TPC of the studied extract was 0.12 µg GAE/ µg of extract (table 2).

Table 2 – Results from DPPH and Folin-Ciocalteu assays with *D.viscosa* aqueous extract

	IC_{50} for DPPH (µg/ml)	TPC (µg GAE/ µg of extract)
DVAE	7.69 ± 0.03	0,12
Gallic acid (Standard)	0.62 ± 0.03	

4.2 – Assessment of DVAE antigenotoxicity

4.2.1 – Assessment of viability of *S. pombe* cells

Primarily to investigate the possible cytotoxic effects of *D. viscosa* aqueous extract, samples of each treatment were collected and stained with a mixture of acridine orange and propidium iodide. Acridine orange and propidium iodide are nuclear stains but while, acridine orange stains the nuclear of live and dead cells, propidium iodide only stains the nuclear material of cells that have lost plasma membrane integrity. They emit green and red fluorescence, respectively, upon excitation, when cells are dead, bright green fluorescence and red fluorescence is emitted due to the compromise of nuclear configuration and cell wall integrity (Agus, Sarp, & Cemiloglu, 2018; Agus, Sengoz, & Yilmaz, 2019).

In this work the majority of cells from the negative control and the extract control, treated and later incubated overnight (overnight, 14 h), displayed green fluorescence, which indicates that they were alive (Fig. 12). On the other hand, more cells that were previously exposed to HU (Fig. 12 C, G and K) exhibited red florescence, which suggests that they were dead. Also, some cells treated with HU and *D. viscosa* extract (Fig. 12 D, H and L) showed red fluorescence but with lower frequency comparing with the cells treated only with HU. In addition, drastic morphological changes were also observed in cells that were formerly treated with HU and both HU and the plant extract.

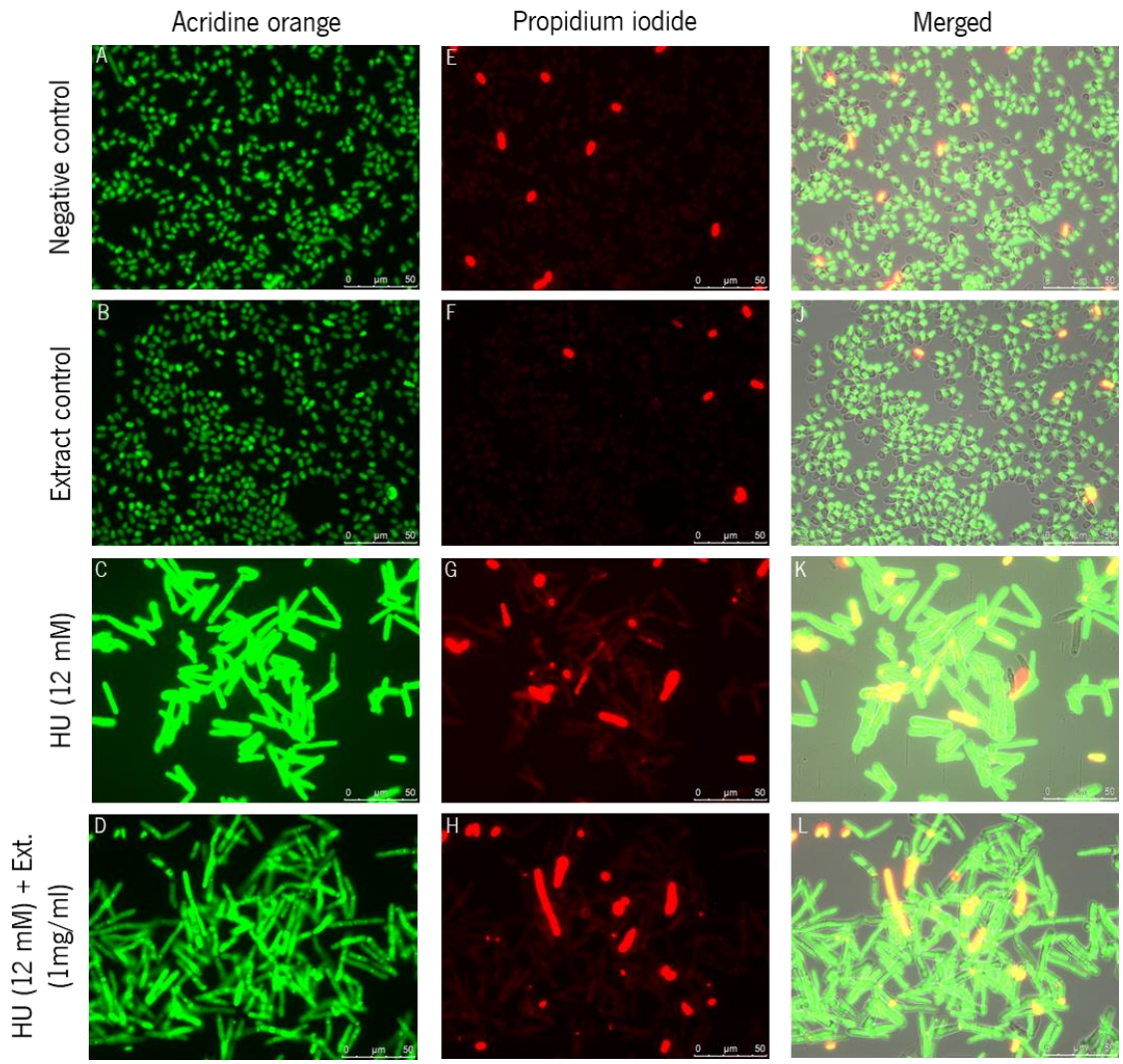


Figure 12. Fluorescence of *S. pombe* cells upon labelling with acridine orange/propidium iodide dual staining. Cells were incubated overnight in YES medium with different treatments, without treatment (A, E and I), 1 mg/ml DVAE (B, F and J), 12 mM HU (C, G and K) and simultaneously to 1 mg/ml DVAE and 12 mM HU (D, H and L). These images are from one representative experiment three independent replicas. Magnification: 400 X. HU: hydroxyurea.

Apoptotic cells and viable cells were quantified, results are depicted in Fig. 13. The percentage of viable cells and cells in apoptosis are practically the same in the negative and the extract control. On the other hand, the percentage of viable cells treated with HU is much lower when compared with both controls and even with cells that were treated HU and the extract. On the contrary, the percentage of dead cells is higher in cells treated with only HU. The percentage of dead cells from the group treated with HU and the extract was higher when compared with both controls but was lower in comparison with the group of cells treated with HU.

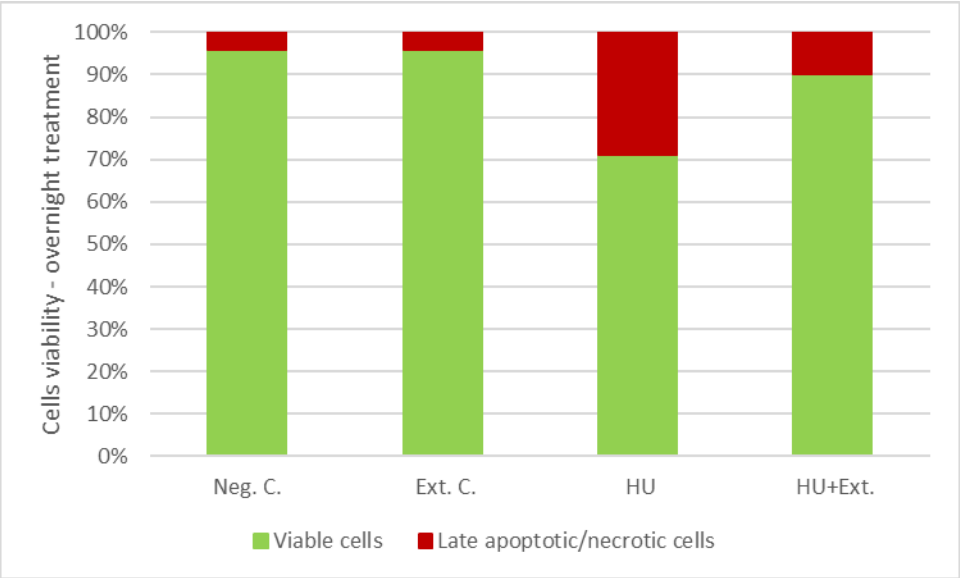


Figure 13. Quantification of viable *S. pombe* cells and dead cells based on staining with acridine orange/propidium iodide after different treatments for 14 h: cells without treatment Neg. C), 1 mg/ml DVAE (Ext. C.), 12 mM HU (HU) or 1 mg/ml DVAE and 12 mM HU (HU+Ext.). Cells were observed by fluorescence microscopy with 400x magnification (see Fig. 12). These results are from one representative representative experiment out of three independent replicas.

To assess the progression of the effect of HU and DVAE, we performed the same experiment and observed the cells after 6 h treatment. Nearly all yeast cells showed green fluorescence, indicating that almost all cells were viable (Fig. 14). Comparing controls (Fig. 14 A, E, I, B, F and J) with treatments (Fig. 14 C, G, K, D, H and L), in terms of apoptosis, no significant differences were detected. Although the number of dead cells was practically the same in all samples, differences in cells morphology were visualized, specifically, in their sizes. Cells that served as control, both negative and positive control (Fig.13 A, E, I, B, F and J), were shorter than the cells that were treated with HU and both HU and the plant extract (Fig.13 C, G, K, D, H and L).

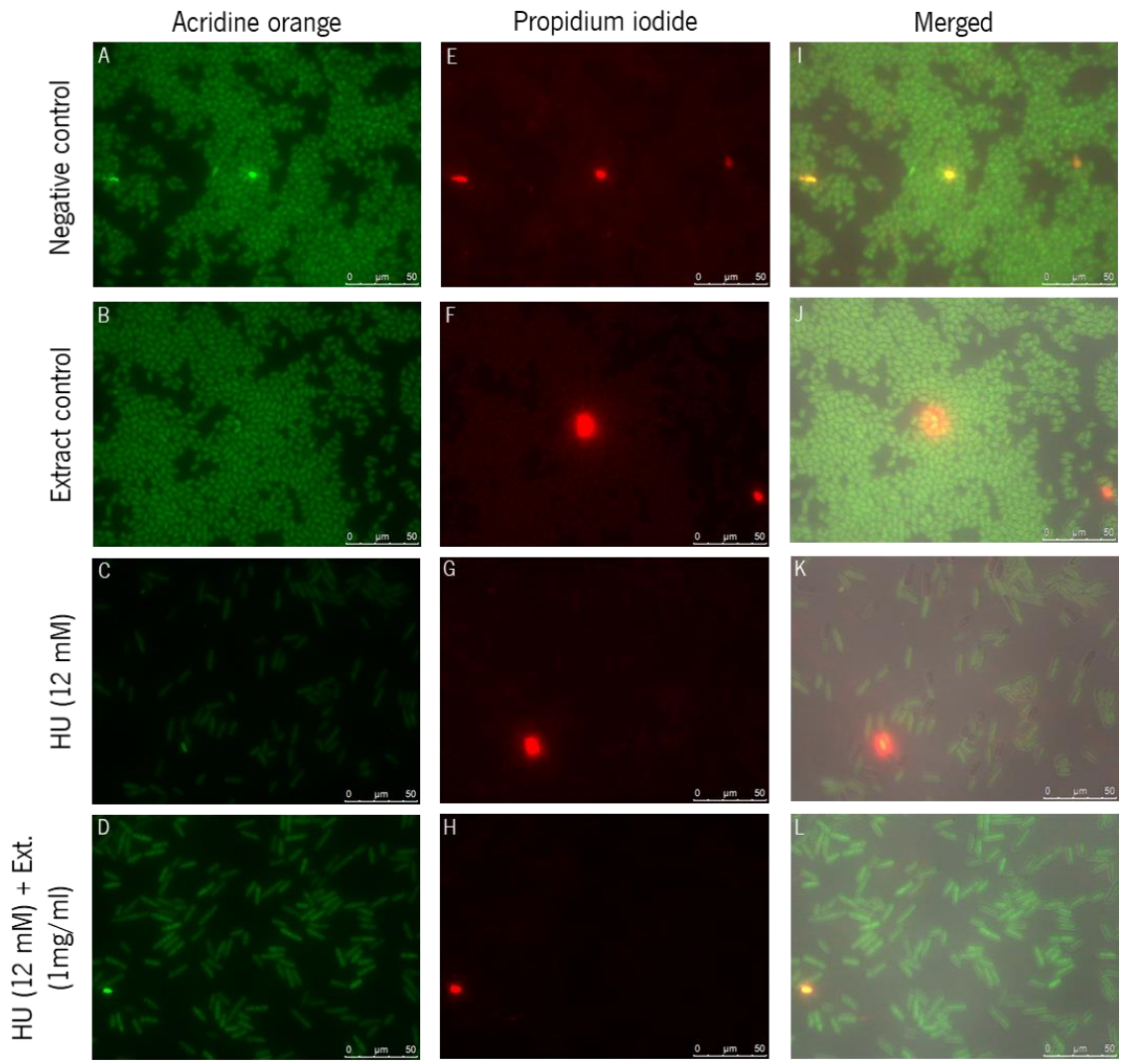


Figure 14. Fluorescence of *S. pombe* cells upon labelling with acridine orange/propidium iodide dual staining. Cells were incubated over 6h in YES medium with different treatments, without treatment (A, E and I), 1 mg/ml DVAE (B, F and J), 12 mM HU (C, G and K) and simultaneously to 1mg/ml DVAE and 12 mM HU (D, H and L). These images are from one representative experiment of three independent replicas. Magnification: 400 X. HU: hydroxyurea.

Results depicted in Fig. 14 were also quantified and a graphic was constructed where the percentage of viable cells and cells in apoptosis, with cells treated for 6h, is represented (Fig. 15). The percentage of viable cells and dead cells is practically the same in the four groups with a slightly difference of dead cells treated with HU.

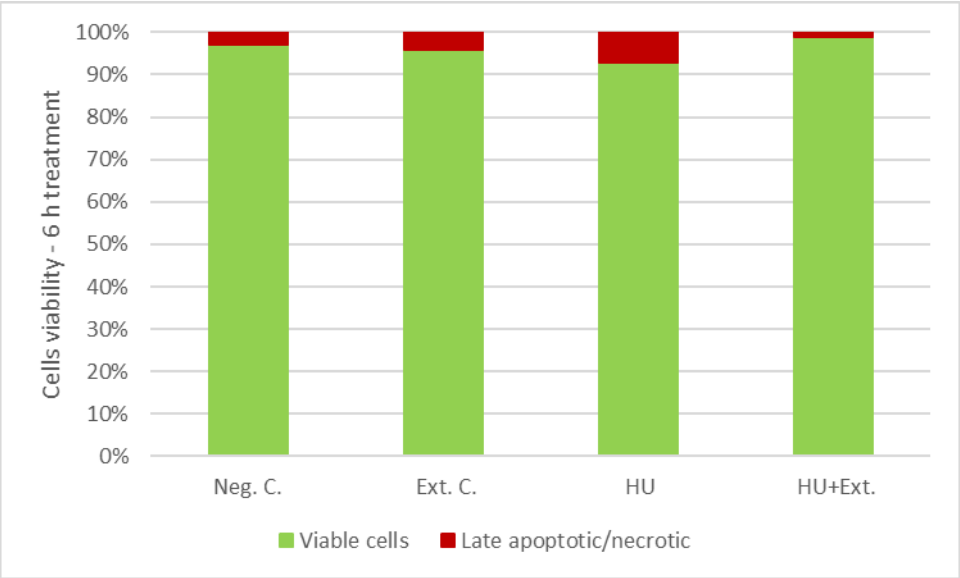


Figure 15. Quantification of viable *S. pombe* cells and dead cells based on staining with acridine orange/propidium iodide after different treatments for 6 h: cells without treatment Neg. C), 1 mg/ml DVAE (Ext. C.), 12 mM HU (HU) or 1 mg/ml DVAE and 12 mM HU (HU+Ext.). Cells were observed by fluorescence microscopy with 400x magnification (see Fig. 14). These results are from one representative representative experiment out of three independent replicas.

Table 3 – Percentage of viable *S. pombe* cells exposed to 1 mg/ml DVAE, 12 mM HU or 1 mg/ml DVAE and 12 mM HU for 6 h and 14 h (overnight). Statistical analyses were made with the program GraphPad Prism 7.00 using the test 1-way ANOVA-multiple comparisons with (a) $p < 0.001$; (b) $p < 0.004$; and (c) $p < 0.0374$. Comparisons were made between the values of experiments with the same incubation time. HU: hydroxyurea.

Treatment (h)	**Percentage of viable cells**			
	Negative control	Extract control	HU	HU + Extract
6	97 ± 1^{a}	97 ± 1^{a}	93 ± 1^{b}	99 ± 1^{a}
14	96 ± 1^{a}	96 ± 1^{a}	71 ± 3^{b}	90 ± 2^{a}

4.2.2 – Evaluation of Nuclear Morphology

To further investigate the effects of DVAE, aliquots of the samples prepared for the apoptosis assays were collected and stained with Hoechst 33342. In the case where fission yeasts cells treated and incubated overnight, cells exposed to HU and the combination of HU and the plant extract, the nuclear morphology was particularly modified in comparison with controls (Fig. 16 and 17). Cells showed DNA fragmentation, dispersed DNA and displaced nuclei (Fig.17). It was also observed nonnucleated and multinucleated cells (Fig. 17). Overall, the nuclear morphology of cells treated for 6 h was apparently normal (Fig. 18 and 19).

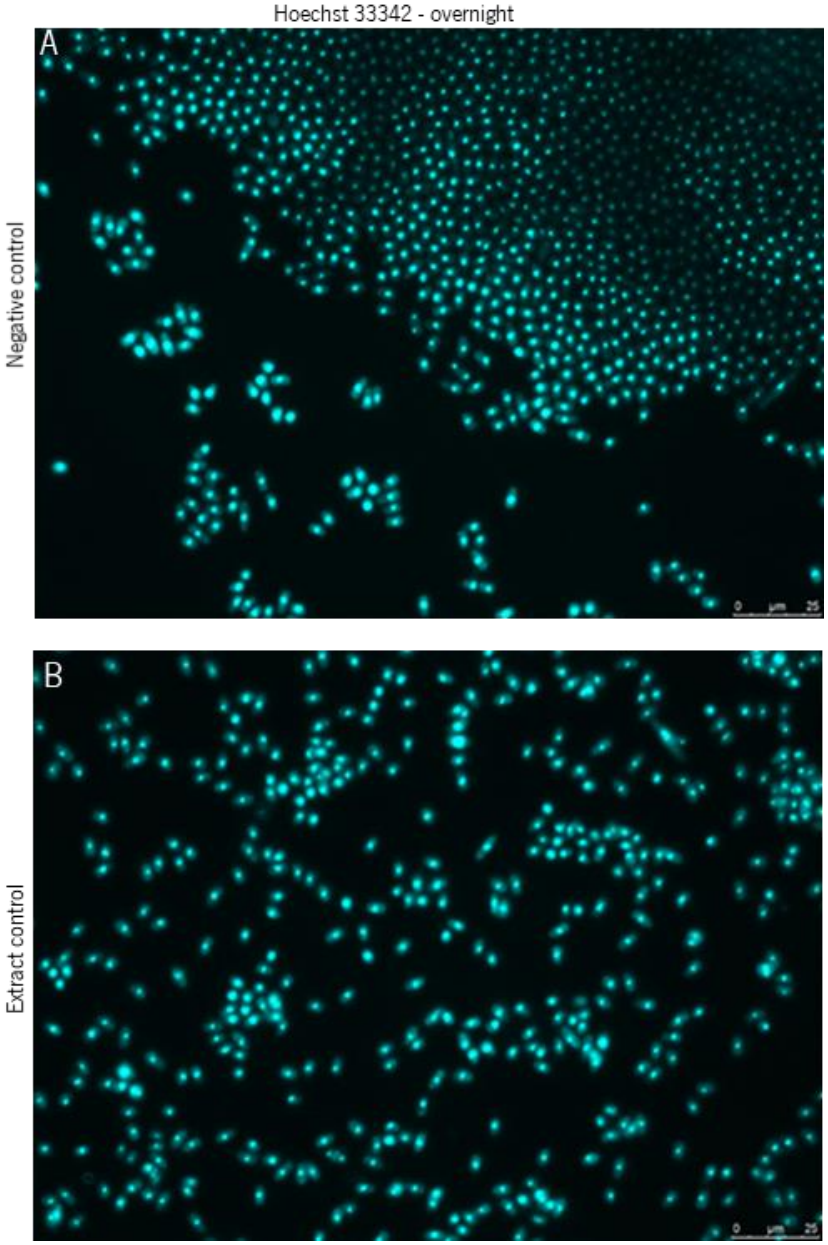


Figure 16. Nuclear morphology of *S. pombe* cells evaluated by fluorescence microscopy, after Hoechst 33342 staining. Initially, cells were incubated in YES medium and afterwards exposed overnight to different conditions: without treatment (A), 1 mg/ml DVAE (B) Magnification: 400 X.

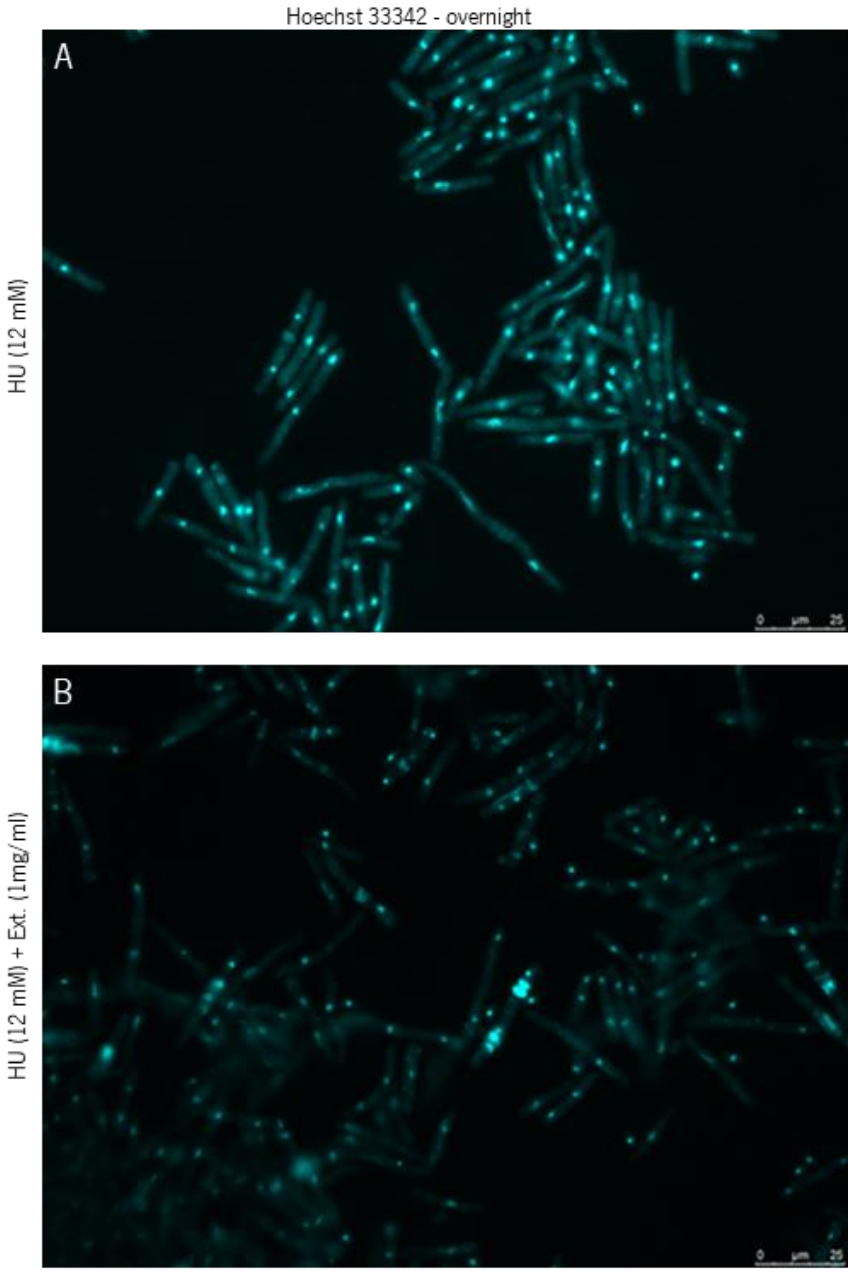


Figure 17. Nuclear morphology of *S. pombe* cells evaluated by fluorescence microscopy, after Hoechst 33342 staining. Initially, cells were incubated in YES medium and afterwards exposed overnight to different conditions: 12 mM hydroxyurea (HU; A) and simultaneously to 1 mg/ml DV extract (Ext.) and 12 mM HU (B). Magnification: 400 X.

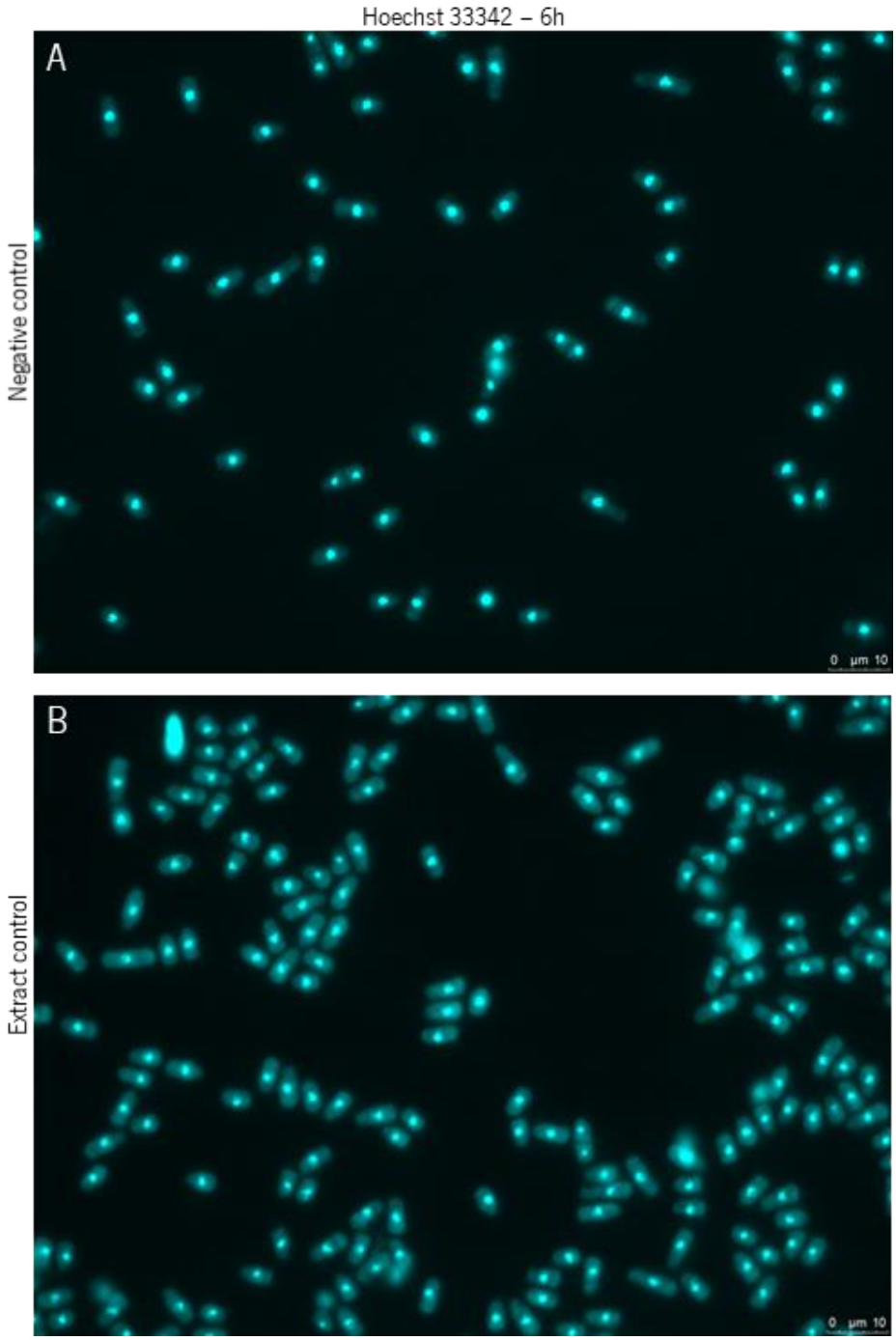


Figure 18. Nuclear morphology of *S. pombe* cells evaluated by fluorescence microscopy, after Hoechst 33342 staining. Initially, cells were incubated in YES medium and afterwards exposed over 6 h to different conditions: without treatment (A), 1 mg/ml DVAE (B). Magnification: 400 X.

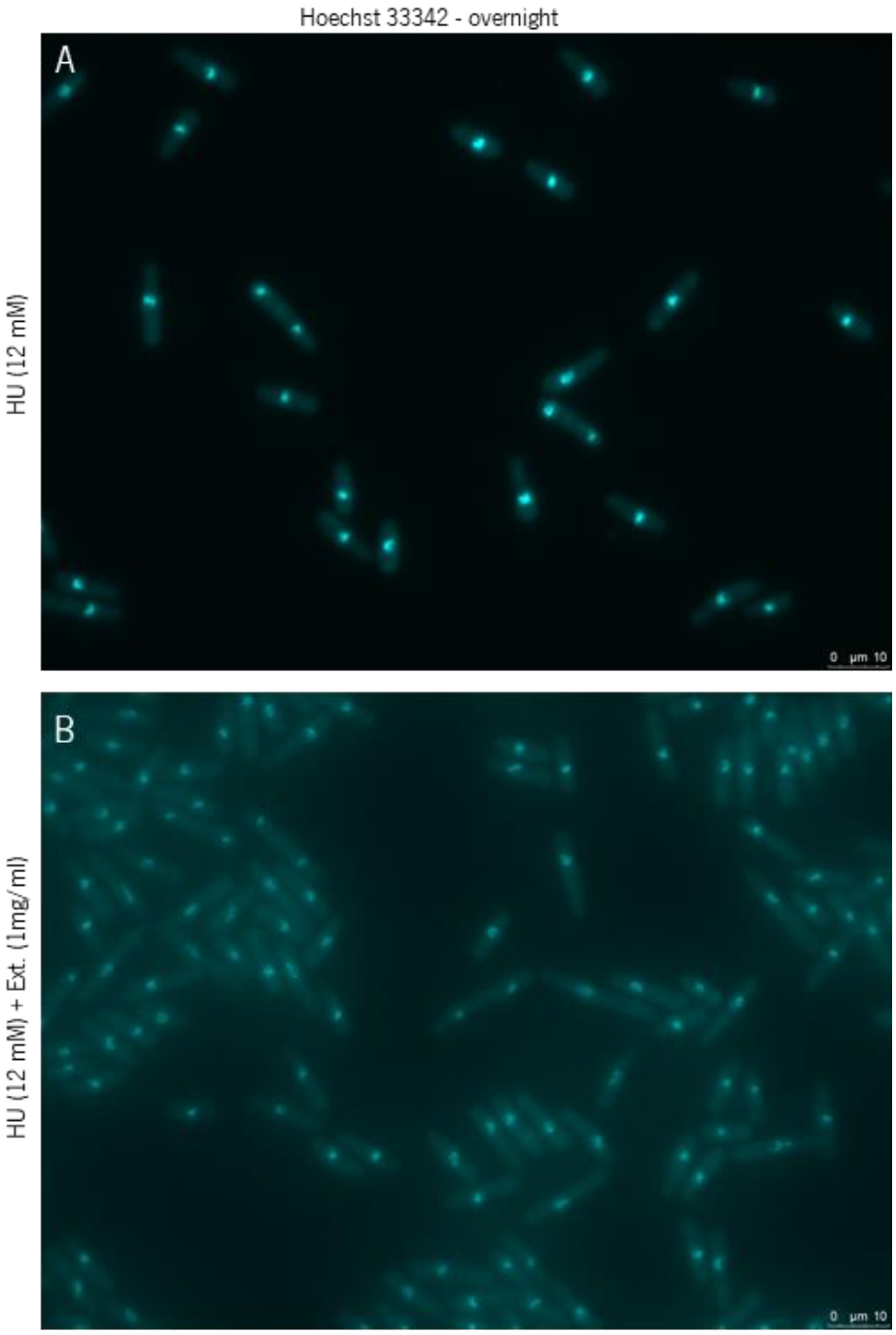


Figure 19. Nuclear morphology of *S. pombe* cells evaluated by fluorescence microscopy, after Hoechst 33342 staining. Initially, cells were incubated in YES medium and afterwards exposed over 6 h to different conditions: HU (A) and simultaneously to 1 mg/ml DV extract and 12 mM HU (B). Magnification: 400 X.

4.2.3 - Assessment of cellular length

Since differences in cells sizes were detected, after exposure to HU only and HU plus DVAE in relation to the controls, the length of the cells were measured in order to determine whether or not progression in

cell cycle was affected (Table 4). Regarding cells treated for 6 h, length was similar in the controls and cells exposed to only HU and HU plus DVAE were lengthier. The length of cells incubated overnight was practically the same in the controls but cells treated with HU and both HU and the plant extract were more than three times longer. Comparing cells exposed to HU and HU combined with DVAE, cells treated only with HU were slightly more elongated. In respect to the length of cells treated for 6 h, significant differences were detected between almost groups, except, between the controls. Concerning the length of cells treated overnight, significant differences were not detected between the controls and the cells treated with HU and both HU and DVAE.

Table 4 – Length of *S. pombe* cells exposed to 1 mg/ml DVAE, 12 mM HU or 1 mg/ml DVAE and 12 mM HU for 6 h and 14 h (overnight). Statistical analyses were made with the program GraphPad Prism 7.00 using the test 1-way ANOVA-multiple comparisons with *(a)* $p < 0.001$; (b) $p <$; and (c) $p <$. Comparisons were made between the values of experiments with the same incubation time HU: hydroxyurea.

Treatment (h)	**Length of cells (µm)**			
	Negative control	Extract control	HU	HU + Extract
6	7.91 ± 0.10^{a}	8.11 ± 0.12^{a}	14.10 ± 0.24^{b}	15.74 ± 0.25^{c}
14	7.14 ± 0.07^{a}	7.51 ± 0.08^{a}	25.37 ± 0.68^{b}	24.04 ± 0.59^{b}

4.2.4 – Cell cycle progression monitoring

The mitotic index measures the proliferation of cell populations, this corresponds to the ratio of dividing cells by the total number of cells that constitute one population. The mitotic index (MI) of populations from both controls were similar, with slight differences. Likewise, the MI of populations treated with HU and the combination of HU and extract were very similar. The MI of the controls was always above the 20%, during the 3 h of the assay. On the contrary, the MI of yeasts previously exposed to HU and HU plus extract, for 3 h, was under the 20%, indicating that most of the cells did not engage in division. The MI of the population from the negative control, that were not treated with HU or/and extract and HU, initially increased until its maximum, 56%, is reached at 1.5 h and then it decreased over time. In the case of the

population treated with the extract a similar pattern was observed, except for a stabilization of MI between 1.5 h and 2.5 h incubation. The population exposed to HU over 3 h decreased the MI over time from 15% to 5% while in the population treated with HU and extract the MI was very stable during all the experiment, never reaching 10 %.

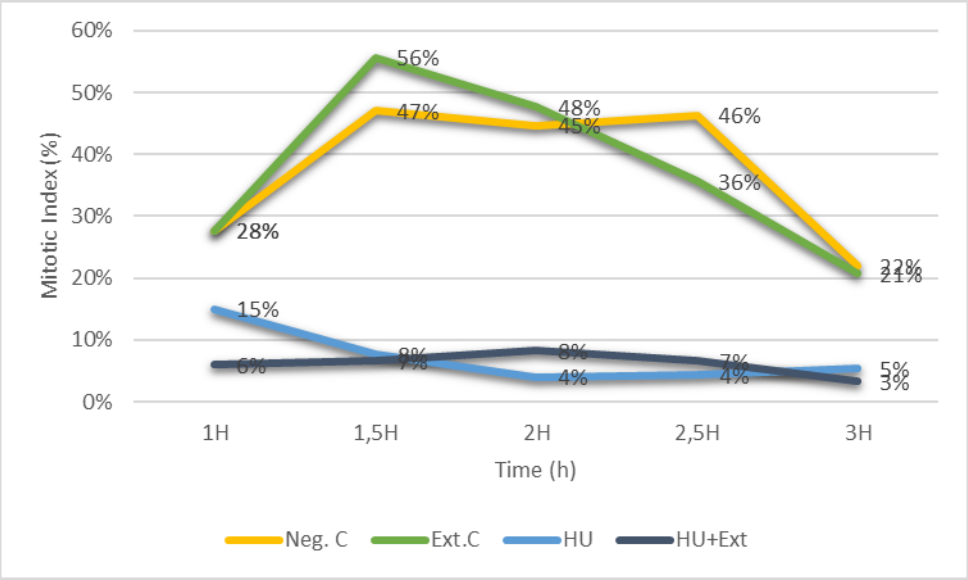


Figure 20 – Graph representing the mitotic index evolution of *S. pombe* cells that were incubated over 3 h in YES medium with different treatments: without treatment (Neg. C), 1 mg/ml *D. viscosa* extract (Ext. C), 12 mM hydroxyurea (HU) or simultaneously to 1mg/ml *D. viscosa* extract and 12 mM hydroxyurea (HU+Ext).

5 - Discussion

In the first instance, *in vitro* tests were done before *in vivo* assays, for the reason that it was necessary to verify if the extract had some sort of biological activity since there is literature testifying that *D. viscosa* extracts have antioxidant activity (Albano & Miguel, 2011; Brahmi-Chendouh et al., 2019; Brahmi et al., 2015; Chahmi et al., 2015; Danino et al., 2009; Kheyar-Kraouche et al., 2018; Wafa Rhimi et al., 2019; Trimech et al., 2014). The antioxidant activity of *D. viscosa* extract was tested using DPPH radical scavenging assay. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical that when reduced to hydrazine (due to the interaction with hydrogen donors like antioxidants), a color transition of purple color to yellow occurs.

The results from this study indicate that the DVAE has antioxidant activity in a dose dependent manner (appendix number 1). In comparison with the standard, gallic acid, more quantity of extract was necessary to inhibit 50 % of DPPH (table 2) Previously, an aqueous extract of *D. viscosa* aerial parts, of plant specimens from Algarve, showed also antioxidant activity with an IC_{50} 3.0 µg/ml (Albano & Miguel, 2011). In more recent studies, antioxidant activity of *D. viscosa* extracts were also reported. For instance, ethanolic extract of *D. viscosa* and also ointments with extract displayed antioxidant activity against DPPH radical, the ethanolic extract being with the highest activity with an EC_{50} of 56,25 ± 1,2 µg/ml (Wafa Rhimi et al., 2019).

It is important to take into consideration that several external factors influence the bioactivities of plant extracts, such as antioxidant activity. Abiotic factors of the site where the plant grows are critical, since they affect seriously plant development. In one investigation of *D. viscosa* with specimens from three different regions of Morocco, Immouzer, Sefrou and Tauounate, distinguished degrees of antioxidant activity were detected by DPPH scavenging assay (Chahmi et al., 2015). The distribution of the bioactive compounds in the plant should be considered in studies of the antioxidant activity of extracts. Very different intensity of activities may be observed for extracts made with the whole plant material or with specific organs. In a study where extracts of different parts of *D. viscosa* were made from flowers, leaves, stems and roots, the roots ethyl acetate extract exhibited the highest DPPH scavenging activity (Trimech et al., 2014). Also, the process of extraction and the solvents that were utilized to perform the extraction process of phytocompounds influence their bioactivities. For example, in the previously mentioned work, Chahmi *et al.* (2015) used as extraction solvent both ethanol or ethyl acetate, the ethanolic extracts showed more antioxidant activity in comparison with the ethyl acetate extracts. Moreover, several extracts of *D. viscosa* with different extraction solvents, diethyl ether ethyl acetate and methanol, the ethyl acetate extracts demonstrated the strongest antioxidant activity (Trimech et al., 2014).

Since the extract used in this work exhibited antioxidant activity, and it is assumed that the phenolic compounds are the principal responsible for the antioxidant activity of this plant, the TPC was quantified. The basis of this method resides in the fact that when Folin & Ciocalteu's reagent reacts with phenols due to its reduction, its yellow color changes to blue. Many studies attested that *D. viscosa* extracts possess phenolic compounds, the total phenolic content ranging between 299.1 mg GAE/g of extract (ethyl acetate extract from *D. viscosa* leaves, plant specimen from Algeria) to 0.42 mg GAE/g of extract (ethyl acetate extract of *D. viscosa* roots, specimen from Crete; (Albano & Miguel, 2011; Brahmi-Chendouh et al., 2019; Kheyar-Kraouche et al., 2018; Wafa Rhimi et al., 2019; Trimech et al., 2014). In the work of Albano and Miguel (2011), the aqueous extract of *D. viscosa* aerial parts, from a plant specimen from Algarve, presented a total phenolic content of 1.12 mg GAE/ml.

Subsequently, *in vivo* assays were performed, with the model organism *S. pombe* for the reason that the *in vitro* assays suggested that the extract has antioxidant activity that is likely to protect genomes of cells against oxidative damage, but on the contrary, the work of Celik and colleagues (Aşkin Celik & Aslantürk, 2010) attested that the extract displayed genotoxicity. Cell cycle possesses four distinct phases, G1, S, G2 and M phase. In general, cell cycle has two main events, the replication of DNA and the equal division of DNA between the two nuclei of the daughter cells. Typically, G1 and S phase are shorter phases, and G2 phase is the longest in fission yeasts (Zhu et al., 2015). Transitions between cell cycle phases, checkpoints, are strongly regulated by different enzymes, such as cyclins and cyclin dependent kinases (cdK; (Moser & Russell, 2000). In checkpoints, several conditions are verified to perceive if the cell is ready to progress to the next phase of the cycle, such as, cell size, DNA integrity, accurate DNA replication and correct chromosome spindle attachment. When these conditions are not in compliance the cell cycle is delayed or blocked until their regularization.

Under the conditions that fission yeasts were cultivated in this work (30 °C, at 200 rpm) one cell cycle is commonly finalized after 2.5 h. Theoretically, yeasts incubated overnight complete 5.5 cycles. Their viability was not severely affected when they were not exposed to any kind of treatment (negative control) or they were exposed to the extract (extract control; Fig. 13). Nevertheless, more dead cells were spotted in populations that were exposed to HU and HU and the plant extract, the number of dead cells being higher in the samples of cells previously exposed to only HU. This is the expected outcome of the HU activity, an inhibitor of RNR enzyme. When RNR activity is repressed, the amount DNA precursors is reduced, and so, not only DNA synthesis is compromised but also DNA damage repair. Furthermore, in recent studies, it was suggested that a long treatment of fission yeasts with HU can generate ROS, which leads to oxidative stress (Singh & Xu, 2016). This can explain partially why the viability of cells treated

with HU and extract simultaneously was not affected at the same extent by assuming the extract exhibited antioxidant activity.

Yeasts exposed overnight to HU or the combination of HU and *D. viscosa* extract exhibited severe cellular and nuclear damage (Figs. 17), consequently, the time of incubation was reduced to 6 h. The viability of these cells was not compromised (Fig. 14).

Fission yeast initiate their cell cycle normally with 8 µm of length, then throughout the G2 phase the cell tips grow until 14 µm. Then growth is slowed until 16 µm is reached and the beginning of cytokinesis takes place. Characteristically, the cytokinesis process of fission yeast happens simultaneously with the end of S phase and sometimes even with the beginning of G2 phase (Zhu et al., 2015). Cell sizes variances were observed, as a result, the cell length of some cells was measured. Fission yeasts cells treated and incubated overnight from the negative and the extract control possessed on average 8 µm in length which corresponds to the beginning of a cell cycle or to the end of it, where two daughter cells are already divided. On the contrary, cells that were in contact with the genotoxic drug and both genotoxic drug and the plant extract showed a great increment in their sizes (table 4), possibly due to DNA damage and the consequent activation of cell cycle checkpoints. In this case the checkpoint of G2 to M phase, where the DNA replication, DNA damage and cell size are checked.

In the case of yeasts that were treated for 6 h, once more, cells from the negative and extract controls on average measured 8 µm and cells exposed to the genotoxic agent and equally to genotoxic agent and the plant extract displayed an increased length. In the case of cells only treated with HU and the combination of HU and DVAE, it appears that the progression of the cycle was only delayed, since their average length was 14 µm and 16 µm, respectively (table 2). Nevertheless, this size difference can indicate that cells exposed to HU and DVAE simultaneously were more advanced in the progression of the cycle then cells only treated with HU.

These results are not in compliance the those obtained by Çelik *et al.* (2010). They found out that aqueous extract of *D. viscosa* (the plant specimen was collected in Turkey) has cytotoxic activity, specifically, on the cell division process. Chromosomal aberrations and micronucleus formation were also detected in onion root tip cells. Moreover, the extract inhibited the root growth of *Allium cepa* (onions), in a dose dependent manner (2.5 mg/ml; 5 mg/ml and 10 mg/ml were the extract concentrations tested, because these concentrations are applied in the traditional medicine).

The mitotic index of controls indicates that the progression of the cell cycle was reestablished after the exposition of cells to HU, to synchronize their cell cycle. But the reentrance on the cell cycle of cells exposed to the extract appears to had been more slow. MI results of cells exposed to HU and both HU

were similar, suggesting that the extract did not affect HU activity. The progression of the cell cycle of these cells was compromised when compared with the MIs from the controls, possibly due to HU action as expected.

6 - Conclusion and Future Perspectives

In conclusion, the results from this work point out that the aqueous extract of *D. viscosa* made from stems and leaves from plant specimens from Algarve possesses antioxidant activity possibly due to its phenolic content. Taking into account all the results from the fluorescence microscopy assays, it appears, that the extract has antigenotoxic activity against HU after a long period of time.

In the future, the chemical composition of the used extract should be analyzed, to understand which compounds are responsible for its antioxidant activity. Also, other in *vitro assays* could be done to verify if this type of extract has other bioactivities.

The effects of different extract concentrations on fission yeast cell cycle should be tested also, to verify if different concentrations exert antigenotoxicity or genotoxicity. The bioactivities of a similar extract used in this work could be explored using fission yeast cell cycle mutants or other model organisms. Investigation of the effect of a similar extract over other genotoxic agents, to assess if the extract is capable of increase or decrease their genotoxicity.

In summary, this kind of studies are important because it is essential to understand the interactions of genotoxic agents and plant extracts in full extent. To develop new therapeutic strategies in which the efficiency of drugs can be synergistically potentiated or to reduce the possible side effects of those drugs, for example in the case of antitumoral therapy or as a prophylactic measure to avoid the development of tumors, where the genome is protected

7 -Bibliography

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8 – Appendix

Appendix number 1 – DPPH Radical Scavenging Activity of DVAE