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Dissertação

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Abstract

Polyphenols are the most abundant phytochemicals in the human diet. It is known that the consumption of polyphenol-rich foods, especially fruits and vegetables, translates into benefits for human health. Polyphenols besides to be free radical scavengers, are now considered to invoke a spectrum of cellular mechanisms of action, such as induction of endogenous antioxidants; modulation of genes related to cell survival/death; cell signaling pathway regulatory activity and regulation of the mitochondrial function. Increasing production of free radicals is one of the major causes of degeneration that can occur via redox-cycling, metabolism of xenobiotics, ageing, environmental toxins and mutant proteins. Agents that prevent these phenomena could be of particular therapeutic interest.

In this study a yeast model of degeneration composed by four *Saccharomyces cerevisiae* strains with different sensitivities to oxidative stress, was implemented. Extracts from fruits and leaves of *Corema album* and *Arbutus unedo* were submitted to an *in vitro* procedure that mimics the gastro-intestinal digestion to obtain the Polyphenol-digested fractions (PDFs). After chemical characterization and toxicity, the PDFs protective effect was evaluated in the yeast model of degeneration. Protective effects were observed for *Δyap1* and *Δsod1* strains due to the treatment with *C. album* fruit and *A. unedo* fruit and leaf PDFs. At the same time alterations on GSH/GSSG ratio for these two strains were also observed in the presence of the PDFs with protective effects. However, it was not observed an effect in the production of reactive oxygen species (ROS) mediated by the tested PDFs, indicating that ROS scavenging is not the mechanism of action of PDFs.

This work provided valuable information about possible mechanisms of protection of polyphenols after gastrointestinal digestion that will be further evaluated in a yeast model of Parkinson's disease already implemented in DSB laboratory to provide more insights.

Keywords: Ageing, ROS, Degeneration, *S. cerevisiae*, Polyphenol-digested fractions.

Resumo

Os polifenóis são os fitoquímicos mais abundantes na dieta humana. O consumo de alimentos ricos nestes compostos, principalmente frutas e vegetais, tem sido cada vez mais associado a efeitos benéficos para a saúde humana. Além disso, estudos indicam que há uma forte associação entre o consumo de alimentos ricos em polifenóis e a diminuição da incidência de doenças degenerativas. Os polifenóis são compostos conhecidos como sendo capazes de eliminar espécies reactivas de oxigénio, no entanto em estudos mais recentemente tem-se vindo a constatar que estes compostos estão também envolvidos num largo espectro de mecanismos de acção celulares, como é o caso da activação de moléculas antioxidantes endógenas, quelação de iões, modulação de genes relacionados com a morte/sobrevivência celular, regulação da activação de genes/proteínas de vias de sinalização celular e ainda na regulação da função mitocondrial.

As reacções redox, que ocorrem no interior das células, estão na base de numerosas vias metabólicas importantes na homeostasia celular. O aumento na produção celular de espécies reactivas de oxigénio, ou de azoto, é uma das principais causas associadas ao desenvolvimento de doenças degenerativas. Este aumento pode ocorrer devido a vários tipos de insultos celulares como o metabolismo de xenobióticos, toxinas ambientais, proteínas mutantes tóxicas para a célula e mais importante, devido a processos de envelhecimento.

A levedura *Saccharomyces cerevisiae* é um dos modelos biológicos mais versáteis para estudar as doenças degenerativas, devido ao facto dos mecanismos relacionados com a disfunção mitocondrial ou proteossomal e a desregulação transcricional serem extremamente bem conservados entre leveduras e humanos, permitindo estudar os mecanismos envolvidos neste tipo de doenças. Para além disso, os mecanismos inerentes à produção de espécies reactivas de oxigénio e de apoptose são reproduzidos com sucesso em levedura.

Neste estudo o principal objectivo consiste em avaliar o potencial protector de metabolitos provenientes da digestão de frutos e folhas de *Corema album* e *Arbutus unedo* num modelo de degeneração em levedura optimizado durante este trabalho. Estudos preliminares do laboratório Biologia da Doença e do Stresse (resultados não publicados) demostraram que as fracções totais não digeridas destas duas plantas apresentam elevada capacidade antioxidante e actividade protectora de células submetidas a stresse.

Para atingir os objectivos propostos para este trabalho foi inicialmente optimizado um modelo de degeneração em levedura induzido pelo H_2O_2. Este modelo é composto por 4 estirpes de *S. cerevisiae* (BY4714, *Δyap1*, *Δsod1* e AD1-8), que apresentam diferentes sensibilidades ao stress oxidativo. Numa primeira fase foram definidos os tempos de geração (Tg) para cada estirpe, sendo de 2 h para BY4741 e *Δyap1* e de 3 h para *Δsod1* e AD1-8 e as concentrações de H_2O_2 a usar tanto em meio sólido como em meio líquido. Em meio solido foram escolhidas as concentrações de 0.15 mM para *Δyap1* e *Δsod1* e 0.5 mM para BY4741 e AD1-8, para incubações prolongadas. Em meio líquido foi escolhida a concentração de 2 mM de H_2O_2 e o Tg de cada estirpe como tempo de incubação. Os resultados obtidos para as curvas de crescimento indicam que baixas concentrações de H_2O_2 na célula podem activar mecanismos de defesa e activação de genes que são importantes para o crescimento e diferenciação celulares.

Os extractos hidroetanólicos provenientes de frutos e folhas de *C. album* e *A. unedo* foram submetidos a um processo de digestão *in vitro* que mimetiza as alterações que ocorrem durante a digestão gastro intestinal e a um passo final de limpeza, para obtenção das fracções digeridas de polifenóis (FDPs). Paralelamente foram também digeridos os extractos totais de *Rubus ideaus* fruto e *Ginkgo biloba* folha para fins comparativos, visto que estas duas espécies estão descritas como tendo capacidade de melhorar o desempenho celular constituindo benefícios para a saúde humana. Numa segunda fase deste estudo foi efectuada a caracterização química dos extractos obtidos em cada etapa do processo de digestão *in vitro,* tendo sido avaliado o conteúdo total em fenóis e a capacidade antioxidante *in vitro*. Esta caracterização permitiu a compreensão das alterações e perdas que ocorrem nos compostos desde que são ingeridos até ao final da digestão no intestino delgado. Em particular neste estudo foram utilizadas as fracções IN, correspondente ao conteúdo que entra passivamente na circulação sanguínea e OUT, que diz respeito ao conteúdo que é atinge o colón, visto que podem ocorrer várias alterações químicas aos compostos.

A terceira tarefa permitiu avaliar a toxicidade inerente a cada FDPs por "spot assays", tendo sido escolhida a concentração máxima de 250 μg GAE.mL^{-1} para a qual foi posteriormente testado o seu efeito protector. Acima desta concentração a FDP de *C. album* folha já apresenta toxicidade. Para testar o efeito protector destas fracções no modelo de degeneração em levedura, sob condições de stresse oxidativo foram utilizadas duas metodologias distintas. Após seleccionadas as FDPs que apresentam melhorias na viabilidade por spot assays, foi avaliado o estado metabólico geral das células pelo ensaio do CellTiter-Blue viability assay. Em ambos os métodos, tanto para a *Δyap1* como para a *Δsod1* foi observada uma capacidade protectora dos metabolitos digeridos do fruto de *C. album* e do fruto e folha de *A. unedo*. Estes resultados permitem concluir que os mecanismos de defesa celulares são activados ou modelados pelos compostos naturais presentes nestas espécies como é o caso dos polifenóis. No caso da FDP dos frutos de *A. unedo* os resultados indicam uma capacidade de activar mecanismos que permitem á célula defender-se mais eficazmente quando submetida a um stresse oxidativo posteriormente.

Numa fase final do trabalho foi avaliado o estado redox da célula. A produção exacerbada de espécies reactivas de oxigénio nas células submetidas está descrita como uma das condições de maior importância no desenvolvimento de doenças degenerativas, foi avaliada a produção destas espécies na presença e na ausência das FDPs, após as células serem submetias a um stresse oxidativo. Estas espécies foram quantificadas pelo método de H_2DCFDA e foi observado um aumento na produção destas espécies quando estas eram incubadas na presença de H_2O_2. No entanto contrariamente ao esperado não foram observadas quaisquer alterações na produção de espécies reactivas de oxigénio, quando as células eram pré-incubadas com os FDPs. Estes resultados contrariam alguns estudos que apontam a eliminação destas espécies como o mecanismo pelo qual os polifenóis melhoram a viabilidade celular mas reforça a ideia que estes compostos possam actuar por outros mecanismos de regulação envolvidos na defesa celular.

Outro parâmetro analisado foi o conteúdo em tióis totais por HPLC. Apenas foram observadas diferenças nos níveis de GSH reduzido para as estirpes *Δyap1* e *Δsod1*, quando submetidas ou não a stresse oxidativo, não se tendo observado diferenças para a WT. O facto de não se observarem

diferenças para a WT dever-se-á ao facto de esta estirpe possuir todos os mecanismos antioxidantes necessários para repor os níveis de GSH reduzido. Avaliou-se então a razão GSH/GSSG para as estirpes mais afectadas, *Δyap1* e *Δsod1,* na presença e na ausência das FDPs. A razão GSH/GSSG é considerado um indicador do estado redox das células.

Na presença das FDPs com capacidade protectora, foi observada uma diminuição no estado oxidado das células para a estirpe *Δsod1*. No entanto, para a estirpe *Δyap1* apenas o fruto de *A. unedo* apresenta capacidade de melhorar significativamente o estado redox da célula, quando esta é submetida à condição de stresse oxidativo, sendo que ainda assim não é capaz de repor os níveis normais de oxidação celular para o *Δyap1*.

Observou-se ainda através das metodologias aplicadas que a estirpe *Δyap1* é a mais afetada pelo stresse oxidativo, o que era espectável, uma vez que o factor de transcrição Yap1p para o qual esta estirpe é mutante, regula vários enzimas antioxidantes como a SOD1 e SOD2. As metodologias aplicadas neste trabalho parecem indicar que os polifenóis presentes nas fracções digeridas têm a capacidade de afectar os mecanismos relacionados com sobrevivência e proliferação celular.

Este trabalho fornece informação importante acerca de possíveis mecanismos de protecção dos polifenóis depois da digestão gastrointestinal, que serão avaliados posteriormente num modelo de PD em levedura já implementado no laboratório da Biologia da Doença e do Stresse.

Palavras-chave: Envelhecimento, Especies Reactivas de Oxigénio, Degeneração, *S. cerevisiae*, Fracções digeridas de polifenóis

General Index

Index of Figures

Index of Tables

Abbreviations

$^{\bullet}$OH - Hydroxyl radical
AAPH – 2,2'-azobis (2-amidopropane) dihidrochloride
Ace1p – metallothionein protein
AD – Alzheimer´s disease
AHP1 – Alkyl hydroperoxide reductase
Aif1p – apoptosis inducing factor
ALS – Amyotrophic lateral sclerosis
Cat1p – Peroxisomal catalase enzyme
CCP1 – Cytochrome c peroxidase gene
Ccs1p – Copper chaperon protein
Crm1 – Yap1p exportin
CSM –Complete supplement mixture
Ctt1p – Cytosolic catalase
CUP1 – metallothionein (copper binding protein) gene
DCF –Dichlorofluorescein
DMSO – Dimetyl sulfoxide
DSB Laboratory – Disease and Stress Biology Laboratory
DT – Doubling time
EGb 761 – *Ginkgo biloba* leaf standard extract
ER – Endoplasmatic reticulum
F.U. – Fluorescence units
G-6-PDH – Glucose-6-Phosphate Dehydrogenase
GAE – Gallic acid equivalents
GIT – Gastrointestinal tract
GLR1 – Glutathione reductase gene
Glr1p – Glutathione reductase
Gpxp – Glutathione peroxidase
Grxp – Glutaredoxins
GSH – Reduced Glutathione
GSH1 – γ-glutamylcysteine synthetase gene
GSH2 – Glutathione synthetase gene
GSSG – Oxidized glutathione
Gstp – Glutathione transferase
H_2DCFDA – 2,7-dichlorofluorescein diacetate
HD – Huntington's disease
IN – Serum available fraction obtain after IVD
ITQB – Instituto Tecnologia Química e Biologica
IVD – *In vitro* digestion
MDR – Multidrug resistance
NADPH – Nicotinamide adenine dinucleotide phosphate
Ndi1p – internal mitochondria NADH dehydrogenase
NEM – *N*-ethyl maleimide
Nrf2p – Mammalian AP-1 transcriptional factor.
OD $_{600\ nm}$ – Optical density at 600 nm
ONOO$^{\bullet}$ – Peroxynitrate
OPA – Orthophtaldehyde
ORAC – Oxygen radical absorbance capacity
OUT – Colon available fraction obtain after IVD
PBS – Phosphate buffer
PD – Parkinson´s disease
PDFs – Polyphenol-digested fractions
Prxp - Peroredoxins
R.F.U. – Relative Fluorescence units
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
SC – Synthetic complete
Skn7p – Transcriptional factor Skn7
SOD – Superoxide dismutase
SOD1 – Cu/Zn Superoxide dismutase gene
Sod1p – Cu/Zn superoxide dismutase protein
SOD2 – Mn superoxide dismutase gene
Sod2p – Mn superoxide dismutase protein
SPE – Solid phase extraction
TE – Trolox equivalents
Tpxp – Thioredoxin peroxidase
Trr1p – Thioredoxin reductase
TRx – Thioredoxins
UPS – Ubiquitin-proteosome system

WT – Wild-type

YAP1 – Yap1p gene

Yap1p – Yeast activactor protein 1

Yca1p – Yeast metacaspase

YPD – Yeast extract, peptone, dextrose

YRE – Yap1p recognition element

YCF1 – Glutathione S-conjugated pump, ATP binding cassette tranporter gene.

1. Objectives

In the developed world, besides the increase of population lifespan, the number of degenerative diseases has also increased associated with the ageing process; this is the case of cancer, cardiovascular and neurodegenerative diseases. It has been demonstrated that plants contain many natural compounds with antioxidant properties, including polyphenols that have been intensively studied as free radical scavengers. Moreover fruits and vegetables rich in polyphenols have been studied as protecting agents in many of the age-related diseases.

S. cerevisiae is one of the most versatile biological systems used as a model for the study of many diseases. In order to evaluate the role of polyphenols with neuroprotective potential from *A. unedo* and *C. album* on the degeneration process, a yeast model was established and optimized for this study.

The main goal of this work is evaluate the protective potential of polyphenols digested metabolites from chosen plants, in a yeast model that mimics the degeneration process after an oxidative insult. To accomplish this goal, 5 tasks were defined with intermediate milestones. The defined tasks were (i) establishment of a yeast model of degeneration induced by H_2O_2 insult on 4 strains of *S. cerevisiae* (BY4741, *Δyap1*, *Δsod1* and AD1-8), (ii) chemical characterization of *in vitro* digested (IVD) fractions by total phenols content and *in vitro* antioxidant capacity, (iii) PDFs toxicity, in the yeast cells, (iv) evaluation of the protective capacity of PDFs in the established yeast model of degeneration, and (v) ultimately understanding the mechanisms of action by which the PDFs interfere with the redox state of cells.

This study is a major contribute for degeneration as a preliminary stage of screening for potential neuroprotective compounds present in plants, to be further tested in a yeast model of Parkinson´s disease, already implemented in DSB laboratory.

2. Theoretical Fundaments

2.1. Degenerative diseases

In the developed world, the population lifespan is increasing, with a concomitant increased incidence of many age-related diseases, such as cancer, cardiovascular and neurodegenerative disorders. However, in what concerns to neurodegeneration, the mechanisms involved in the behavioural deficits during ageing remain not totally clear [1, 2]. The brain, due to its high metabolic rate, low enzymatic activity, as well as the high proportion of polyunsaturated fatty acids, becomes a tissue particularly susceptible to oxidative damage [3, 4].

Several studies have been supporting the influence of environmental and genetic factors in degeneration processes, becoming these disorders difficult to study. Substantial evidence also indicates that excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells plays an important role in the regulation of redox reactions, that in high concentrations lead to an increase of lipid peroxidation, oxidative damage to DNA and proteins and decreased levels of GSH. All this phenomena have as consequence the ageing process and finally degeneration. (Fig. 1) [1-3].

In chronic degenerative diseases, apoptosis is the predominant form of cell death. Several pathways such as protein misfolding and aggregation, alterations of the endoplasmatic reticulum (ER) and cytoskeleton, altered RNA metabolism, dysfunction of the ubiquitin proteasome system (UPS), and mitochondrial dysfunction have a role in many of these disorders, leading to programmed cell death (Fig. 1) [4-6].

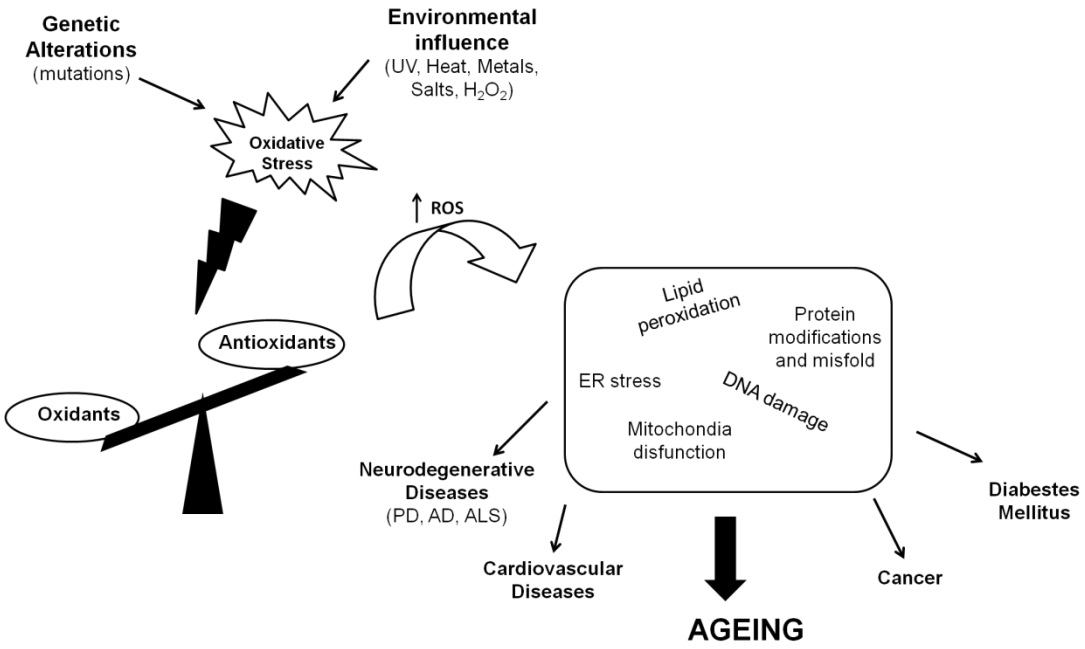

Figure 1. Representation of the phenomena that lead to oxidative stress damage in ageing process. Both genetic alterations and environmental influences that leads to oxidative stress, characterized by ROS increase and cellular components damage. The final consequence is the appearance of several diseases associated with ageing. Adapted from Uttara and co-workers, 2009 [1].

During ageing, the alterations in energetic metabolism and mitochondrial function reflect either in a decreased mitochondrial turnover and mitochondrial damage by accumulation of ROS [1, 5, 7]. Oxidative stress is generally caused by the excessive accumulation of ROS and RNS in cells, which has been implicated in the development of many diseases, including Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) [1, 2, 8].

2.1.1. Oxidative stress and degeneration

Classically oxidative stress is described as an imbalance between production of ROS by cell metabolism and its elimination by antioxidants systems to restore the redox balance. Aerobic organisms have the capacity to respond to chemical, environmental or physiological stress through widespread changes in macromolecular synthesis, degradation, trafficking, signal transduction pathways and also by operating an antioxidant defense system capable of neutralizing and removing ROS. Besides, cells have the ability to eliminate cellular damaged constituents and repair the damage. Several pathological conditions such as neurodegenerative disorders, cancer, atherosclerosis, and cardiac disease have been linked to impairment of the antioxidant machinery [2, 3, 5, 9].

Molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^{\bullet}$), are not free radicals, but can originate them through various chemical reactions. Free radicals and a variety of molecules derived from reactions in the presence of molecular oxygen (O_2) are classified as ROS due to their ability to promote oxidative changes in the cell. Moreover O_2, is not very reactive, but when in is reduced form, the superoxide anion (O_2^-), becomes the precursor of most ROS. Dismutation of O_2^-, spontaneously or catalyzed by superoxide dismutases (SOD) produces H_2O_2 which may be fully reduced to H_2O by cytosolic and peroxisomal catalases (Ctt1p, Cta1p) respectively, however partial reduction generates the hydroxyl radical ($^{\bullet}OH$), one of the strongest oxidants in nature [7, 10].

The ROS production is considered the principal cause of oxidative stress and cell death [3, 5, 10, 11]. Mitochondrial dysfunction has been attributed as one of the causes of ageing associated diseases, since the theory of ageing focus on mitochondria as the principal free radicals generator, formed during a number of cellular processes including respiration and dopamine metabolism [1, 4, 10, 12].

Cells communicate and can respond to extracellular insults stimuli through cell signalling and signal transduction, which allow the transmission of information from outside of cells to functional elements inside the cells such as transcriptional factors responsible for gene expression. Molecules such as ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living cells, due his physiologic role in a number of cellular signalling cascades related to mitogenic response [7, 13, 14]. When production of ROS exceeds the ability of cells to defend themselves, the apoptotic pathways are induced. As defense mechanism against oxidative stress induced by ROS, low concentrations of these species have the ability of induce the expression of endogenous antioxidant enzymes and anti-apoptotic proteins [13].

Under physiological conditions, small amounts of ROS have been proven to be involved in important physiological functions such as signal transduction pathways. ROS control the gene expression that regulates cell proliferation, redox state and activation of several proteins involved in

adaptation. Cell homeostasis is achieved through a highly coordinated mechanism of sensing and transduction stress signal, a genetic reprogramming involving several transcription factors. These mechanisms leads to cellular changes, such as the decrease in the expression of housekeeping genes and protein synthesis; increase of the expression of genes encoding stress proteins (including molecular chaperones responsible for maintaining protein folding), membrane transporters, proteins involved in DNA repair and regulating the degradation and detoxification pathways and nutrient metabolism [7, 11, 15].

Endogenous antioxidant systems are essential for the maintenance of the redox balance of eukaryotic cells. In mitochondria an abnormal increase of ROS can also lead to an increase oxidation of GSH and consequently to lower GSH/GSSG levels, being GSH the most important non-enzymatic antioxidant molecule for the maintenance of mitochondrial integrity [1, 5, 16].

Proteins are the main effectors in the cell, playing the most essential functions in all biological processes. A fine balance between protein synthesis and degradation rate must be maintained. Normally, the cell is fully equipped with a surveillance system mediated by UPS that rapidly eliminates unfolded and/or abnormal proteins, which are unwanted in the cell. Proteins with non-native or aberrant structures are not observed in normal cells, since they are rapidly removed, but if the cell ability to degrade abnormal, mutated or oxidized proteins is exceeded or a UPS dysfunction occur, proteins tend to aggregate. Proteolytic stress could be due to the over production of various toxic molecules such as ROS or mutanted proteins (Fig. 2) [4, 17-19].

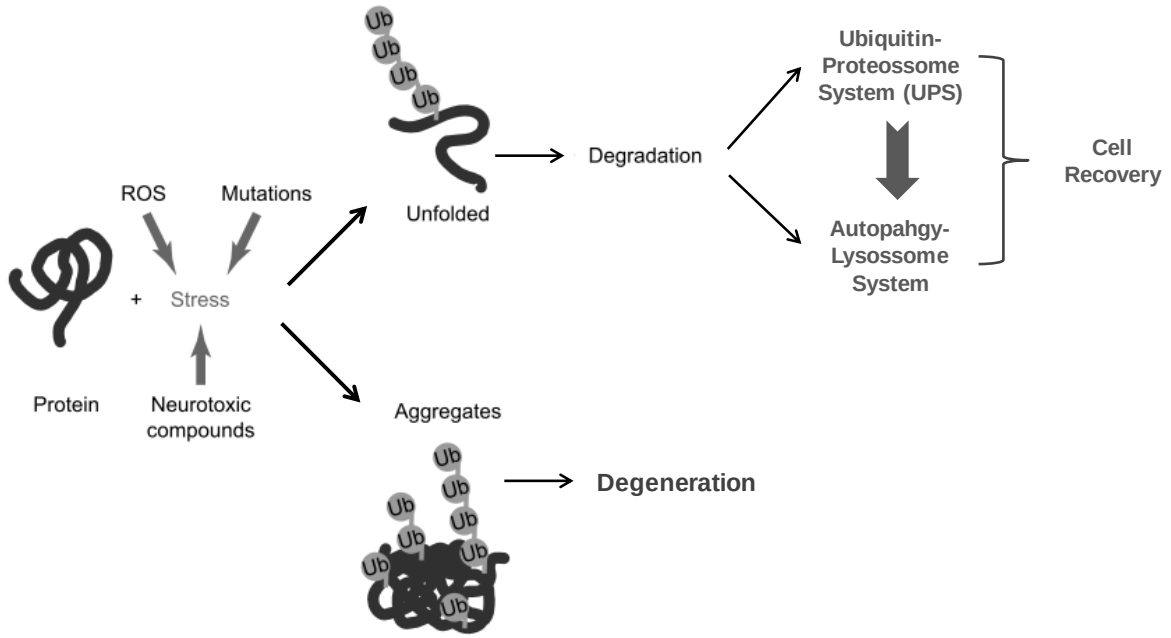

Figure 2. Protein misfolding and ubiquitylation processes induced by an external stress. A fine balance between protein synthesis and degradation rate must be maintained. Proteins with non-native or aberrant structures are rapidly removed inside the cells, but if the cell ability to degrade abnormal proteins is exceeded they tend to aggregate leading to degeneration processes (Adapted from Alves-Rodrigues and co-workers, 1998 [20]).

Despite the strong connection between protein misfolding, aggregation and disease, the mechanism by which these proteins form aggregates and their pathological significance are yet unknown [4, 17-19].

The UPS plays a major role in all stress responses and in protein homeostasis The degradation of proteins by the UPS is mediated by an important post-translational modification, the ubiquitylation, that plays a regulatory role in a broad range of biological processes such as cell cycle regulation, DNA repair, cell signaling, metabolic adaptations, development and differentiation. Severe or prolonged oxidative stress can promote partial unfolding of the target proteins leading to irreversible protein damage and aggregation (Fig. 2). Aggregates consist in insoluble, unfolded, ubiquitylated polypeptides that fail to be degraded by the 26S proteasome that stimulates a series of oxidative stress biological signaling pathways and inflammatory responses [4, 6, 17, 18, 21].

Under physiological conditions protein aggregation is mediated by both autophagy-lysosome pathway and UPS, however when cell are injured by oxidative stress the UPS can be overwhelmed and the autophagy-lysosome pathway is then required to increase its activity and to compensate for the increased protein damage (Fig. 2) [12, 22, 23].

Autophagy is physiologically important to maintain the balance between organelle biogenesis, protein synthesis and their clearance. This process mediates pathological responses and engages in cross-talk with ROS and RNS in both cell signalling and protein damage. The mechanisms that relate oxidative stress, mitochondrial impairment and autophagy in cell are not well understood, but possibilities include accumulation of toxic proteins and decrease of mitochondrial function [12, 22, 24].

2.2. *Saccharomyces cerevisiae*

2.2.1. Yeast as a model of degeneration

Degenerative disorders in humans can be modelled in laboratory using standardized procedures that provides an indispensable tool for basic research, or even eukariotic models of human disorders that allow the investigation of therapeutic options and molecular mechanisms of the disease. Since the common pharmacological therapies are limited to a treatment of the symptomatology of degenerative disorders without arresting the course of the diseases, it is extremely important to develop experimental models that replicate several aspects of degeneration process [25].

The yeast *Saccharomyces cerevisiae* is a lower eukaryote, also known as baker’s or budding yeast that is very useful to study fundamental cellular processes. Yeast models have been use as instrument for our current understanding of conserved cellular mechanisms such as cell division, DNA replication, metabolism, protein folding, intracellular transport and metabolic pathways associated with the response to oxidative stress. Yeast cells cannot be used to study neurodegeneration directly, but despite the obvious absence of a nervous system, yeast has proven to be effective to reveal information about basic mechanisms and pathways underlying degenerative diseases (mitochondrial dysfunction, transcriptional deregulation, trafficking defects and proteasomal impairment), since these mechanisms are well conserved between yeast and higher eukaryotes. One greatest advantage of yeast as a model system lies in the ability to rapidly perform genetic manipulations and screen for induced phenotypes [8, 11, 26, 27].

The involvement of ROS in oxidative stress and ageing process has been studied in the yeast *S. cerevisiae.* Ageing yeast cells, either chronologically or replicatively, accumulate ROS, indicating

that oxidative stress defenses plays a major role in governing the key pathways involved in ageing-induced apoptosis (Fig. 3) [10, 28-30].

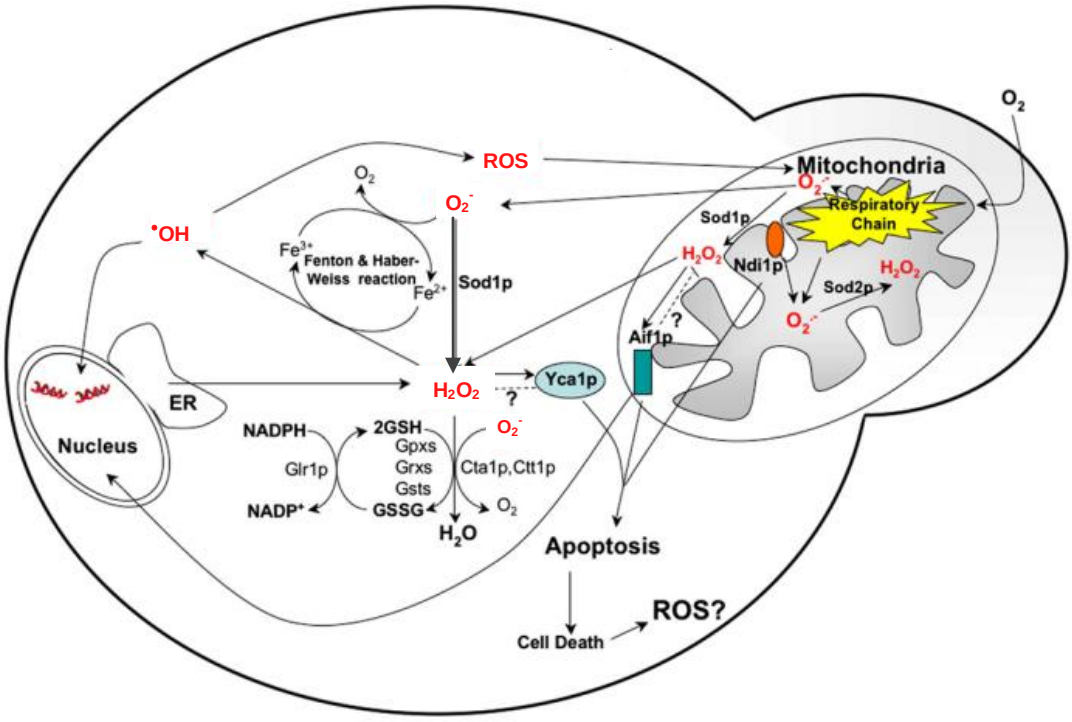

Figure 3. Sources of ROS and its influence in the mechanisms of apoptosis in *S. cerevisiae*. In red are intracellular sources of reactive oxygen species. Superoxide dismutases (Sod1p and Sod2p) represent the conversion of O_2^- to H_2O_2 and O_2 in cytosol and mitochondria respectively. The detoxification of H_2O_2 is mediated by catalases (Cta1p, Ctt1p) into H_2O. Glutathione systems are represented as fundamental elements for ROS elimination: Glr1p (glutathione reductase), Gpxp (Glutathione peroxidase), Grxp (Glutaredoxins) and Gstp (Glutathione transferase). Is represented later the oxidation of GSH into GSSG and consequent reduction of GSSG into GSH by Glr1p. Processes such as protein misfolding, ER stress, mitochondrial dysfunction and Fenton & Haber-Weiss reaction are all represented. Ndi1p is an internal mitochondria NADH dehydrogenase, Aif1p is apoptosis inducing factor and Yca1p that is a yeast metacaspase. Adapted from Perrone and co-workers [30].

2.2.2. Adaptive response against oxidative stress

Adaptation to efficient respiratory maintenance ensures that aged cells still display a functional replicative capacity. Indeed, yeast longevity is characterized by overexpression of the transcription factors which increases the expression of numerous respiratory genes, or overexpression of mitochondrial NAD-dependent dehydrogenases [7, 31]. All enzymes involved in the pentose phosphate metabolic pathway such as glucose-6-phosphate dehydrogenase (G-6-PDH), are crucial for the production of cellular reducing power in the form of NADPH. Enzymatic response against ROS, energetic metabolism and glutathione systems requires NADPH as reductant (Table 1) [28, 32].

Yeast contains two classes of oxygen regulated nuclear genes: aerobic and hypoxic genes. Transcriptional factors of these genes are altered as a response to ROS increase. In stress conditions due to increase of ROS, the glycolytic flux is also inhibit being the genes coding for glycololytic enzymes downregulated [7, 15].

Cells possess both enzymatic and non-enzymatic defense systems to protect their cellular constituents and maintain cellular redox state. Enzymatic machinery is constituted by SODs, reductases, catalases, peroxiredoxins (Prxp) and glutaredoxins (Grxp) (Fig. 3), whereas the non-enzymatic defense systems are typically consisted by small molecules such as ascorbic acid, glutathione, ubiquinone and vitamin E and C (Table 1). These molecules are soluble in either aqueous or lipid environment and act in general as radical scavengers, being oxidized by ROS and removing oxidants from solution [30].

Yeast cells have two groups of enzymatic strategies, one group of enzymes that act directly as ROS detoxifiers (e.g. Sod1p, Sod2p, Ctt1p, Cat1p) and a second group consisting of several enzymes which act as redox regulators of protein thiols contributing to cells defense against ROS (e.g. γ-glutamylcysteine synthetase (Gsh1p),Glutathione redutase (Glr1p)) (Table 1) [10, 28, 33, 34].

Table 1. Representation of the most important antioxidant defenses in *S. cerevisiae* relationated with ROS detoxification. The three main categories of defense: Antioxidant metabolites, Enzymes and Transcriptional factors. Adapted from Perrone and co-workers [30].

	Gene(s)	Gene Product	Role
Antioxidant metabolites			
Glutathione			Aqueous redox buffer; excretion of xenobiotics
Ubiquinone			Lipid soluble antioxidant, respiratory chain component
Enzymes			
Superoxid Dismutase Cytosolic	SOD1	Sod1p	Dismutation of O_2^- to $H_2O_2 + O_2^-$
Superoxid Dismutase mitochondial	SOD2	Sod2p	
SOD Chaperon	CCS	Ccs1p	Require for leading cooper into Sod1p
Catalase T cytosolic	CTT1	Ctt1p	Dismutation of H_2O_2 to $H_2O + O_2$
Catalase A peroxisomal	CTA1	Cta1p	
Cytosolic Thioredoxin	TRX2	Trx2p	Deoxyribonucleotide Synthesis, sulphate assimilation, cofactor for some peroxidases, redox control
γ-glutamyl-cystein syntethase	GSH1	Gsh1p	Stepwise synthesis of GSH
Glutathione reductase	GLR1	Glr1p	Reduction of GSSG to GSH
Metal binging proteins	CUP1		Copper binding protein
Glucose-6-phosphate dehydrogenase	ZWF1		Generation of NADPH for recycling of glutathione, glutaredoxins and thioredoxins
Transcriptional factors			
Yeast activator protein 1	YAP1	Yap1p	Oxidative stress, resistance to xenobiotics and metals
Skn7p	SKN7	Skn7p	Auxiliary transcriptional factor, functions with Yap1p for some oxidative stress and also acts in osmoregulation
Cup2p	ACE1/CUP2	Ace1p	Cu-binding transcriptional factor activates CUP1 at higher copper levels

S. cerevisiae exhibits the classic markers of apoptosis (nuclear fragmentation, chromatin condensation, DNA cleavage, phosphatidylserine externalization, and the accumulation of ROS), when in response to a stimulus such as H_2O_2, salts or metals. ROS can play an important role as

secondary messenger in protein phosphorylation regulation and transcriptional factor activation, to mediate the early response to oxidative stress [15, 35]. Cells can adapt to become more resistant to a subsequent lethal exposure, by induction of cell growth and differentiation, but at higher doses of ROS the cell activates gene expression mediated mainly by Yeast activator protein 1 (Yap1p) and Snk7p transcription factors, that in normal conditions are negatively regulated by glucose and promotes cellular growth [30, 36]. These two transcriptional factors control expression of protective genes that repair DNA damage, stress proteins (e.g. chaperons), arrest the proliferation of damage cells and induce apoptosis (Table 1) [15, 30, 37].

2.2.2.1. Yeast activator protein (Yap)

In yeast, the basic leucine-zipper transcription factor, Yap1p regulates most of the known cellular antioxidant genes and plays a major role in the adaptive response to oxidative stress [28, 35, 38]. The adaptive response to oxidative stress involves a change in the expression of sereval proteins, as well as a rapid and widespread genomic response suggesting the existence of specific control pathways. Mutants deficient in Yap1p have reduced activities of several enzymes related with antioxidant defense such as SOD, G-6-PDH, and Glr1p, suggesting that this transcription factor is involved in the regulation of enzymes which protect against oxidants [10, 28, 39]. Yap1p is a transcription factor that controls the oxidative stress adaptive response through the expression of genes encoding most of the yeast endogenous antioxidants and components of the cellular thiol-reducing pathway, including the SOD1, SOD2, TRx (Thioredoxin), GLR1, CTT1, CCP1 (cytochrome c peroxidase), TRR1 (Thioredoxin redutase), AHP1 (alkyl hydroperoxide reductase) and GSH1 genes [10, 37, 39].

The Yap1p target-genes include important activities in the maintenance of cellular redox homeostasis and in cellular xenobiotic detoxification (ATP-binding cassettes transporters, GSH transferases, GSH biosynthetic pathway) [28, 39-41]. Yap1p activates an oxidative stress adaptive response by redox sensory mechanisms which detect changes in the intracellular redox balance caused by ROS, oxidized thiols and metals. Yap1p can activate gene expression through binding to Yap1 Recognition Elements (YRE) present in the majority of gene of response to oxidative stress promoters [10, 28, 39].

Yap1p can be activated by H_2O_2, thiol oxidants, electrophilic compounds and metals. ROS (hydroperoxides and the superoxide anion) and chemicals with thiol reactivity (electrophiles and divalent heavy metals cations) both inhibit Yap1p nuclear export by disrupting its interaction with the nuclear export receptor Crm1, leading to gene transcription. The response of Yap1p to O_2^- was suggested by the identification of SODs as target genes. Yap1p might also protect yeast from the combined oxidative and phytochemical induced stress, since several phytochemicals have electrophilic properties [38-40].

The similarity between the cysteine thiol reactivity of Yap1p and the mammalian AP-1, Keap1–Nrf2 systems makes of Yap1p a good model for investigating the general mechanisms of oxidative stress response in mammalian cells [42].

2.2.2.2. Glutathione defense system

Sulfhydryl groups are important components of cellular defense against oxidative stress and for the maintenance of the redox homeostasis of cells. Glutathione is present in most living cells from microorganisms to humans and can occur in the cells under the form of GSH (reduced glutathione), GSSG (oxidized glutathione), and mixed disulfides, GSS-CoA and GSS-Cys. GSH is a low-molecular-mass thiol existing in cells in the concentration range of 1–10 mM and function as a redox buffer in many cellular processes including protection against xenobiotics, carcinogens and damaged by ROS. GSH is essential in eukaryotes; studies in yeast have shown that GSH is required as a reductant to remove endogenously-derived toxic metabolites.Glutathione is the best-known example of a non-enzymatic defense system that acts as a radical scavenger to maintain redox homeostasis. The redox active sulphydryl group of this thiol reacts with oxidants to produce GSSG. GSH is synthesized in two ATP-dependent steps catalyzed by Gsh1p and Gsh2p (Glutathione synthesize) (Table 1). The biological importance of GSH is dependent upon the redox-active free sulfydryl moiety of its cysteine residue [9, 31, 41, 43].

ROS detoxification through GSH is catalysed by Glutathione peroxidase (Gpxp), which converts two molecules of GSH into one molecule of GSSG, while the enzyme Glr1p is responsible for the reduction of GSSG that is very important to maintain the GSH/GSSG ratio in cells. This process utilizes NADPH as a reducing power, which is generated by the G-6-PDH and is found not only in the cytosol of cells but also in the mitochondrial matrix (Fig. 3 and 4). In cells this system is equilibrated by a GSH/GSSG ratio of approximately 50 [28, 36, 41, 44].

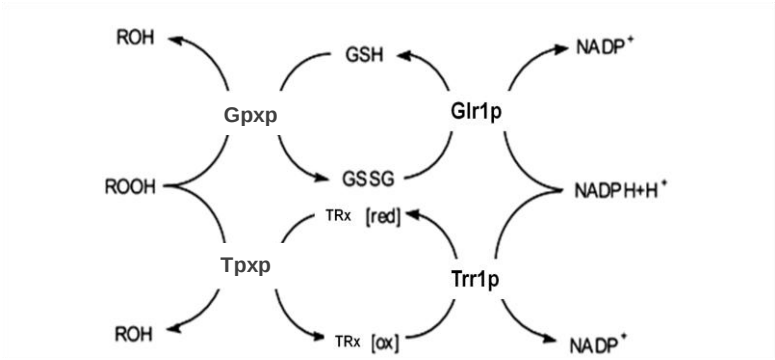

Figure 4. Cooperative action of the two main enzymatic systems of ROS modulation on *S. cerevisiae,* to the mantainance of redox homeostasis. Glr1p - glutathione reductase; **Gpxp** – glutathione peroxidase; **Trr1p** – thioredoxin reductase; **TRx** - thioredoxins; **Tpxp** – thioredoxin peroxidase. (Adapted from Costa and co-workers [7].

During respiration, mitochondrial thiols are the principal ROS targets. GSH is synthesized in the cytosol and must be transported into the mitochondria via an active energy-requiring process; however, GSSG formed in the mitochondrial matrix is unable to exit of this compartment and must be reduced by Glr1p. Mitochondrial and cytosolic forms of Glr1p are both encoded by a nuclear gene and expressed via alternative start site selection, a mechanism that is conserved in mammalian cells. In stress conditions, the levels of GSH decrease, with a consequent increase in GSSG. When GSSG is formed, cells must, in order to maintain redox homoeostasis induce the regeneration of GSH. In parallel the thioredoxin system also contributes for the balance on redox state (Fig. 4) [7, 31, 32, 45].

GSH also has strong nucleophilic properties allowing the conjugation with xenobiotics with electrophilic properties. In living tissues and also in yeast GSH assumes a pivotal role in the regulation of sulphur metabolism, being most of the excess of sulphur incorporated into GSH. It is also important in bioreductive reactions, transport, enzyme activity and integrity of mitotic apparatus [41, 43].

2.2.2.3. Superoxide dismutase (SOD)

SODs catalyze the disproportionation of O_2^- to H_2O_2 and O_2, but their activity requires redox active metal ions. Yeast cells, like other eukaryotes, possess two SODs, the Cu/ZnSod (Sod1p) encoded by SOD1 gene and MnSod (Sod2p) encoded by SOD2 gene. Both Sod1p and Sod2p play a role in detoxification of superoxide anion generated in the mitochondrial respiratory chain. Sod1p comprises 90% of the total SOD and is widely distributed in the cell being in more quantity in the cytoplasm. The Sod1p, the major enzyme involved in the remotion of O_2^- from the cytoplasm and possibly also from the peroxisome, appears to be a key enzyme involved in the regulation of intracellular levels of ROS and in protecting cells from the exogenous toxicity of oxidant agents. On the other hand Sod2p, localized in the mitochondria, has the physiological role of protect the mitochondria from O_2^- generated during respiration. These proteins have different metal binding ability, distribution in cell compartments and sensitivity to various reagents [11, 44, 46].

Sod1p is synthesized in a non-active form; being functional after addition of Cu^{2+} at the active site by the copper chaperone protein (Ccs1p) [11, 44, 46]. Oxidation state of sulfhydryl groups is important for Sod1p activation, essential for the folding and for import to mitochondria. Grxp protect Sod1p from oxidation by catalyzing both the formation and reduction of intermolecular disulfides between Sod1p and low molecular weight thiols such as GSH [44, 47].

In eukaryotic cells, MnSOD is strictly a mitochondrial enzyme located in the inner membrane but is synthesized by nuclear genes. A larger precursor form is produced in the cytosol and transported into the mitochondria by an energy dependent process. For its translocation CUP1 (that codifies for metallothionein, a copper binding protein) and SOD1 genes are coordinately regulated through Ace1p (metallothionein protein) and copper (Table 1) [44, 47].

SODs have great physiological significance and therapeutic potential, being involved in diseases frequently associated with ageing, being ALS the best study. Mutations in the gene coding for the antioxidant enzyme Sod1p are the most frequent cause of familial ALS. Oxidative crosslinks at cysteine residues play an important role in Sod1p aggregate formation, accumulation of this protein in motor neuronal cells would interfere with axonal transport, UPS and mitochondrial function, resulting in neuronal cell death of ALS patients [46].

2.3. Natural phytochemicals and human health

Intake of fruit and vegetables, increase cell survival and life span, in mammals through the reduction of oxidative stress damage Natural phytochemicals present in food prevent oxidation of proteins, lipid peroxidation and generation of ROS. Significant evidences show beneficial effects on human health and the reduction of the risk of cardiovascular diseases, cancer, type II diabetes and other disorders associated with ageing [2, 3, 48].

The protective effect of fruits and vegetables may be due to the biological activities of its phytochemical compounds, defined as bioactive non-nutrient components. Berry extracts are widely consumed as dietary supplements due to their potential human health benefits [49, 50]. It has been demonstrated that plants contain many natural phytochemicals such as carotenoids, polyphenols, alkaloids and other nitrogen-containing compounds; which have been identified as free radicals or active oxygen scavengers. In addition, intake of these phytochemicals in the diet may prevent diseases by modulating signalling pathways, decreasing the DNA damage or through enhanced DNA repair by genes activation [51-53].

2.3.1. Polyphenols

The primary function of polyphenols is the protection of plants against ROS produced during photosynthesis, and from herbivores attack. The main dietary sources of polyphenols are berries, cereals, dry legumes, chocolate, and plant-derived beverages, such as tea, coffee, and wine. Several hundreds of different polyphenols have been identified in foods, constituting a large group of phytochemicals such as: flavonoids (anthocyanins, flavonols, and flavanols), tannins, proanthocyanidins, ellagitannins, gallotannins, stilbenoids, and phenolic acids [3, 48, 53-55]

Total dietary polyphenols intake could be as high as 1 g per day, which is much higher than that of all other classes of phytochemicals from diet [48, 54, 56]. Flavonoids are themselves distributed among several classes: flavones, flavonols, flavanols, flavanones, isoflavones, proanthocyanidins, and anthocyanins. Proanthocyanidins, common in many fruits, such as apple, grape, or cocoa, are responsible for their characteristic astringency or bitterness [48, 57].

Anthocyanins are polyphenol pigments responsible for the attractive colors red, blue, and purple of fruits, vegetables, flowers, and other plant tissues that are important to their large role of bioactivities including antioxidant, anticancer, and anti-inflammatory. Anthocyanins are major dietary components mainly found in berries [48, 57]. Ellagitannins, which are complex derivatives of ellagic acid, identified in tea, medicinal plants, and several fruits, also have general scacenging effects [49, 58].

For many years, polyphenols and other phenolic compounds were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, phytochemicals are described as also possesing anti-inflammatory, antimutagenic, anti-atherosclerotic, anticarcinogenic, antibacterial and antiviral activities and can exert their effects through different mechanisms. Cells also respond to polyphenols through direct interactions with receptors or enzymes involved in signal transduction or modulating the activity of enzymes involved in ROS modulation, which may result in modification of the redox status [50, 58, 59]. Numerous pathways have been reported as being targets of phenolic compounds, thereby demonstrating the broad spectrum of targets and strengthening their usefulness in addressing multifactorial diseases [2, 3].

2.3.2. Berries

Berries such as blackberries, strawberries, raspberries and blueberries provide a rich, diverse and specific source of dietary phytochemicals, especially polyphenols [49, 58]. Levels of

proanthocyanidins or ellagitannins, vary considerably among berries and among commonly consumed berries, blueberries and cranberries contain predominantly proanthocyanidins whereas blackberries, raspberries, and strawberries contain predominantly ellagitannins [58, 60, 61].

The *A. unedo* and *C. album* berries and leaves were tested in this study for being still poorly characterized for protective effects in degeneration processes. *G. biloba* leaf and *R. ideaus* fruit are two plant matrixes very well studies that can serve as comparison.

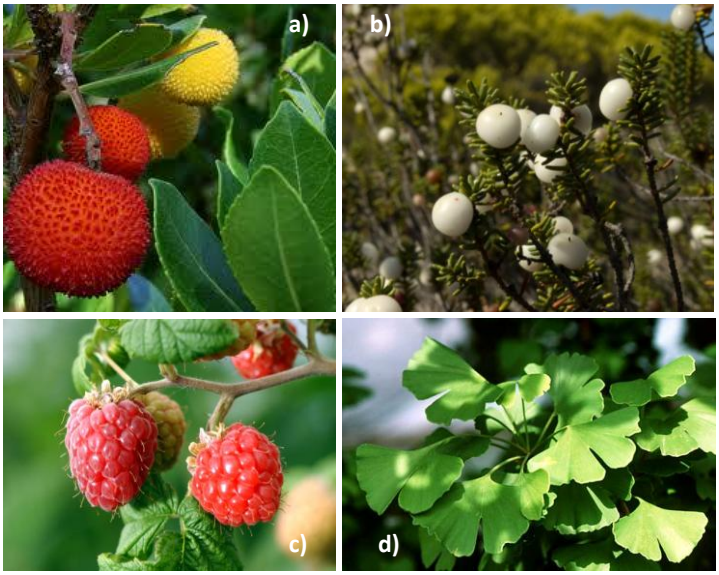

Figure 5. Plants used in this work. a) *Arbutus unedo* (L.) *Ericaceae* family commonly known as strawberry tree; **b)** *Corema album* (L.) D. Don *Empetraceae* family commonly kwon as Portuguese crowberry; **c)** Red raspberry (*Rubus idaeus* L.), *Rosaceae* family; **d)** *Ginkgo biloba* tree (Maidenhair tree, *Ginkgoaceae)*

Arbutus unedo

Arbutus unedo (L.) commonly known as strawberry tree is an evergreen shrub, a native Mediterranean species that belongs to *Ericaceae* family. Its fruits (berries) are spherical and dark red. These berries are rarely eaten as fresh fruits but have importance in local agricultural communities which use them for the production of alcoholic beverages, jams, jellies and marmalades. In Portugal, the strawberry tree is mainly implanted in the south, being however present throughout all of the country in a dispersed way (Fig. 5 a)) [51, 62, 63].

A. unedo fruit has been used in folk medicine as antiseptics, diuretics and laxatives, while the leaves have long been employed as an astringent, diuretic, urinary anti-septic agent, depurative and, more recently, in the therapy of hypertension, inflammatory diseases and diabetes. Studies showed that fruit and leaf extracts contain several phenolic compounds, like tannins, flavonoids, phenolic (e.g. anthocyanins), gallic acid derivatives, tannins, vitamins C and E, and carotenoids, which make them a good source of phytochemicals [51, 62-64].

Corema album

Corema album (L.) D. Don, belongs to *Empetraceae* family. It is also designated by camarinha or Portuguese crowberry, is a dioecious shrub endemic from Iberian Peninsula and Azores Islands

(ssp. azoricum) that grows in sand dunes and coastal cliffs of the Atlantic coast. It produces edible berries, which are white when ripe and have a sugary and water-rich pulp. The fruits take months to ripen while flowering occurs from February to April, with fruits ripening from June to September. This fruit is not currently commercially explored and very few studies were performed with this plant, however might be a potential important source of nutrients and phytochemicals (Fig. 5 b)) [65, 66].

Rubus ideaus and *Ginkgo biloba*

Berries belonging to *Rosaceae* family, namely, raspberry (*Rubus idaeus* L.), provide delicious fruits with high content in phenolic compounds. The genus Rubus, one of the most diverse in the plant kingdom, contains approximately 740 species. These diverse species are native on six continents and have been found from the tops of mountains to coastal locations at sea level. Subgenus Idaeobatus includes the European raspberries (*Rubus idaeus* L.)(Fig. 5 c)) [67, 68].

Raspberries are common in cool temperate regions of the northern hemisphere and its cultivation only became widespread in European countries by the 16th century. Nowadays raspberries are of economical importance, this fruits are widely consumed fresh, frozen, or in processed forms such as jellies, jams, and juices [67, 69]. Raspberry is a source of a variety of potentially healthy compounds. Studies recognized possible health benefits from their natural phytochemicals. Rubus fruit are considered a healthy and nutritious food, containing phenolics, vitamin C, α-tocopherol, carotenoids, linoleic acid and linolenic acid [60, 69, 70].

Red raspberries (*Rubus idaeus*) presents among fruits one of the highest scavenging capacity due to a unique phytochemical profile rich in ellagitannins and anthocyanins that distinguishes them from other fruits. Besides, raspberries contain a variety of beneficial compounds, including essential minerals, vitamins, fatty acids, and dietary fiber [60, 69-71].

The *Ginkgo biloba* tree (Maidenhair tree, *Ginkgoaceae*) is described as a "living fossil" since it represents the only surviving species of the order Ginkgoales that existed when the dinosaurs roamed the earth more than 200 million years ago. Modern Chinese pharmacopoeias introduced *Ginkgo* leaves for treating dysfunctions of the heart and lungs, and currently extracts of these leaves represent one of the most common phytomedicines in the world (Fig. 5 d)) [72].

Extracts of *G. biloba* leaf are widely used in herbal medicine for the treatment of mild to moderate cognitive disorders and dementia. A standardized extract of leaves from *G. biloba* named EGb 761 that is constituted by flavonol glycosides, terpene, lactones flavonoids, inkgolides, bilobalides, proanthocyanidins and organic acids is efficient in the treatment of clinical disorders with multifactorial origins. This extracts have been suggested by several studies to possess numerous beneficial properties, including free radical scavenging, antiapoptotic, antiageing and antigenotoxic. They also have been described to regulate gene expression and attenuate AD symptoms [3, 72-74].

2.3.3. Polyphenols bioavailability

Phenolic compounds present relatively low levels in circulation due to its reduce bioavailability. The ability of polyphenols to influence cellular function and affect health is dependent of their absorption from the gastrointestinal tract (GIT) to the blood and delivery to the target tissues. These compounds are extensively metabolized in the body tissues and can be absorbed in stomach and at small intestine level, by diffusion or transport. Variable amounts of flavonoids that are not absorbed in the upper GIT, reach the colon, where they are subjected to colonic microflora action and the microbial catabolites could be absorbed into the circulatory system from the large intestine. The real biological response to these compounds is greatly determined by the bioavailability of active molecules (Fig. 6) [49, 53, 75].

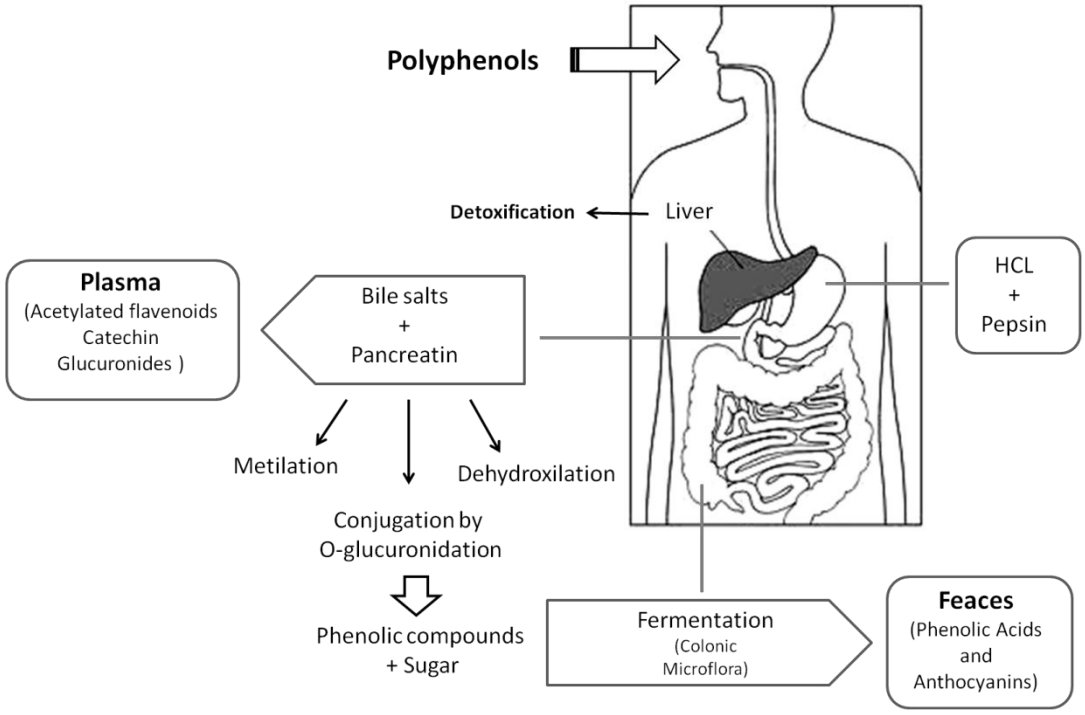

Figure 6. Bioavailability of polyphenols after alterations occurring during metabolization in the human body. Representation of the three main steps of polyphenols digestion: Stomach conditions where occur the degradation of sugars, vitamins and minerals; Small intestine where the majority of phytochemicals are modify and colon where occur the modifications by microflora. (Adapted from [2, 57, 76-78])

Bioavailability studies determine which are the: best absorbed polyphenols; modifications that polyphenols suffer after absorption; active metabolites and the polyphenols that lead to the active metabolites. Therefore, the knowledge of polyphenols bioavailability and metabolization is essential to identify those most predisposed to exert protective effects. The most absorbed in humans are isoflavones and gallic acid, followed by catechins, flavanones, and quercetin glucosides. The least well-absorbed polyphenols are the proanthocyanidins and the anthocyanins [49, 54, 58, 75, 76].

Evaluation of metabolites as they exist in plants or the effect of single purified phenolic compounds, ignoring the chemical alterations with a consequential impact on bioavailability and bioefficacy, leads to a loss of possible synergetic/cooperative or competitive activities between phenolic compounds that affect their final form present in serum (Fig. 6). These compounds may

persist *in vivo*, accumulate in target tissues, and contribute significantly to the prevention and treatment of chronic human diseases [2, 48, 58, 68]. In addition, there are also large variations in polyphenols bioavailability observed among individuals due to nutrigenetic and nutrigenomic effects. [50, 54, 77]. For these motif is important evaluate the food metabolites in the circulating form in the body.

In vitro *digestion (IVD)*

In order to evaluate the potential role of plant phytochemicals in the human body, should take into account the physiochemical changes occurring in the GIT [2, 48, 58, 68]. Only the compounds that can reach the serum and be distributed by all organs contribute significantly to the biological effects [49, 54, 58]. For these reasons is important an IVD process that mimics the physic and chemical transformations occurring in stomach and gut during disgetion before pass to serum, to provide essential informations on the relative potential bioavailability of different polyphenolic components in this specific phase of digestion. [52, 55, 57].

Despite the relatively high amounts of anthocyanins consumed in the diet and the reported biological activities, little is known about the *in vivo* biological activity of anthocyanins including bioabsorption. The instability of anthocyanins in procedures that mimic the physiochemical and biochemical changes that occur in the upper GIT has been study by Gordon M. and collaborators, that demonstrate that the major part of anthocyanins is degraded in GIT or goes out into colon, remaining only a small part in serum. [52, 53, 57, 76, 79].

Chemical and structural modifications due to gastrointestinal absorption and metabolization have not been taken into account in many previous studies. Information about absorption, distribution, metabolism, and excretion of individual flavonoids is still scarce and is important to understand the molecular mechanism(s) underlying the effects of food on human health and their biological activities [2, 52, 57].

3. Material and Methods

3.1. Samples preparation

3.1.1. Plant material

For this study were selected fruits and leaves, from *C. album (Corema album* (L.) D. Don) and *A. unedo (Arbutus unedo* (L.)). *R. idaeus* (*Rubus idaeus* L. *cv polka*), fruit and *Ginkgo biloba* leaf were also chosen due to their well known health benefits, to comparison purposes.

All the berries were harvested, frozen, grinded and then freeze-dried. Fruits and leaves of *C. album* and *A. unedo* were collected by random sampling in Comporta (southern region of Portugal) and in an extensive area of Arrábida Natural Park, respectively. *R. ideaus cv polka* fruit was harvested at Fataca experimental field (Odemira, Portugal). Leaves from *G. biloba* were from plants keep at ITQB (Instituto Tecnologia Química e Biológica) green house.

3.1.2. Hydroethanolic extraction

Secondary metabolites were extracted, using clean solvents, as previously described by Tavares and co-workers [80]. Briefly, to 1 g of lyophilised powder 12 mL of hydroethanolic solution (50% (v/v) ethanol/water) were added. This mixture was shaken for 30 min at room temperature in the dark and centrifuged at 12400 *g* for 10 min at room temperature. The supernatant was then filtered through 0.2 μm cellulose acetate membrane filters and the resulting extracts were stored at - 80 ºC.

3.1.3. *In vitro* digestion (IVD)

Phytochemical alterations occurring during digestion were mimicked using an adapted IVD model, accordingly with Tavares and co-workers [2]. This methodology was performed by a colleague of the Disease & Stress Biology (DSB) Laboratory in collaboration with Gordon McDougall and Derek Stewart from The James Hutton Institute, Dundee, Scotland.

This method consists in two sequential steps that represent an average time for gastrointestinal transit; an initial pepsin/HCl digestion for 2 h at 37 ºC, that simulate the gastric conditions, and a final bile salts/pancreatin digestion for 2 h at 37 ºC, that simulate small intestine conditions.

The original extract was adjusted to pH 1.7 with 5 M HCl and then the pepsin (Sigma Product number P6887) was added at 315 units.mL^{-1} and incubated at 37 ºC in a heated water bath for 2 h with shaking at 100 rpm. The remainder solution was placed in a glass beaker and 4.5 mL of a mixture consisting of 4 $mg.mL^{-1}$ pancreatin and 25 $mg.mL^{-1}$ bile salts were added. Then a segment of cellulose dialysis tubing, containing sufficient 0.1 M $NaHCO_3$ to neutralize the samples titratable acidity, was added and the beaker sealed with parafilm. Diffusion of $NaHCO_3$ out of the dialysis tubing represents the simplest and most convenient means to mimic the gradual rise in pH that occurs to the stomach contents when entering the small intestine.

After another 2 h of incubation at 37 ºC, the solution inside the dialysis tubing (IN), representing the serum available material and the solution outside the dialysis tubing (OUT), that mimics the material remained in colon, were mixed together to be representative of the total digestion process and the solution was frozen [76].

3.1.4. Solid Phase Extraction (SPE)

A Solid Phase Extraction (SPE) protocol was carried out to concentrate polyphenols of digested extracts, using a GIGA tubes 20g/60mL (1000 mg capacity, Phenomenex® Ltd.), C18-E units, after the IVD process. This protocol was performed accordingly with Tavares and co-workers [80] to afford complete separation of total phenolics compounds from the bile salts presents in the sample [52].

Briefly, after pre-equilibration of the column with 0.5% (v/v) (CH_3COOH/H_2O), the soluble materials from IVD were diluted in the equilibration solution and loading into the column C18-E. A wash step was performed with two volumes of 0.5% (v/v) (CH_3COOH/H_2O). Finally the bound fraction

that is enriched in polyphenols was eluted and recovered by adding a solution of 0.5 % (v/v) (CH_3COOH/CH_3CN).

Fractions were concentrated in a Speed-Vac to a final suitable phenol concentration and frozen at -80 ºC. To test in *S. cerevisiae*, PDFs (polyphenol-digested fractions) obtained after the IVD-SPE procedure were unfrozen and dissolved to the final concentration to test, in a (v/v) $CH_3COOH/CH_3CN/H_2O$ (0.05/0.995/99.9) solution.

3.2. Total phenol content

Determination of total phenolic compounds present in PDFs was performed by the Folin–Ciocalteu method, adapted to a 96 microplate reader, as described by Tavares and co-workers [80].

Phenolic compounds are excellent oxygen radical scavengers because of its electron reduction potential. Folin-Ciocalteu reagent is a solution of polymeric complex ions, formed by phosphomolybdic and phosphotungstenic acid. The phenolic compounds in the samples react with this colorimetric reagent, which allows measurement in the visible portion of the spectrum. The transfer of electrons in alkaline medium from phenolic compounds to oxidized phosphomolybdic/phosphotungstic acid complexes forms blue complexes that are determined spectroscopically at approximately 760 nm, due to its reduction. Sodium carbonate provides the alkalization [81].

To determine the total phenolic compounds in our samples along all steps of the digestion protocol, in each well of a 96-well plate, 235 µL of water, 5 µL of sample, 15 µL of Folin-Ciocalteu reagent (Fluka®) and 45 µL of saturated Na_2CO_3 (Riedel-deHaën®), were added. The microplate was incubated for 30 min at 40 ºC and the absorbance read at 765 nm in the end.

In case of the blank and standards we added 5 µL of water or gallic acid (concentrations between 0.05 and 0.7 $mg.mL^{-1}$), respectively, instead of sample. All measurements were performed with three replicates and the results were expressed as mg of gallic acid equivalents (mg GAE) and as % of original fraction.

3.3. Antioxidant capacity assay

Peroxyl radical-scavenging capacity of the samples in study was determined by the ORAC (Oxygen Radical Absorbance Capacity) method. This assay was carried out following a modified procedure described by Tavares and co-workers [59], adapted from Cao and co-workers [82], to 96-well plates.

This assay measures the ability of the antioxidant components in samples to inhibit the decline in fluorescein fluorescence, induced by a peroxyl radical generator, AAPH (2,2'-azobis (2-amidopropane) dihydrochloride) (Sigma®). The peroxyl radicals generated from thermal decomposition of AAPH in aqueous buffer, react with fluorescein, to form a non-fluorescent product which allow the quantification of fluorescein fluorescence decline. [82]

Briefly, the reaction mixture was prepared in a 96-well plate containing 150 µL of sodium fluorescein (0.2 nM) (Uranine, Fluorescein Sodium Salt® TCI Europe), 25 µL of sample and 25 µL of

AAPH (41.4 $g.L^{-1}$) per well. All solutions were prepared in 75 mM phosphate buffer (pH 7.4) and instead of standards/sample, the blank contained 25 µL phosphate buffer (75 mM, pH 7.4). To perform the standards 25 µL of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Fluka®) with concentrations ranging between 10 - 50 µM, were assayed.

Fluorescent emission at 515 nm was monitored kinetically during 30 min at 37 ºC, after excitation at 493 nm using FLx800 Fluorescence Microplate Reader (Biotek). All measurements were performed with three replicates. The final results were calculated and expressed using the differences in area under the fluorescence decay curves between the blank and the Trolox/sample, and were expressed as µM trolox equivalents (µM TE) and µmol of trolox equivalents per phenol unit (µmol TE. mg GAE^{-1}).

3.4. *Saccharomyces cerevisiae*

In this study four different strains of *S. cerevisiae* were used; the WT BY4741 (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and its isogenic mutants, *Δsod1* (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *Δsod1::KanMX4*) and *Δyap1* (*MAT*a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0 Δyap1::KanMX4*), which do not possess a functional gene corresponding to the cytosolic superoxide dismutase (Sod1p) or a critical transcription factor involved in the oxidative stress response (Yeast Activator Protein 1, Yap1p), respectively. These strains were acquired from Euroscraf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) and carry a KanMx4 deletion cassette replacing the gene in question.

In addition, AD1-8 strain was also used. This strain features eight deletions in MDR genes (multi drug resistance genes) (*Δyor1, Δsnq2, pdr5-Δ2, Δpdr10, Δpdr11, Δycf1, pdr3 -Δ2, Δpdr15*) that encode ABC transporters involved in xenobiotic efflux capacity. This strain does not have efflux systems, facilitating the uptake of compounds and allow us to verify the effect of a prolonged exposition to compounds from PDFs.

All strains used present a different sensitivity to oxidative stress and were routinely maintained in YPD (Yeast extract, Peptone, Dextrose) solid medium composed of yeast extract 1% (w/v) (HiMedia), glucose 2% (w/v) (Sigma), peptone 2% (w/v) (HiMedia) and agar 2% (w/v) (HiMedia), at 4 °C, after growth at 30 °C for 48 h [11].

For all experiments, a pre-inoculum was prepared, in syntetic complete (SC) liquid medium, composed of yeast nitrogen base without amino acids 0.67% (w/v) (Difco), glucose 2% (w/v) and complete supplement mixture (CSM) 0.79 $g.L^{-1}$ (QBiogene). A colony was growth until the optical density at 600 nm ($OD_{600\ nm}$) reached approximately 0.4-0.6 (which corresponds to the middle of log phase) at 30 ºC under constant shaking, to a final volume of 3 mL (ratio of 1:3 flask/medium volume). Afterwards, to prepare the inoculum, cells were diluted in the same medium, in order to obtain all strains in log phase ($OD_{600\ nm}$ aprox. 0.6) and the same conditions of pre-inoculum were repeated. Also was taken into account the doubling time (DT) of each strain, using a DT of 2 h for BY4741 and *Δyap1* and 3 h for *Δsod1* and AD1-8 according with what was defined by previous growth curves. Therefore OD readings were performed in the plate spectrophotometer Power Wave XS (Biotek) and all experiments were done with three biological replicates.

For long period storage, stocks of these strains were frozen at - 80° C in a solution consisting of 50% (v/v) glycerol and YPD liquid medium.

3.4.1. Growth curves

To study the different sensitivities of each strain to H_2O_2, the growth of all strains (BY4741, *Δyap1, Δsod1*, AD1-8) was monitored by $OD_{600\ nm}$ reads during 24 h, to achieve the growth curve and growth parameters corresponding to each strain. The growth curves were performed, in presence and absence of several concentrations of H_2O_2.

After inoculum preparation, previously described in section 3.4, cells were diluted in SC liquid medium to $OD_{600\ nm}$ of 0.03 and H_2O_2 was added to the yeast culture at final concentrations between 0 and 1 mM in a 96-well plate, to a final volume of 100 μL. The H_2O_2 concentrations used were determined based in previous unpublished studies of DSB Laboratory.

All strains were incubated at 30 °C for 24 h, with constant shaking and yeast growth was kinetically monitored hourly by $OD_{600\ nm}$ readings in a spectrophotometer microplate reader (PowerWave XS Biotek).

All experiments were performed with three replicates. For each strain and condition, in the end of growth, the final biomass was estimated by $OD_{600\ nm}$ readings at 24 h. DT and *lag* phase times were determined based on the linear regression of log growth phase equation, obtained after logarithmic transformation of OD values.

3.4.2. Spot Assay

Spot assays was the methodology chosen to test three different conditions: 1) The susceptibility of yeast strains to oxidative stress induced by H_2O_2; 2) The range of non-toxic concentration of PDFs and 3) The protective potential of PDFs in the presence of oxidative stress.

After an inoculum preparation, yeast cells were incubated with different concentrations of H_2O_2. In a 96-well plate, cells were diluted until $OD_{600\ nm}$ of 0.4 in SC liquid medium and H_2O_2 was added in concentrations between 0 - 10 mM, to a final volume of 100 μL. The microplate was incubated during 1 h at 30 ºC, with constant shaking. Subsequently 30 μL of cells were withdrawn to a new 96-well plate and the $OD_{600\ nm}$ adjusted to 0.1 in PBS (phosphate buffer) pH 7.4. Finally, five-fold serial dilutions were made, in PBS pH 7.4 and 5 μL of each well was spotted in YPD solid medium and incubated for 48 h at 30 ºC. At the same time 5 μL of the same cells growth in the absence of H_2O_2 were spotted in SC solid medium with glucose supplemented with different concentrations of H_2O_2, between 0 and 1 mM.

The PDFs toxicity was evaluated by the same procedure, a 96-well plate was prepared but instead H_2O_2, the PDFs were added in final concentrations between 0 and 1000 μg $GAE.mL^{-1}$. The initial $OD_{600\ nm}$ was 0.2 and the microplate was incubated during 4 h at 30 ºC, with constant shaking. At the end 5 μL of cells were spotted in SC solid medium with glucose.

After PDFs toxicity assay, in order to evaluate the protector potential of PDFs under an oxidative stress condition, cells were grown first in the presence of PDFs and subsequently were submitted to an oxidative stress induced by H_2O_2.

Cells were inoculated in a 96-well plate with an initial $OD_{600\ nm}$ of 0.2 and in presence of PDFs non-toxic concentrations, previously determined. The microplate was incubated at 30 ºC during 4 h, with constant shaking. Finally the same serial dilutions described above were performed and 5 μL of cells were spotted in SC solid medium with glucose supplemented with the previously selected H_2O_2 concentration according with the strain (BY4741 0.5 mM and *Δyap1, Δsod1* 0.15 mM). Cells were incubated for 48 h at 30 ºC.

Spot assays were prepared in the same technical conditions and always with three biological replicates, being the most representative showed. Images were acquired using Quantity-one® software from Chemidoc and were obtained in the end of 48 h of growth.

3.4.3. Resazurin-based assay

To evaluate the protective effect of PDFs based on mitochondrial metabolism integrity the resazurin-based assay methodology was used, adapted from CellTiter-Blue® Cell Viability Assay (Promega) protocol, to *S. cerevisiae*. This is a sensitivity test that has been used for estimating viability based on cell energy metabolic activity. Resazurin is a blue weakly fluorescent indicator dye that changes into highly fluorescent pink resorufin, in response to irreversible chemical reduction inside the cell [83].

Resazurin can penetrate cells and acts as an intermediate electron acceptor in the electron transport chain. This allows enzymatic reduction in the mitochondria due to the activity of enzymes and subsequently a pink resorufin that is also a fluorophor, can diffuse from cells and back to surrounding medium, which results in fluorescence increase [83].

To establish the number of cells (represented by $OD_{600\ nm}$ readings) to use in this methodology, in a 96-well plate each strain was inoculated with $OD_{600\ nm}$ between 0.2 and 0.02. To each 100 μL of total volume of cell suspension, 20 μL of CellTiter-Blue® reagent were added. Incubations of 45 min were performed with resazurin at 30 ºC and in the end of each time fluorescence was measured.

To evaluate if PDFs can affect the metabolic answer of yeast cells in the presence of oxidative stress, the inoculum was diluted to a final $OD_{600\ nm}$ of 0.2 in 96-well plate, with SC liquid medium with glucose, in the same conditions described in section 3.4.2. After 4 h incubation with PDFs, yeast cells were harvested by centrifugation at 1000 *g* for 5 min, rinsed twice in PBS pH 7.4 and diluted to a final $OD_{600\ nm}$ of 0.2, with the same medium supplemented with 2 mM H_2O_2, for all strains, in a new 96-well plate. The microplate was incubated for 1 DT according with the strain and in the end 30 μL of cells were withdrawn and diluted in SC liquid medium with glucose until $OD_{600\ nm}$ of 0.02 and CellTiter-Blue reagent was added. Before fluorescence reading the microplate was incubated for 45 min at 30 ºC with constant shaking.

All fluorescence readings were performed using a FLx800 Fluorescence Microplate Reader (Biotek) using excitation and emission wavelengths of 560 and 590 nm, respectively. The experiences

were done with three biological and two technical replicates and results were expressed as fluorescence units (F.U.) and percentage of viable cells relative to the control.

3.4.4. Dichlorofluorescein assay

To determine the levels of ROS produced, intracellular ROS production was measured by H_2DCFDA (2,7-dichlorofluorescein diacetate) method, adapted from Priault and co-workers [84]. H_2DCFDA is added to cells, which penetrates easily the plasma membrane and is hydrolyzed inside the cells by non-specific esterases, being converted to the non-fluorescent H_2DCF that in presence of ROS is oxidized to fluorescent dichlorofluorescein (DCF), a highly fluorescent compound and can be measured fluorimetrically. Thus, measurement of DCF concentrations is a valid measure of oxidation processes inside the cells [84-86].

H_2DCFDA (Sigma-Aldrich®) stock solution was prepared in dimethyl sulfoxide (DMSO Sigma-Aldrich) and stored at −80 °C.

To test the effect of PDFs in the boost of ROS induced by H_2O_2, the incubation with PDFs was performed exactly in the same conditions described for CellTiter-Blue viability assay. Then yeast cells were harvested by centrifugation at 1000 *g* during 5 min, diluted to a final $OD_{600\ nm}$ of 0.2 in SC liquid medium with glucose. H_2DCFDA was added in a final concentration of 100 μM and cells were incubated at 30 ºC for 15 min, in the dark with constant shaking, to allow the uptake of the probe by cells. After the 15 min incubation, $OD_{600\ nm}$ reading was performed and H_2O_2 added to a final concentration of 2 mM for all strains.

Fluorescence intensity was always measured at every 10 min, during 1 DT for each strain with constant shaking, using a FLx800 Fluorescence Microplate Reader (Biotek). Fluorescence excitation and emission used were of 488 and 520 nm, respectively. All experiments were performed with three biological replicates and the results were expressed as percentage of increase in fluorescence per well, normalized for total cell number represented by $OD_{600\ nm}$.

3.4.5. Glutathione determination

To analyze the redox state of cells, GSH and GSSG were quantified by HPLC after derivatization with orthophthalaldehyde (OPA), accordingly to the already described in Tavares and co-workers [2].

Briefly, yeast cells were harvested by centrifugation at 1000 *g* during 5 min and rinsed twice in PBS pH 7.4. Then cold 10% (w/v) metaphosphoric acid was carefully added to samples, into 1.5 mL propylene tubes (50 µL for determination of GSH and 200 µL for determination of GSSG). Glass beads were added and cells were lysed by vortexing, 6 cycles of 1 min in the vortex followed by 1 min at 4 ºC. The final mixture was centrifuged at 2700 *g* during 5 min, the supernatant was removed and centrifuged again at 2700 *g* during 30 min and the final supernatant was collected. After samples preparation the final supernatant was derivatizated accordingly to Kand'ar and co-workers [87], adapted from Hissin and Hilf [88].

For GSH analysis 1 mL of 0.1% (w/v) EDTA in 0.1 M sodium hydrogenphosphate, pH 8.0, was added to 50 µL of supernatant. To 20 µL portion of this mixture, 300 µL of 0.1% (w/v) EDTA in 0.1 M PBS, pH 8.0, and 20 µL of 0.1% (w/v) OPA in methanol, were added. Tubes were incubated at 25 ºC for 15 min in the dark. The reaction mixture was then stored at 4 ºC until analysis. In parallel, for GSSG analysis, 200 µL of supernatant was incubated at 25 ºC with 200 µL of 40 mM *N*-ethyl maleimide (NEM) for 25 min in dark. To this mixture, 750 µL of 0.1 M NaOH was added. A 20 µL portion was taken and mixed with 300 µL of 0.1 M NaOH and 20 µL of 0.1% (w/v) OPA. Tubes were incubated at 25 ºC for 15 min in the dark and stored at 4 ºC until analysis.

Chromatographic analysis was accomplished using isocratic elution on C18 analytical column (YMC- PACK ODS-A, HPLC 200Å, 5 µm) at 37 ºC on an Acquity TM Ultra performance LC system (Waters). The mobile phase consisted of 15% (v/v) methanol in 25 mM PBS (v/v), pH 6.0. The flow rate was kept constant at 0.7 $mL.min^{-1}$. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak area using Empower® Pro 2.0 software [13]. The concentration of GSH and GSSG in the samples was determined from standard curves with ranges 0 - 250 µM for GSH and 0 - 25 µM for GSSG, prepared in parallel with the samples. All experiments were performed with three biological and two technical replicates. The results were expressed as µM of GSH normalized for total number of cells ($OD_{600\ nm}$) (GSH / $OD_{600\ nm}$) and as the ratio (GSH/GSSG).

3.5. Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Regressions presented were obtained using Microsoft® Excel 2007 tools. Differences among treatments were detected by analysis of variance with Tukey HSD (Honest Significant Difference) multiple comparison test (α = 0.05) using SigmaStat 3.10 (Systat).

4. Results and Discussion

4.1. Oxidative stress in a yeast model of degeneration

4.1.1. Establishment of the yeast model of degeneration and optimization of methods

Growth curves and parameters

In this work the yeast model of degeneration is constituted by 4 different *S. cerevisiae* strains submitted to an oxidative injury that can mimics the metabolic changes that occur during ageing processes and degeneration. For this model were chosen the WT, BY4741, 2 isogenic mutants (*Δyap1* and *Δsod1*) and AD1-8. The *Δyap1* strain does not possess a critical transcription factor involved in the oxidative stress response, the *Δsod1* strain does not possess a functional gene codifying to the cytosolic superoxide dismutase and AD1-8 strain that features eight deletions in MDR genes, encoding ABC transporters involved in xenobiotic efflux capacity. All these strains together allow the achievement of important information about their differential behaviour in presence of H_2O_2

and about the metabolic pathways involved in the deveplment of degeneration process. To implement the yeat model of degeneration several oxidative stress conditions were induced in the 4 strains.

The facility of mutation isolation, the rapid growth on defined media and the fact of being a well defined system makes of yeast a model very attractive to study. The most common method to study microbial growth is through batch culture, allowing the monitorization of yeast growth and the detection of small phenotypic changes or growth defects based on genetic mutation of strains. This pattern of changes in the cell growth parameters, either due to mutant restrictions or presence of a stress, could provide important information concerning the mode of action of tested chemicals [8, 89]. The analysis of cell growth in liquid cultures are particularly suitable [89].

In a typical cell growth analysis in liquid medium, the number of yeast cells in a growing population is monitored by the optical density at 600 nm ($OD_{600\ nm}$) and the relevant growth parameters, such as *Lag* phase time, doubling time (DT) and final biomass are extrapolated from the growth curve. *Lag* time is determined based on the linear regression of log growth phase equation obtained after logarithmic transformation of $OD_{600\ nm}$ values, and corresponds to the time of adaptation required by cells to respond to environmental changes. DT is also obtain based on a logarithmic transformation of $OD_{600\ nm}$ values of log growth phase and is used to characterize the kinetic growth of a population under a set of growth conditions and is an indicator of the microorganism response to a specific environment. The growth rate varies typically with the strain and is strongly influenced by the growth medium. The rate of growth together with stationary-phase OD increments reflects quantitatively the minor changes in growth phenotype, and is shown by cell density or final biomass in the end of growth. [8, 89, 90].

In order to determined growth curves and parameters, as a first approach in this optimization, tree different H_2O_2 concentrations were tested in liquid medium. The main propose of this task was to select the H_2O_2 concentration that represent about 50% of lost of viability in a chronic exposure and to define the growth conditions in liquid medium to use in the further tasks. To perform the growth curves, yeast cells of each strain were grown in SC liquid medium with glucose and supplemented with 0, 0.25, 0.5 or 1 mM of H_2O_2 and finally the growth was monitored hourly during 24 h (Fig. 7 A).

The effects of H_2O_2 on cell growth at low concentration or transient concentrations stimulate growth and enhanced survival [7, 15]. The results of growth curves (Fig. 7 A) show that the WT strain (BY4741) is the most resistant to H_2O_2 as expected. For this strain, when the growth parameters (Fig. 7 B) are considered, the most significant change is denoted on the doubling time when cells are treated with 0.25 mM of H_2O_2 or higher. One possible reason for this could be the fact that 0.25 mM of H_2O_2 induces retardation on growth, increasing the Lag time (Fig. 7 B). However, cells are capable to induce mechanism of defense that are reflected by the increase in final biomass (Fig. 7 B), contrarily to what is observed with 0.5 or 1 mM of H_2O_2 that reflects in a reduction of final biomass.

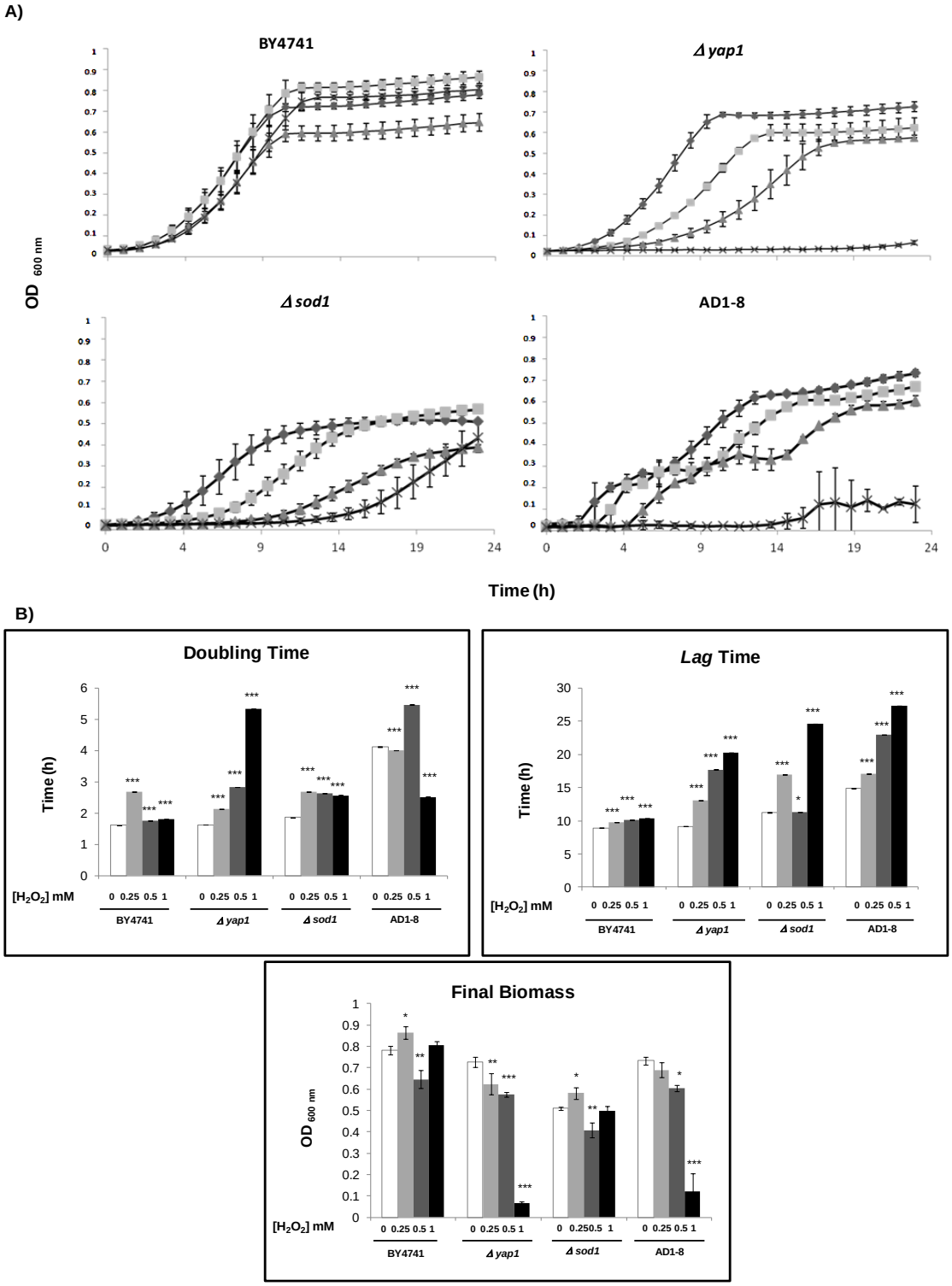

Figure 7. Growth curve and growth parameters of the *S. cerevisiae* strains in presence of H_2O_2. *S. cerevisiae* strains BY4741, *Δyap1*, *Δsod1* and AD1-8 were grown in SC liquid medium with glucose and supplemented with 0.25 (■), 0.5 (▲) and 1 mM (×) of H_2O_2 or without H_2O_2 as control (♦), for 48 h at 30 ºC with constant shaking. A) Growth was kinetically monitored hourly by OD measurements at 600 nm and growth curve represented. B) Growth parameters were determined based on the growth curve being represented the 0.25 (■), 0.5 (■) or 1 mM (■) of H_2O_2 or without H_2O_2 as control (□). Doubling time (h), representing the time necessary to cells duplicate, determined by logarithmic transformation of raw $OD_{600\ nm}$ from growth curves. *Lag* times (h), representing the adaptation time, determined by logarithmic transformation of raw $OD_{600\ nm}$ from growth curves. Final Biomass ($OD_{600\ nm}$), representing the final number of cells is calculated based on the measurements of OD at 24 h of growth. Results represent the mean ± SD of three independent biologic replicates. *, ** and *** represent statistically significance for a control of cells without H_2O_2, for a $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

It is possible to observe that for both BY4741 and *Δsod1* cells growth in presence of 1 mM of H_2O_2 seem to suffer a strong adaptation that allows these cells to restore the normal viability, demonstrated by the number of cells in the end of 24 h, e.g. by the final biomasss (Fig. 7 B). These results may indicate that ROS production have a preconditioning effect on cells at low concentrations. These results indicate that at high concentrations of H_2O_2 (1 mM) the *Δsod1* strain, besides present the longest adaptation phase (*Lag* phase) can activate mechanisms of defese that allow to growth and adapt contrarily to *Δyap1* and AD1-8 that present a inhibition of growth in the same conditions (1mM) (Fig 7 A and B).

In general the most affected mutant by oxidative stress is *Δyap1* (Fig. 7 A and B). This is revealed based on the increasing amount of time needed by cells to start replication (*Lag* time) and reaching the *log* phase (maximum growth rate). Was also observed that *Δyap1* strain present almost total growth inhibition at 1 mM of H_2O_2.

In normal conditions *Δyap1* and *Δsod1* present a lower number of cells in the end of the 24 h of growth than BY4741, represented by final biomass values. For *Δyap1* and AD1-8 the final biomass is the more affected at 1 mM of H_2O_2, while that for BY4741 and *Δsod1* strains the final biomass is more affected at 0.5 mM of H_2O_2. BY4741 and *Δsod1* show a similar metabolic adaption in these conditions, in what regards to final biomass parameter (Fig. 7 B)).

When is induced the oxidative stress, even at 0.25 mM of H_2O_2 all strains are affected, however the H_2O_2 concentration needed to induce more metabolic alterations is different according with the strain mutation. Furthermore, the pattern of changes in the cell growth parameters could reveal important information concerning the mode of action of the tested agents and the involved pathway.

These results were essential to define the incubation times in liquid medium according with the strain and also the H_2O_2 concentrations to test in solid medium by spot assays in different type of exposures. With the growth curves results was possible to define the time of incubation according with the strain, being defined 2 h of DT for BY4741 and *Δyap1* and 3 h of DT for *Δsod1* and AD1-8 (results that are corroborated with previous results from DSB laboratory). The definition of at least 1 DT of incubation in liquid medium for each strain is important for next studies. Based on these results was also defined the H_2O_2 concentrations to test in solid medium of until 2 mM or 0.5 mM of H_2O_2 according with acute or chronic exposures respectively. In the next section was defined the conditions to use in solid medium by spot assays.

Viability evaluation under oxidative stress by spot assays

In general, phenotypes such as defective growth are used to determine the sensitivity of cells to chemical compounds. Plating or spotting assays on solid media can be used to screen non-toxic concentrations of chemicals that affect principally the replicative viability. Although often valuable, the qualitative interpretation of colony formation does not identify whether changes occured during the lag-time after the insult or during growth. Spot assays involve the visual inspection of colony size, number and the thickness of cells, which allows a preliminary visual evaluation of colonies growth and showing

just the cells that are still capable of replication. Therefore, spot assays are used as a methodology to access total cellular viability [89, 90].

In order to evaluate the strains behaviour to the presence of H_2O_2 in solid medium was decided to analyse the impact of incubation time (1 h/1 DT) and most importantly the difference between a complete medium and a medium constituted only by amino acids (YPD or SC) respectively. To achieve these insights, were tested the same two different types of exposure to oxidative stress (chronic and acute) in solid medium. These procedures allow to complete the information obtain based on growth curves.

Yeast cells were grown in SC liquid medium with glucose supplemented with 0, 1 or 2 mM of H_2O_2, during 1 h or 1 DT and in the end of this incubation cells were spotted in both rich solid medium (YPD) or in SC glucose solid medium (Fig. 8A, 8C and 8E), for 48 h at 30 ºC. In parallel yeast cells were growth at least during 1 DT in SC liquid medium with glucose and finally spotted in SC solid medium with glucose supplement with 0, 0.1, 0.2 or 0.4 mM of H_2O_2 for the mutants *Δyap1* and *Δsod1* and 0, 0.1, 0.2, 0.4 or 0.5 mM for BY4741 (WT) and AD1-8 (Fig. 9A). The used concentrations were different for BY4741 and AD1-8 since that these 2 strains present more resistance to oxidative stress induced by H_2O_2.

The results, demonstrate that in tested conditions, cellular viability of all strains was not significantly affected by H_2O_2 until the 2 mM when cells were incubated during for 1 h in YPD medium (Fig. 8B). Although some mechanisms implied in growth retardation suffer modifications and are visible by growth curves, by spot assays the effects are not totally clear. This lack of alterations compared with growth curves can be explained by the fact that submitting the cells to an oxidative stress in a liquid medium during 24 h represent a chronic exposure, while in this experiment the oxidant is administrated by a short period of time, representing an acute exposure, and then cells are incubated during 48 h in solid rich medium (YPD) that allows the recovery of cells. Another curious observation in this result is that both *Δyap1* and *Δsod1* are almost not affected by stress oxidative to H_2O_2 concentrations at 1 h of incubation, since this are the most sensitive strains. These results indicate that the tested conditions were not the most appropriated to denote the differences of growth.

Since this methodology is based on visual inspection of colonies is also important to have significant differences in the number of spot between treatments in order to detect possible viability changes. Besides, these differences are also important in further tasks to analyse viability improvements after incubation in presence of extracts from selected plants. For these reasons cells were incubated during 1 DT. The exposure time to H_2O_2 was rearranged to show significant differences on viability since that cell division is one of the most important steps on cell cycle, indicating if the cells are able or not to create a new generation. Based on the results obtain it is possible to observe that the treatment with 2 mM of H_2O_2 during 1 DT for each strain already represent significantly changes in the viability of cells above 1 mM (Fig 8D and 8F), demonstrating that this exposure maximize the visualization of significant differences in viability, relatively to 1 h of exposure. The *Δyap1* is the most affected strain followed by *Δsod1*, an expected result, in agreement with the results obtained from growth parameters analysis.

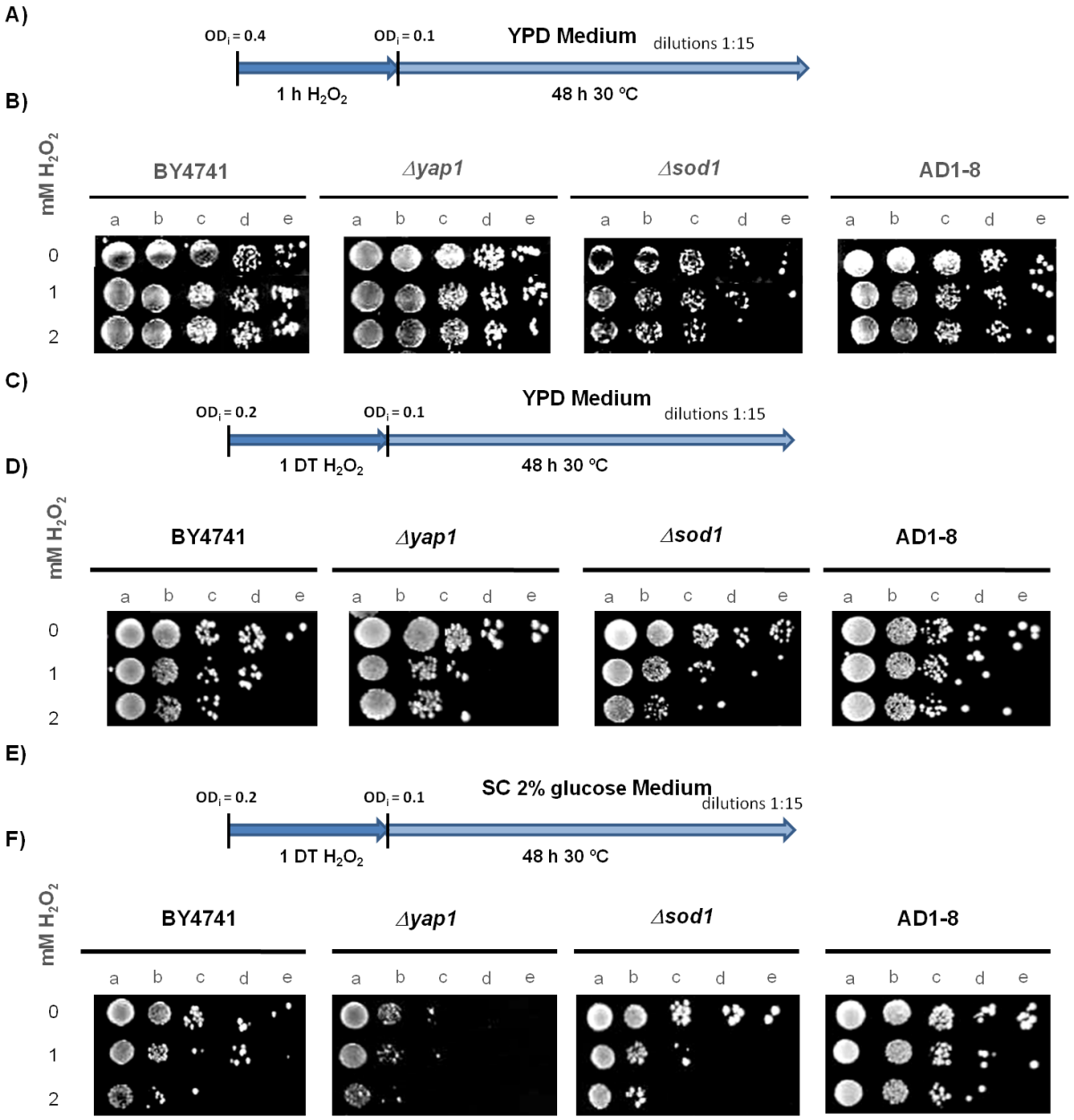

Figure 8. Spot assays of *S. cerevisiae* strains BY41741, *Δyap1*, *Δsod1* and AD1-8 grown in the presence of oxidative stress induced by H_2O_2. A) Schematic representation of cells growth for 1 h and spotted in YPD medium. **B)** Spot assays for *S. cerevisiae* strains in presence of 0, 1 or 2 mM of H_2O_2. Cells were grown in SC liquid medium with glucose supplemented with the concentrations of H_2O_2 during 1 h at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an OD $_{600\ nm}$ of 0.1 and subsequently 5 μL of each dilution were spotted in YPD medium and incubated for 48 h at 30 ºC. **C)** Schematic representation of cells growth for 1 DT and spotted in YPD medium. **D)** Spot assays in the presence of 0, 1 or 2 mM of H_2O_2. Cells were grown in SC liquid medium with glucose supplemented with the concentrations of H_2O_2 during 1 DT according with the strain: 2 h (BY4741 and *Δyap1*) and 3 h (*Δsod1* and AD1-8) at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an OD $_{600\ nm}$ of 0.1 and subsequently 5 μL of each dilution were spotted in YPD medium and incubated for 48 h at 30 ºC. **E)** Schematic representation of cells growth for 1 DT and spotted in SC solid medium with glucose. **F)** Spot assays in the presence of 0, 1 or 2 mM of H_2O_2 in SC solid medium with glucose. Cells were grown in SC liquid medium with glucose supplemented with the concentrations of H_2O_2 during 1 DT according with the strain: 2 h (BY4741 and *Δyap1*) and 3 h (*Δsod1* and AD1-8) at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an OD $_{600\ nm}$ of 0.1 and subsequently 5 μL of each dilution were spotted in SC solid medium with glucose and incubated for 48 h at 30 ºC. a - e represent the serial dilutions. Image acquisition was made in the end of 48 h of growth with Chemidoc XRS and Quantity-one software and the most representative of the biological replicates is show.

Spot assays in the two different medium do not present significant differences of growth, as expected. Nevertheless SC medium was chosen since that this medium does not allow cells recover so quickly after the oxidative insult and allow emphasizing the growth differences. Moreover SC was the medium selected solubilisation, since preliminary assay reveal that PDFs interfere with the components of YPD medium promoting precipitations. At this stage to establish the yeast model of degeneration in liquid medium, was decided that incubate cells at least during 1 DT in presence of 2 mM of H_2O_2, for being the procedure that maximize the differences between treatments.

A cellular state of disease is represented by a situation where cells are constantly in contact with toxic compounds, such as oxidants, like the case of degeneration. For this reason, in parallel a chronic exposure to oxidative stress during 48h in SC solid medium with glucose was tested, since this type of exposure mimics the physiologic conditions of degenerative diseases (Fig. 9A). The incubation time of 4 h before chronic exposure to oxidative stress was chosen since the future incubations in presence of extracts will be also of 4 h, ensuring more than 1 DT.

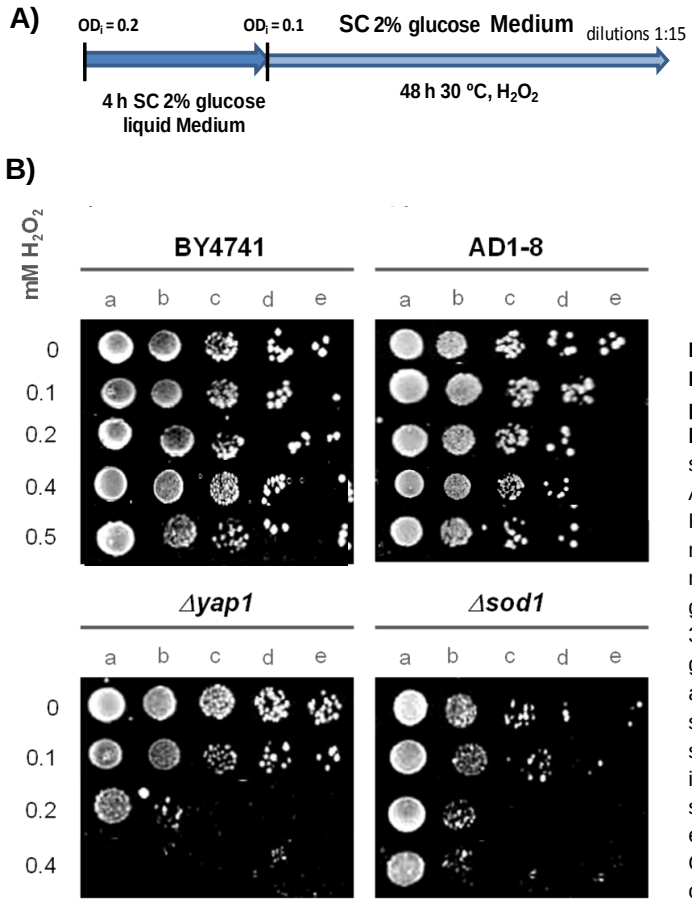

Figure 9. Spot assays of *S. cerevisiae* strains BY41741, AD1-8, *Δyap1* and *Δsod1* grown in the presence of a chronic oxidative stress induced by H_2O_2. A) Schematic representation of cells spotted during 48h in presence of H_2O_2. B) Spot Assays in presence of 0, 0.1, 0.2, 0.4 or 0.5 mM of H_2O_2 for BY4741 and AD1-8 and 0, 0.1, 0.2 or 0.4 mM of H_2O_2 for *Δyap1* and *Δsod1* in SC solid medium with glucose. *S. cerevisiae* strains were grown in SC liquid medium with glucose for 4 h at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an $OD_{600\ nm}$ of 0.1 and subsequently 5 µL of cells of each dilution spotted in SC solid medium with glucose supplemented with the concentrations of H_2O_2 and incubated for 48 h at 30 ºC. a – e represent the serial dilutions. Image acquisition was made in the end of 48 h of growth with Chemidoc XRS and Quantity-one software and the most representative of the biological replicates is show.

The results indicate that BY4741 and AD1-8 even in presence of a high concentration of H_2O_2 are not as sensible to oxidative stress, contrarily to *Δyap1* and *Δsod1* strains, maximizing the identification of metabolic differences between WT and the 2 most sensitive mutants (Fig. 9B). These differences are important to understand the role of PDFs on viability in further studies. Based on these

results was decided that the most appropriated medium to spot cells for further evaluations is SC solid medium with glucose and with chronic exposure to H_2O_2.

According with these observations was also decided use a concentration of 0.15 mM of H_2O_2 for *Δyap1* and *Δsod1* and 0.5 mM of H_2O_2, for BY4741 and AD1-8, in the further studies. The concentration of 0.15 mM of H_2O_2 was chosen for *Δyap1* and *Δsod1* (results not shown) since that in presence of 0.2 mM of H_2O_2 *Δyap1* cells viability is too low and *Δsod1* also shows a significant decrease on viability. For further studies was decided to use the same concentration of H_2O_2 for the two mutants directly involved in oxidative stress defense (*Δyap1* and *Δsod1*), since that differential alterations on viability of cells submitted to the same growth conditions could provide important information about the affected mechanism.

For both BY4741 and AD1-8 was decided to test only until 0.5 mM of H_2O_2 since that AD1-8 not denoted a significant decrease on viability between 0.1 and 0.5 mM of H_2O_2 concentrations and BY4741 is not affected significantly by any tested concentration. As expected the WT strain demonstrate to be the more resistant in both liquid and solid medium to H_2O_2. The differences demonstrated on viability must be due to the fact of BY4741 and AD1-8 has all the antioxidant defenses that are not present in *Δyap1* and *Δsod1* mutants.

All the experiments described in this section were important to optimize this model already implemented in DSB laboratory for further studies of this work implicated in cells protection by plant extracts. Finally the selection of the most appropriate conditions must be taking into account the type of experiment and the type of exposure (chronic or acute).

The *Δyap1* and *Δsod1* mutants present different responses to a wide range of oxidant molecules what becomes difficult to determine which events lead to loss of viability of cells following damage. These responses depend on the dose, at very low doses the cell can adapt to become more resistant to a subsequent lethal exposure while at higher doses the cell activate various antioxidant functions including a program of gene expression mediated mainly by several transcription factors, like for example Yap1p. These distinct responses include expression of proteins, differentially induced by H_2O_2 or menadione, as a source of superoxide [21, 29, 37, 40, 42, 91].

CellTiter-Blue viability assay optimization

Cell viability and growth was also monitored by CellTiter-Blue viability assay that is based on a blue weakly fluorescent indicator dye named resazurin.

Resazurin can enter in viable cells and already inside of cells undergoes enzymatic reduction into a highly fluorescet pink fluorophor resorufin, in mitochondria, due to the activity of enzymes such as: flavin mononucleotide dehydrogenase, flavin adenine dinucleotide dehydrogenase, nicotinamide adenine dehydrogenase, and cytochromes. Resorufin after irreversible reduction reactions is excreted from cells to the medium.

The CellTiter-Blue viability assay is described to be used in human cell lines. For this reason was essential the optimization of the methodology parameters and sensibility according with the model and type of cells, being significantly different when applied to yeast, since that yeast presents a much faster metabolim. Due to the fact of CellTiter-Blue viability assay being based on the quantification of a

fluorescent indicator produced during cells metabolism is important to obtain linear fluorescence detection and avoid over metabolization of resazurin in resorufin. In order to accomplish these requirements, the appropriated number of cells for inocullum and time of incubation were determined. Both a high or low number of cells cannot be measured due to the limits of detection of the fluorimetter. When occur an over metabolization inside the cells of resazurin in resorufin is visible a change on color of the medium from blue to pink.

To determine the appropriate number of cells, *S. cerevisiae* strains $OD_{600\ nm}$ inoculum of 0.025, 0.05, 0.1 or 0.2 were performed and cells incubated in presence of resazurin and fluorescence measured. The selected incubation time of 45 min was the time that allowed the maximization of the differences between treatments.

For BY4741, *Δyap1* and *Δsod1*, the results (data not show) demonstrate that the inoculums with $OD_{600\ nm}$ of 0.025 is the most appropriated, being used in the further studies. The other tested inoculum present an imediatly change of color of medium to pink, denoting over metabolization. The viability of AD1-8 strain cannot be evaluated by this methodology, since the $OD_{600\ nm}$ of 0.025 for this strain presents over metabolization (data not shown) and above 0.02 of $OD_{600\ nm}$ the spectrophotometer is not capable of detect correctly the number of cells.

Since AD1-8 strain features eight deletions in multidrug resistance (MDR) genes, the efflux of metabolites is compromised leading to an over metabolization of several compounds. This represents one possible explanation for the results obtained by CellTiter-Blue viability assay. Besides this observation, the spot assays results for AD1-8 strain do not present significant changes on viability in presence of oxidative stress, showing a similar performance to BY4741.

The *S. cerevisiae* transcription factor Yap1p also plays an important role in MDR by activating target genes involved in cellular detoxification, such as YCF1, that codifies for an ATP binding cassette (ABC) transporter and functions as a glutathione S-conjugate pump. Besides, AD1-8 strain presents the capacity of keep the PDFs inside of cell is an approach to prolong the protective effects or even an eventually scavenging capacity [40]. For these reasons AD1-8 strain was initially chosen to be part of this model. However due to this strain behaviour in CellTiter-Blue viability assay and poor sensibility to H_2O_2 stress, the AD1-8 strain was discarded in following studies.

4.2. Characterization of polyphenol-digested fractions (PDFs)

In this study polyphenol fractions were chemically characterized before and after an *in vitro* digestion process. The total phenolic content and *in vitro* antioxidant capacity of the fractions obtained during the main steps of IVD process were performed to understand the role of digestion in polyphenols protection capacity. This chemical characterization was performed to three different fractions. The original fraction obtained after hydroethanolic extraction before digestion (Orig.); the digested fraction obtained in the end of IVD and that is composed by IN fraction representing the serum available part plus the OUT fraction representing the colon available part of polyphenols content (Diges.) representing IN+OUT; and finally the fraction that were tested in the yeast model of degeneration, the digested fraction after a SPE cleaning process to eliminate all digestion compounds such as bile salts and pancreatin (PDF).

For this study were selected fruits and leaves, from C. *album* (L.) D. Don (Empetraceae) also known as Portuguese crowberry and *A. unedo* (L.) commonly known as strawberry tree (*Ericaceae* family), based on previous unpublished results from DSB laboratory. Undigested extracts from both species reveal neuroprotective portential, in previous studies on a yeast model of Parkinson´s disease (yeast overexpressing α-synuclein), unpublish results from DSB laboratory. *R. idaeus cv polka* fruit (*Rosaceae* family) or red raspberry and *G. biloba* leaf (Maidenhair tree, *Ginkgoaceae*) were also chosen due to their well known health benefits, to comparison purposes, for fruits and leaves, respectively [51, 67, 72]

To understand how digestion affects the properties of polyphenols, the PDFs were chemical characterized and their bioactivity analysed in the yeast model of degeneration established. The chemical characterization includes the total phenolic content and *in vitro* antioxidant capacity performed by Folin-Ciocalteu and ORAC methods respectively, also the LC-MS profile was obtain and is currently under analysis in collaboration with Derek Stewert from James Hutton Institute, Dundee, Scotland.

According to Folin-Ciocalteu it is possible to observe that there is a significant loss on total phenolic content during the 2 main steps of the IVD (Digest. and PDF extracts) compared with the original (Orig.). The results demonstrate that leaves present higher levels of phenolic content than fruits for almost all the fractions analysed (Fig. 10). The total content in phenolic compounds is represented in mg of acid gallic equivalent (mg GAE) normalized for the total volume obtain for each fraction, to demonstrate the quantity of phenolic compounds that are collected, independently of the volumes loss during this process.

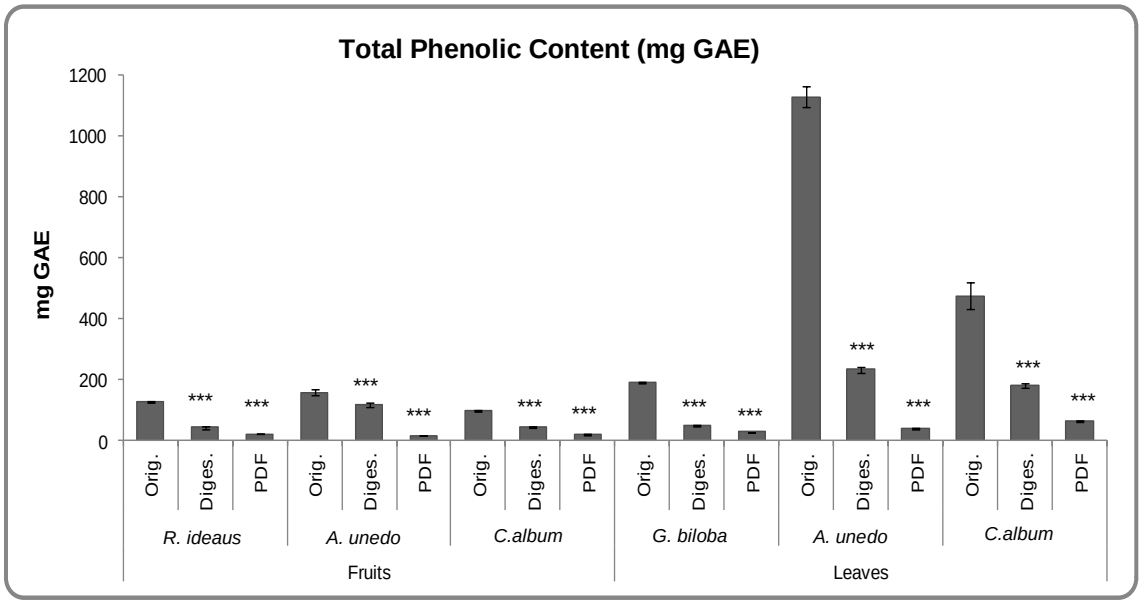

Figure 10. Total phenolic content for polyphenol extracts in each step of the IVD process. Total phenolic content was quantified by Folin-Ciocalteu method: Orig. (original fraction before IVD), Diges. (Digested fraction after IVD representing IN and OUT fractions together) and PDF (polyphenol-digested fraction after SPE column). Values represent the mean ± SD of three independent measurements in mg GAE. *** represent statistical significance for $p < 0.001$ compared with the Orig. fraction of the respective specie.

The highest value was obtained for the orig. fraction of *A. unedo* leaf, with 1127 ± 33 mg GAE. Regarding to the fruits, *A. unedo* presents the highest levels for all fractions, having the Orig. fraction 156 ± 10 mg GAE.

The decrease on total amount of compounds in both Diges. and PDF fractions, comparing to the Orig. was expected since digestion is an aggressive transformation that implies chemical changes. For this reason the percentage of phenols lost were also evaluated and compared with Orig. fractions by specie (Fig. 11 and 14), for fruits and leaves respectively. Due to the fact of digested fraction had pass through a cleaning step that is always responsible for losses, lower values for PDFs were also expected.

Folin-Ciocalteu method was an initial screening that allows the comparison between the fractions chosen for this study with *R. ideaus* and *G. biloba*, for fruits and leaves, respectively, since this species are described as having health benefits. These results show that the chosen fruits for this study *A. unedo* and *C. album* present approximately the same among of phenolic compounds than *R. ideaus* (Fig. 10).

Phytochemical studies showed that both fruit and leaf extracts from *A. unedo* contain several classes of naturally phytochemicals such as phenolic compounds, like tannins, flavonoids, phenolic glycosides, anthocyanins and other flavonoids such as gallic acid derivatives. *A. unedo* berries are already known as a very good dietary source of phytochemicals based on their chemical composition and high antioxidant capacity [51, 59, 62]. For *C. album* a few studies were performed and is not totally known its composition and biological effects on degeneration.

4.2.1. Fruits

The percentage of original was calculated based on values of mg GAE (Fig. 10), for each species separetly. The association of total phenol content in Orig. Fraction with the other two (Diges. and PDF) allows the understanding if the losses of polyphenols is related with the specie and the different sensibilities of the respective phenolic content to the IVD process.

Relative to the percentage of total phenolic content in original fraction for fruits no more than 19% is recovered, after digestion, being *C. album* the fruit with the higher percentage of recover for PDF (Fig.11). However *A. unedo* fruit PDF do not seem suffer many alterations during IVD since that Diges. fraction have a high percentage of original, however after SPE procedure the percentage of phenolic compound recovered in PDF fractions is significantly lower that in Orig. Fraction. Contrarily, *R. ideaus* do not show the higher percentage of loss on PDF but is the fraction that represents more losses due to digestion process since that the Diges. fraction have the lower percentage of original compared with same fractions of the other tested fruits (Fig. 11). These results can be explained by the fact of *R. ideaus* be very rich in anthocyanins, that are very unstable, contrarily to *C. album* that have no anthocyanins. All the fruits seem to present a similar decrease on phenolic compounds in end of the IVD process (PDF fractions) [71].

Other methodology that is used to study the antioxidant properties of extracts is ORAC. This methodology is based on the antioxidant capacity of metabolites in the presence of a strong oxidant,

the AAPH that is added to the sample to generate oxidant radicals. When these radicals are eliminated by scavenging a decrease on fluorescence is observed. The results for *in vitro* antioxidant capacity of fruits extracts are represented in concentration μM of Trolox equivalents (μM TE), a well known antioxidant (Fig. 12A). In parallel with the antioxidant capacity was also evaluated the antioxidant potential (μmol TE.mg GAE^{-1}) determined by a ratio between *in vitro* antioxidant capacity (μM TE) and total phenol content (mg $GAE.mL^{-1}$) that represents the antioxidant capacity for phenolic unit. Antioxidant potential demonstrates the scavenging capacity of the phenolic content presents in the extract (Fig.12B).

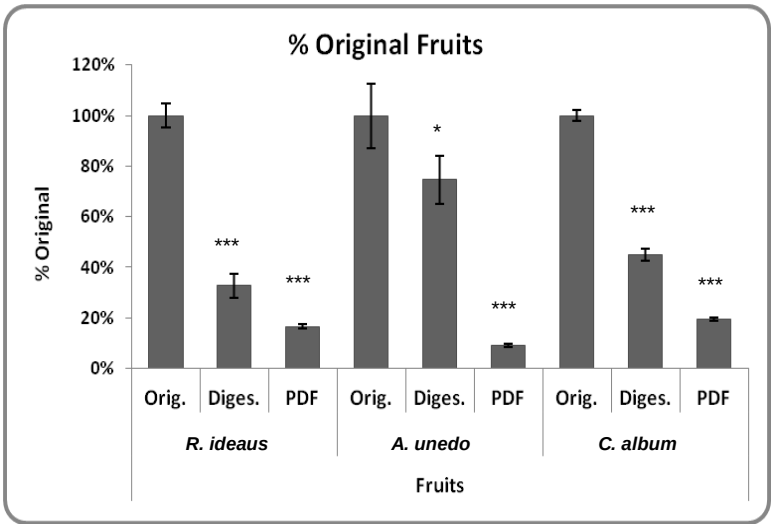

Figure 11. Percentage of the original of total phenolic content for polyphenol fruit extracts in each step of the IVD process. Total phenolic content of fruits was quantified by Folin-Ciocalteu method. The percentage of phenolic compounds present on each fraction was calculated based on original: Orig. (original fraction before IVD), Diges. (Digested fraction after IVD, representing IN and OUT fraction together) and PDF (polyphenol digested fraction after SPE column). Values represent the mean ± SD of three independent measurements. * and *** represent statistic significance for p <0.05 and p <0.001 respectively compared with the Orig. fraction of the respective species.

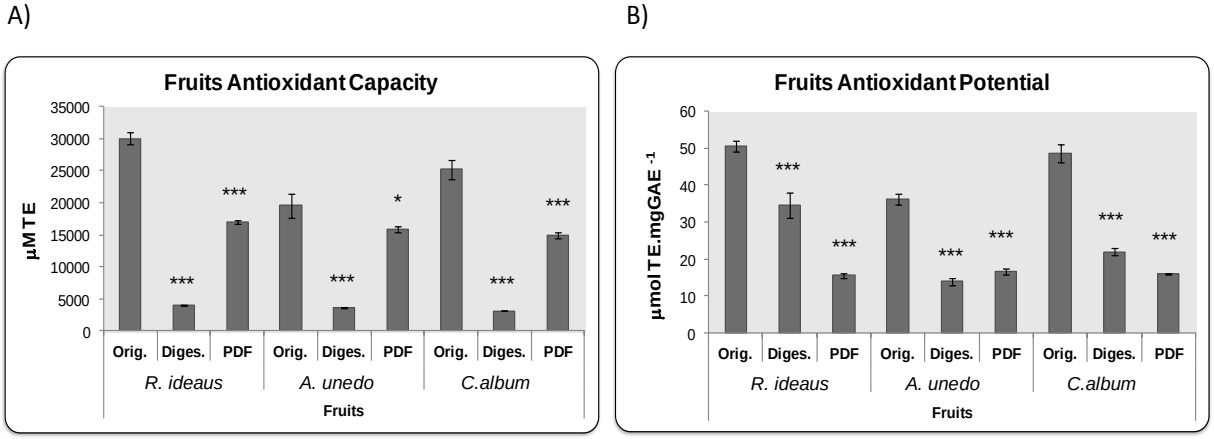

Figure 12. Antioxidant properties of polyphenol fruit extracts in each step of the IVD process. A) *In vitro* antioxidant capacity of fruits was quantified by ORAC method in μM TE. B) Antioxidant potential represented by the ratio between *in vitro* antioxidant capacity represented by μM TE and total phenolic content represented by mg $GAE.mL^{-1}$. *In vitro* antioxidant capacity was quantified by ORAC method and total phenolic content by Folin-Ciocalteu method. Values represent the mean ± SD of three independent measurements in μmol TE. mg GAE^{-1}. Orig. (original fraction before IVD), Diges. (Digested fraction after IVD, representing IN and OUT fraction together) and PDFs (polyphenol-digested fractions after SPE column). * and *** represent statistic significance for P <0.05 and p <0.001 respectively compared with the Orig. fraction of the respective species.

The results obtained for antioxidant capacity of the fruits, only indicated that occur a decrease of antioxidant capacity after the first step of digestion process demonstrated by the Digest. fraction

(Fig. 12), but it seems to have a recovering after SPE, showed by PDF values for all fruits. The decrease on antioxidant capacity demonstrated for Diges. fraction can occur due to loss/transformation of polyphenols during IVD, corroborating the total phenolic content results.

Antioxidant potential results demonstrat that the loss of phenolic compounds in Diges. fraction is accompanied by a loss of antioxidant capacity, since that antioxidant potential is a value normalized for the content in phenolic compouds (Fig. 12B). The results obtained for antioxidant potential in each step of IVD are different according with the species probably due to different compositions in phenolic compounds. *R. ideaus* is the fruit that presents the higher antioxidant potential for Orig. and Diges. fractions, with 50.6 ± 1.4 μmol TE.mg GAE^{-1} and 34.5 ± 3.4 μmol TE.mg GAE^{-1}, respectively. However, according with the antioxidant potential, *R. ideaus* is the fraction that suffers more losses during SPE process, presenting the lower levels for PDF comparing with the other fruits (Fig. 12B).

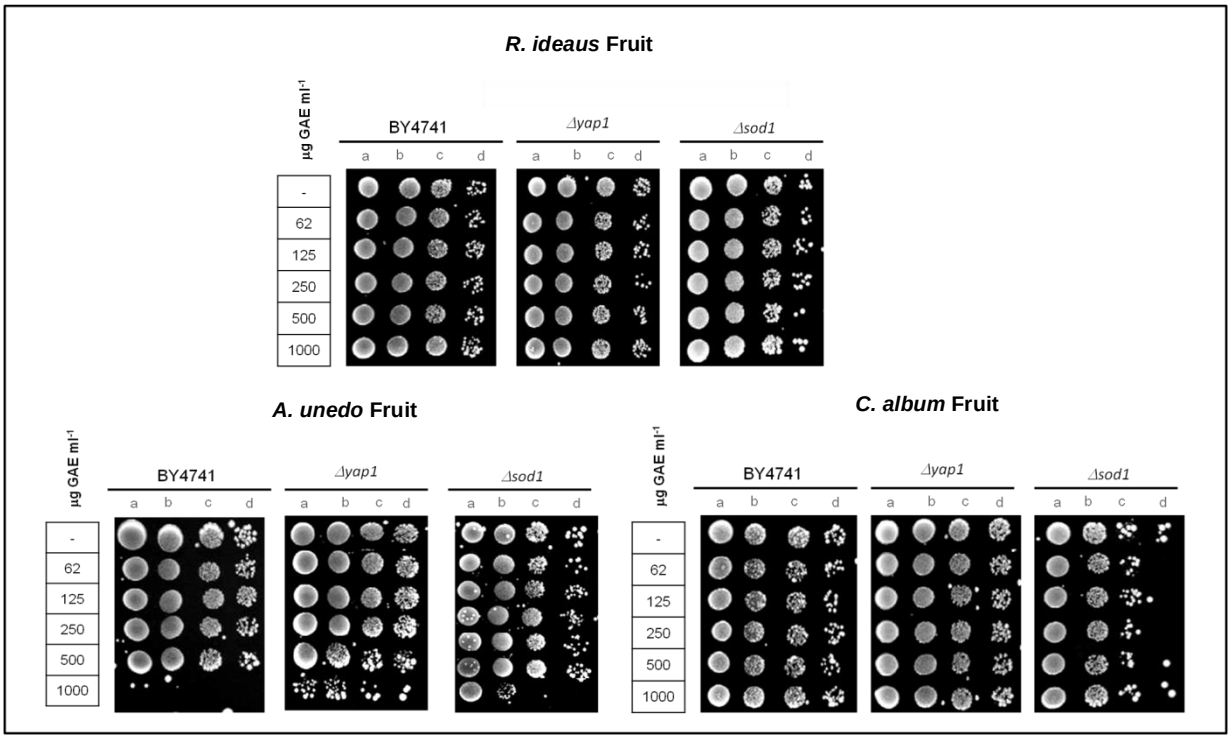

Figure 13. PDFs toxicity evaluation by spot assays for *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1* exposure to PDFs from fruits. BY4741, *Δyap1* and *Δsod1* were grown in SC liquid medium with glucose supplemented with concentrations of 0, 62, 125, 250, 500 or 1000 μg GAE $.mL^{-1}$ of PDFs from fruits (*R. ideaus* fruit, *A. unedo* fruit and *C. album* fruit), for 4 h at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an $OD_{600\ nm}$ of 0.1 and subsequently 5 μL of cells of each dilution spotted in SC solid medium with glucose and incubated for 48 h at 30 ºC. a - e represent the serial dilutions. Image acquisition was made in the end of 48 h of growth with Chemidoc XRS and Quantity-one software.

The direct correlation between antioxidant potential or phenolic content and the biological role of extract compounds inside of the cells cannot be done. Because of this it is important to evaluate the direct influence of extracts in the cellular metabolism through different methodologies. The non-toxic range of PDFs concentrations in the yeast strains BY4741, *Δyap1* and *Δsod1* were determined, as well as the concentrations that was used in the protective assays in the degeneration model.

In order to achieve the more appropriated concentrations of PDFs from all characterized species, to use in further tasks, the PDFs toxicity was first evaluated by spot assays as described in

section 3.4.2. The *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1* were grown in presence of 0, 62, 125, 250, 500 and 1000 μg GAE.mL^{-1} of the PDFs to determine the maximum concentration that not induce death (Fig. 13 and 16) for fruits and leaves respectively. The tested concentrations were chosen based on unpublished results from DSB laboratory.

Previous toxicity assays were performed for undigested fractions by spot assays and no significant inibithion of growth was observed until 1000 μg GAE.mL^{-1} (data not shown). In this work was tested the toxicity of Diges. and PDF fractions and a clearly increase of toxic effects was detected, being more accentuated for the Diges. fractions without the cleaning step by SPE than for PDF fractions (data not shown).

It was expected that digestion affects the metabolites toxicity possible due to the type of phenolic compounds or on the alterations suffer by this compounds during digestion. The results show that *C. album* fruit and *R. ideaus* PDFs do not demonstrate toxicity until concentrations of 1000 μg GAE.mL^{-1}. Contrarily, the PDF from *A. unedo* fruit present some toxicity after IVD process at higher concentrations than 500 μg GAE.mL^{-1} (Fig. 13).

The possible metabolic influence of these fractions in yeast cells, in general did not lead to inhibition of growth in solid medium, for fruits, above of 500 μg GAE.mL^{-1}. This is an important result for selection of the higher PDFs concentrations that not represent growth inibithion, to performe the protection evaluation.

The fruits PDFs present all a similar antioxidant capacity and potential. However *A. unedo* fruit PDF is the fraction that presents the higher antioxidant potential and is the only fraction from fruits showing toxicity above 500 μg GAE.mL^{-1}. This result indicates that the toxicity observed for *A. unedo* PDF could be related with its chemical composition. Besides, this PDF affects selectivity the three tested strains. Curiously the *Δyap1* is the less affected by *A. unedo* fruit PDF suggesting that this PDF present the capacity of improve *Δyap1* phenotype even in normal conditions (Fig. 13). One interesting result observed for *Δsod1* when incubated in presence of *C. album* fruit PDF, is the fact that this PDF seems to have the capacity of induce some toxicity that not increase with the increasing concentrations of PDF (Fig. 13).

4.2.2. Leaves

In the case of leaves, the percentages of recovery of total phenols demonstrate that *G. biloba* and *C. album* present the higher percentage of recovery for Diges. fractions, being 39 ± 1% for *G. biloba* and 38 ± 5% for *C. album* (Fig. 14). The percentage of recovery for PDF fractions from leaves, of *C. album* is slightly lower than *G. biloba*. Comparatively, very few phenolic compounds were recovered in the PDF from *A. unedo* leaf after digestion process representing the lower percentages of recovery for PDF frations. Based on these results both *G. biloba* and *C. album* leaf seem more promising that *A. unedo* leaf (Fig. 14).

Antioxidant capacity for Orig. fractions is much higher in general for leaves then for fruits, being the maximum value obtained for Orig. fraction of *A. unedo* leaf (Fig. 15A) compared with

minimum value obtained for Orig. fraction of *A. unedo* fruit (Fig. 12A). The antioxidant capacity results also suggest that the major part of the phenolic compounds naturally existent in leaves suffer more alterations (Fig. 15A). These phenomena can also be observed based on the percentage of original that seems slightly lower for Diges. and PDF fractions from leaves than from fruits. Besides the fact of leaves show higher values for antioxidant capacity than fruits, in general the antioxidant potential is similar or even lower, probably to the fact that leaves also present an higher content on phenolic compounds. At the same time is also notable that leaves suffer more alterations during IVD than fruits, being the differences between antioxidant capacities for Diges. or PDF compared with Orig. fraction more accentuated in leaves (Fig. 15A and 15B). *In vitro* antioxidant capacity showed that the original fraction from *A. unedo* leaf and *C. album* leaf are higher than *G. biloba*, however the antioxidant potential demonstrate that Orig. fraction of *A. unedo* leaf have a lower antioxidant potential, presenting only 18.20 ± 0.82 μmol TE.mg GAE^{-1}.

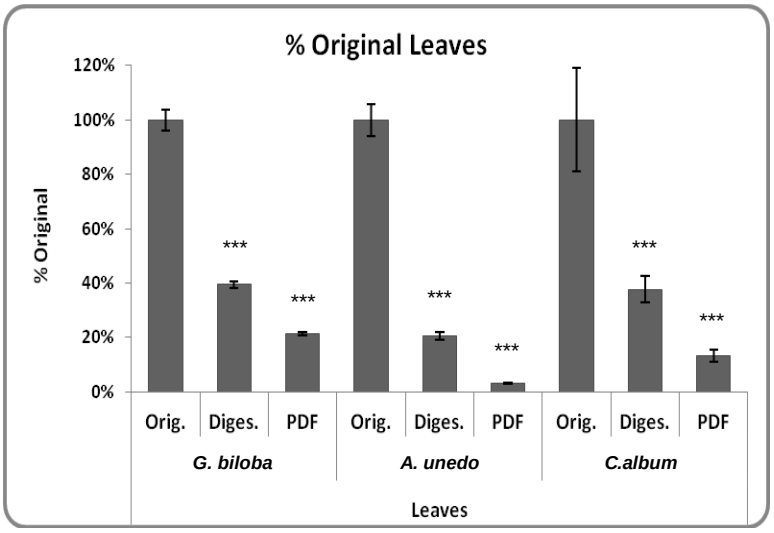

Figure 14. Percentage of the original of total phenolic content for polyphenol leaf extracts in each step of the IVD process. Total phenolic content of leaves was quantified by Folin-Ciocalteu method. The percentage of phenolic compounds present on each fraction was calculated based on original: Orig. (original fraction before IVD), Diges. (Digested fraction after IVD, representing IN and OUT fraction together) and PDF (polyphenol digested fraction after SPE column used in the model). Values represent the mean ± SD of three independent measurements. *** represent statistic significance for p <0.001 compared with the Orig. fraction of the respective species.

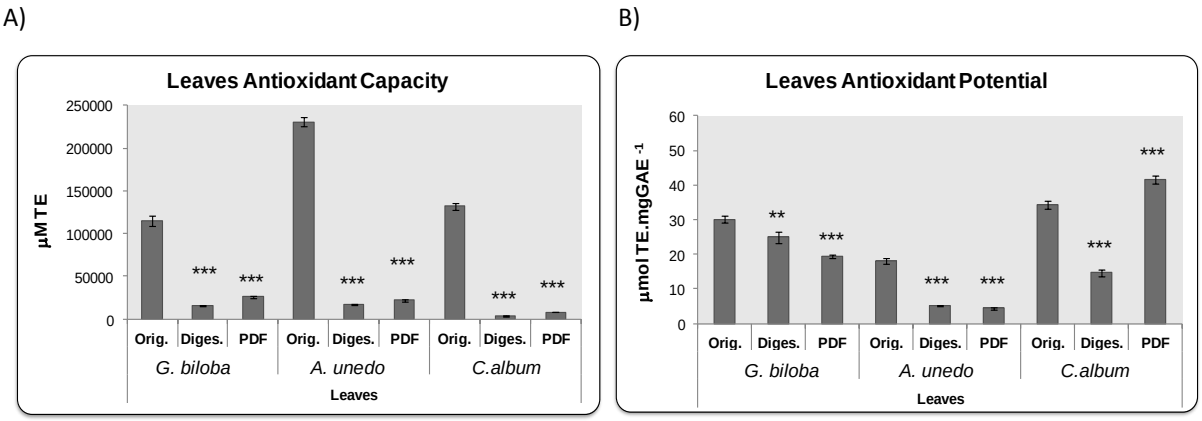

Figure 15. Antioxidant properties of polyphenol leaf extracts in each step of the IVD process. A) *In vitro* antioxidant capacity of leaves was quantified by ORAC method in μM TE. B) Antioxidant potential represented by the ratio between *in vitro* antioxidant capacity represented by μM TE and total phenolic content represented by mg $GAE.mL^{-1}$. *In vitro* antioxidant capacity was quantified by ORAC method and total phenolic content by Folin-Ciocalteu method. Values represent the mean ± SD of three independent measurements in μmol TE. mg GAE^{-1}. Orig. (original fraction before IVD), Diges. (Digested fraction after IVD, representing IN and OUT fraction together) and PDFs (polyphenol-digested fractions after SPE column).). ** and *** represent statistic significance for p <0.01 and p <0.001 respectively compared with the Orig. fraction of the respective species.

The antioxidant potential for *A. unedo* leaf present the lower levels for all the three characterized fractions, including for Orig. fraction, despite of be the leaf that has the higher *in vitro* antioxidant capacity for Orig. fraction.

Fruits and leaves present a similar pattern for antioxidant capacity of PDFs, since these fractions also present higher values of antioxidant capacity than Diges. frations. Antioxidant potential results demonstrate that *G. biloba* is the plant that seems to have this parameter less affected by digestion process, since that the Diges. fraction for this leaf is the fraction that show less differences compared with Orig. fraction. However after SPE occur a significantly lost of phenolic compounds (Fig. 15B), result corroborated with percentage of original of total phenol content (Fig. 14). The PDF fraction of *C. album* leaf, contrarily to fruit is the only fraction that has higher values than Orig. fraction, for antioxidant potential, which can indicates that SPE clean substances that could interfere with Folin-Ciocalteu method (Fig. 15B).

To access the PDFs toxicity from leaves, PDFs were tested by spot assays in the same concentrations of fruits, between 0 – 1000 μg GAE.mL^{-1}. The results represented show that independently of possible changes in cellular metabolism of yeast only *A. unedo* leaf PDF does not present any toxicity until concentrations of 1000 μg GAE.mL^{-1} (Fig. 13), contrarily to observe for *A. unedo* fruit PDF that induces death at concentrations higher than 500 μg GAE.mL^{-1} (Fig. 16).

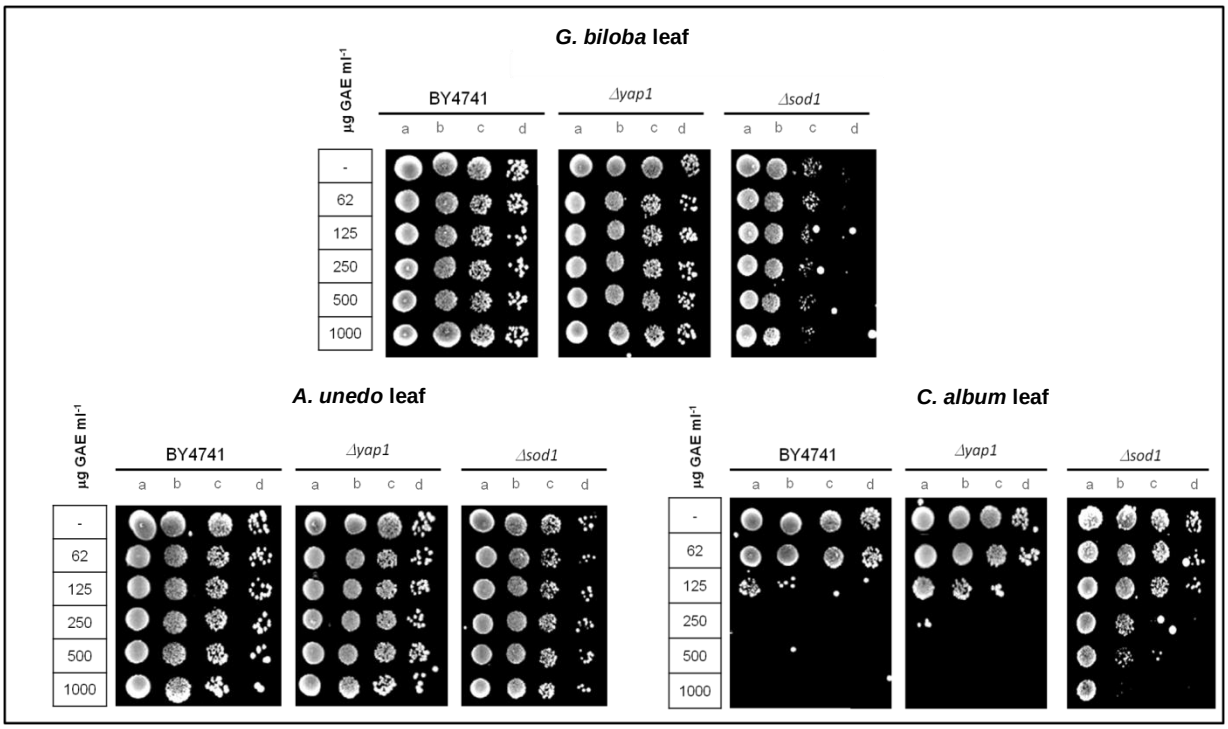

Figure 16. PDFs toxicity evaluation by spot assays for *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1* exposure to PDFs from leaves. BY4741, *Δyap1* and *Δsod1* were grown in SC liquid medium with glucose supplemented with concentrations of 0, 62, 125, 250, 500 or 1000 μg GAE.mL^{-1} of PDFs from leaves (*G.biloba* leaf, *A. unedo leaf* and *C. album* leaf), for 4 h at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an OD $_{600\ nm}$ of 0.1 and subsequently 5 μL of cells of each dilution spotted in SC solid medium with glucose and incubated for 48 h at 30 ºC. a - e represent the serial dilutions. Image acquisition was made in the end of 48 h of growth with Chemidoc XRS and Quantity-one software.

For this type of matrix *C. album* leaf PDF is toxic for yeast cells, showing lost of viability for *Δsod1* at concentrations higher than 250 μg GAE.mL^{-1} and for *Δyap1* and BY4741 higher than 125 μg GAE.mL^{-1}. The differences on toxicity of analised fractions can indicate that phytochemical compounds with electrophilic properties can influence the viability of mutants differentially, probably due to the alteration of redox homeostasis of cells (Fig. 16). Interestingly in the case of leaves, *C. album* PDF is the fraction that show higher values for antioxidant potential, comparing with all other fractions from leaves, chemically characterized.

For *G. biloba* leaf, these results indicate a slightly decrease on viability with the increase of PDF concentration, however this changes on viability just become more accentuated for *Δsod1* in concentrations higher than 500 μg GAE.mL^{-1} (Fig. 16). *G. biloba* PDF also present a high antioxidant capacity comparing with *A. unedo* leaf PDF, however the value of antioxidant potential is not higher than antioxidant potential of fruits. In general fruits are less toxic than leaves, a result consistent with the fact of all the results presented in this section.

Based on these results together with the results obtain for the fruits, it was chosen 0, 62, 125 and 250 μg GAE.mL^{-1} of PDFs fractions as the concentrations to use in further protective evaluation by spot assays for all the species except for *C.album* leaf PDF. This fraction was tested until the concentration of 125 μg GAE.mL^{-1}, since that 250 μg GAE.mL^{-1} of this PDF is already too toxic for yeast cells.

Like in fruits the toxicity analisys was compared for Diges. without SPE and PDF fractions. The results obtained for this toxicity screen reveal that the Diges. fractions without SPE are very toxic for yeast cells for all tested fractions demonstrated to be very toxic. The other tested Diges. fractions present a similar behaviour (Data not shown). The cleaning step by SPE allowed the cleaning of the digestion compounds keeping only polyphenols that are available in serum and in colon. This demonstrate to be very important to ensure polyphenol bioactivities analisys without digestion resiudes that could interfere. After SPE column the PDFs obtained were tested in the yeast model of degeneration for protection (Fig. 15 and 18), demonstrating that in fact after this procedure the major part of toxic compounds are eliminated and almost all fractions did not present toxicity until 1000 μg GAE.mL^{-1}.

4.3. Selection of polyphenol-digested fractions with protective potential

4.3.1. Viability analysis by Spot Assays

The protective potential of PDFs was carried out by two different methods. Viability was determined by spot assays and the general cellular state evaluated by resazurin-based assay. To achieve further insight into the bioactivities of PDFs from fruits and leaves in the yeast model of degeneration, cells were incubated during 4 h with 0, 62, 125 or 250 μg GAE.mL^{-1} of all PDFs except *C. album* leaf PDF that was tested only until 125 μg GAE.mL^{-1}, as defined in section 4.1. (Fig. 16).

Finally cells were spotted in SC solid medium with 0.5 mM of H_2O_2 for BY4741 and 0.15 mM of H_2O_2 for *Δyap1* and *Δsod1*, during 48 h.

Spot assays were performed as a first approach to select the PDFs with protective potential and to understand the form by each PDF affect the viability according with the strain. Each strain of this model represents a defect on metabolism and possible changes on growth conditions by the presence of PDFs can reflect the different roles of PDFs in oxidative metabolic pathways.

In presence of *C. album* fruit PDF, BY4741 do not show any difference on viability but contrarily to what was expected was observed that this PDF present a curiously result, since that seems to affect *Δyap1* and *Δsod1* differentially. This fraction is not able to improve *Δyap1* cells viability and seems to induce some lost of viability in the yeast model of degeneration that was not demonstrate to be toxic for yeast cells. However for *Δsod1* is not totally clear if is capable of provide any viability improvement concretely (Fig. 17). For these reason *C. album* fruit PDF was evaluated in further methodologies.

A. unedo fruit and leaf PDFs show a clear increase of viable cells after being subjected to an oxidative stress for 48 h. *A. unedo* fruit is the only PDF providing viability improvements for the two mutant strains *Δyap1* and *Δsod1.* The PDF from *A. unedo* leaf can ameliorate the H_2O_2 induced damaged only for *Δyap1* mutant (Fig. 17).

Cells growth in presence of *G. biloba* PDF seems differently affected according with the strain, being *Δsod1* the only strain that present a slightly improve on viability (Fig. 17). Contrarily, *R. ideaus* PDF does not ameliorate the damage induced by the H_2O_2 for any strain, an interesting observation since that several studies based on phytochemical composition of this berries point their benefitial effects in some ageing disorders including neurodegeneration [69]. Gordon, M. and co-workers evaluated the effects of IVD in anthocyanins compounds, showing that this polyphenols suffer many alterations during this process [76]. Since red raspberry is rich in anthocyanins, the observed results for protecting assay could suggest that the IVD possible alter the protecting capacity of this berries.

With these results is possible to conclude that not all PDFs have the capacity of improve the viability by protecting cells during a chronic exposure to oxidative stress, such as *R. ideaus* and *C. album* leaf PDFs. On the other hand *A. unedo* leaf and fruit PDFs, were the fractions with more potential to be explored. *Δyap1* was the strain that was more responsive to improvements on viability by PDFs (Fig. 17).

The results obtained for *A. unedo* leaf PDF are surprising since the antioxidant capacity per unit of phenol content, shown in section 4.2.1. is lower than the other PDFs. One possible explanation for these results is that this PDF can improve viability not through antioxidant mechanisms but functioning thought other molecular mechanisms. To confirme if ROS scanvenging is involved in the protective mechanisms induced by these fractions, ROS production was evaluated after cells growth in presence of PDFs with protective potential.

The PDFs from *A. unedo* fruit and leaf exhibit a rising protecting capacity with the increase of PDFs concentration until the tested 250 μg GAE.mL^{-1}, demonstrating a dose response. This increase on viability promoted by *C. album* fruit PDF is not clear and for this reason becomes interestingly to evaluate its biological role in cells by other methodologies.

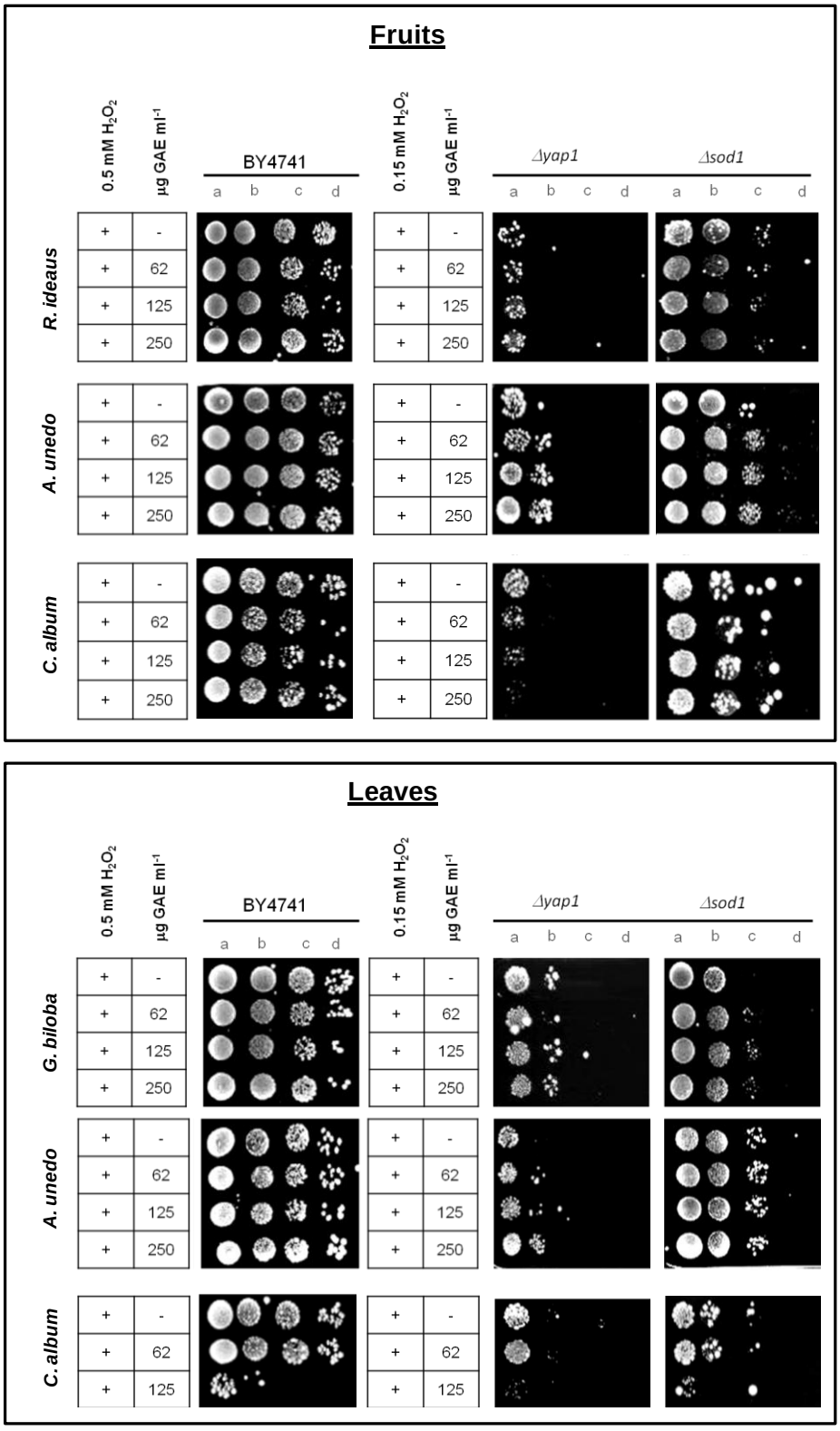

Figure 17. PDFs protective potential evaluation by spot assays for *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1* exposure to PDFs and subjected to an oxidative stress. BY4741, *Δyap1* and *Δsod1* were grown in SC liquid medium with glucose supplemented with concentrations of 0, 62, 125 or 250 µg GAE.mL^{-1} of all PDFs, except for C. album leaf tested only until 125 µg GAE.mL^{-1}, for 4 h at 30 ºC, with constant shaking. Cells in *lag* phase of growth were diluted starting with an $OD_{600\ nm}$ of 0.1 and subsequently 5 µL of cells of each dilution spotted in SC solid medium with glucose supplemented with 0 mM of H_2O_2 or 0.5 mM of H_2O_2 for BY4741 and 0.15 mM of H_2O_2 for *Δyap1* and *Δsod1*; cells were incubated for 48 h at 30 ºC. a – e represent the serial dilutions. Image acquisition was made in the end of 48 h of growth with Chemidoc XRS and Quantity-one software.

4.3.2. CellTiter-Blue viability assay analysis

Many recent studies have proven that mitochondria are one of the cellular components with the most impact in ageing disorders influenced by oxidative stress to keep cell homeostasis. To understand how the most promising PDFs, demonstrated by spot assays results (Fig. 17) influence the mitochondrial state we also assay for viability evaluation by CellTiter-Blue viability assay that is based on mitochondrial functional metabolism. CellTiter-Blue viability assay is method that also reflects the mitochondrial state of cells, since resazurin is reduced by mitochondrial enzymes in viable cells. The fact of resazurin became reduced due to the activity of mitochondrial enzymes associated with some of the most signalling pathways on cell, allow the understanding the metabolic sate of cells and consequently the viability.

Based on protective effect results by spot assays, only *A. unedo* fruit and leaf and *C. album* fruit PDFs were evaluated in the next tasks of this work (Fig. 17). The previous results also allow the choosing of 0 and 250 μg GAE.mL^{-1} concentrations of the PDFs, since that 250 μg GAE.mL^{-1} is the higher concentration of phenolic compounds showing protective capacity. *S. cerevisiae* strains were grown in SC liquid medium with glucose supplemented with or without PDFs and subsequently grown in the same medium supplemented with 2 mM of H_2O_2 to induce the oxidative stress condition. The results are shown in percentage of viability, normalized for the number of cells, after cells being incubated during 45 min in presence of the resazurin compound (Fig.18).

This approach is important to understand the cellular state of each strains according with the treatment, which can not be inferred by spot assays. The percentage of viability implies a relation between enzymatic activity of cells in normal conditions, to infer the alterations of this activity in cells submitted to the tested condition. One the other hand provides information about how the different mutants lead with oxidative stress and which pathways can be possible more affected by the presence of PDFs.

When the percentage of viability is calculated for BY4741 it is not observed any differences in cells viability after incubation with 250 μg GAE.mL^{-1} of PDFs. At the same time a decrease on viability is observed for cells incubated only in the presence of H_2O_2 for this strain, suggesting that could occur mechanism related with mitochondria, such as mitofagy and that is not verify by spot assays.

In these results is also possible to observe a slight improvement for the BY4741 viability in the presence of PDFs from *A. unedo* fruit and leaf, when cells are submitted to an oxidative stress. For *A. unedo* leaf the improvements observed in the cellular viability demonstrate that this is the most favourable fraction, presenting higher viability values (Fig. 18)**.** Interestingly the improvements demonstrated by this methodology for BY4741 in the presence of *A. unedo* fruit and leaf are not clear by spot assays (Fig. 17 and 18), suggesting that PDFs beneficial effect may be occur through direct/indirect amelioration of enzymatic mitochondrial activity. The fact of the percentage of viability for *Δyap1* do not demonstrate significant difference for BY4741 in presence of H2O2 could indicate a mechanism of degeneration in commun for both strains that not implies Yap1p activation, such as autophagic process. However more studies must be performed.

The results indicate that in normal conditions the *Δyap1* strain has the lower viability, as expected, due to lower levels of percentage of viability when submitted to oxidative stress. These

results also allow to inferred relevant information for *Δyap1,* by CellTiter-Blue viability assay (Fig. 18) that was not detected by spot assays (Fig. 17).

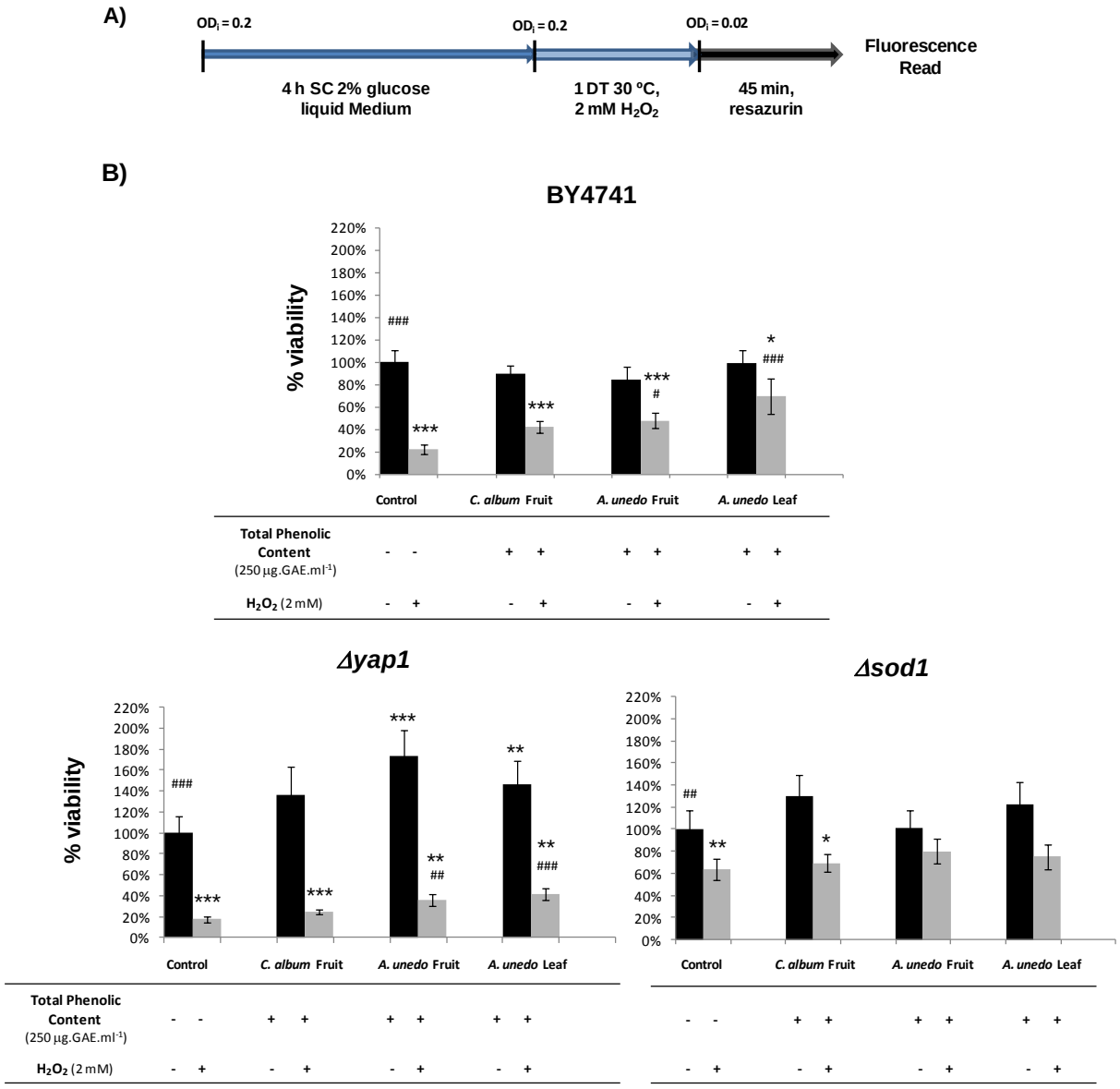

Figure 18. Percentage of viability of *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1* growth in the presence of PDFs and subjected to an oxidative stress. A) Schematic representation of tested conditions in this experiment. B) Results expressed as percentage of viability with comparison to control (cells without PDFs and H_2O_2). Strains were grown in SC liquid medium with glucose supplemented with 0 or 250 mg GAE. mL^{-1} of *C. album* fruit, *A. unedo* fruit and *A. unedo* leaf PDFs for 4h at 30 ºC, with constant shaking. Cells were harvested and grown in the presence of 0 mM (Black bars) and 2 mM (Grey bars) of H_2O_2 for 1 DT according with the strain (2 h for BY4741 and 3 h for *Δyap1* and *Δsod1*) at 30 ºC. Viability was accessed using CellTiter-Blue viability assay. Values represent mean ± SD of three independent biological replicates. *, ** and *** represent statistically significance for a control of cells without PDFs and H_2O_2, for a $p<0.05$, $p<0.01$ and $p<0.001$ respectively. #, ## and ### represent statistically significance for a control of cells without PDFs and in presence of 2 mM of H_2O_2 for a $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

When analyzed the percentage of viability of *Δyap1* cells not submitted to oxidative stress, it is observed a significant improvement in cells growing with *A. unedo* fruits and leaf PDFs (Fig. 18). This mutant does not possess an important transcriptional factor that regulates the majority of the

antioxidant enzymes; consequently the enzymatic activity is normally low, leading to a lower viability. When *Δyap1* cells are incubated with the PDFs from *A. unedo,* the percentage o viability values obtained rise significantly, above the values obtain for cells in normal conditions. These indicate that the compounds constituents of *A. unedo* fruit and leaf PDFs may be have the capacity of ameliorate the *Δyap1* phenotype. After this study could be interesting analyse if in presence of these PDFs the cell state can reaches the WT phenotype (Fig. 18).

However in the presence of an oxidative stress condition PDFs have not the same capacity to improve the viability of this mutant to a control condition. Nevertheless is observed a viability increase when cells are incubated with *A.unedo* fruit or leaf (Fig. 18).

The *Δsod1* strain show fewer differences with the several treatments applied in comparison to the control. One important result was obtained for *C. album* fruit PDF, representing an improvement on viability in cells when incubated with this PDF. This observation is not clear in spot assays results (Fig. 17), demonstrating that the small differences observed by CellTiter-Blue viability assays can represent a general cellular metabolism improvement (Fig. 18). For the other PDFs there are not visible significant differences on viability for *Δsod1*, but interestingly when *Δsod1* cells are incubated in the presence of *A. unedo* fruit PDF and submitted to oxidative stress, the percentage of viability values show that cells have not a much different viability from cells not submitted to H_2O_2. These results indicate that *A. unedo* fruit PDF could have a preconditioning role in *Δsod1* cells, which allow to the cells be more responsiveness to oxidative stress (Fig. 18).

In general, cells in presence oxidative stress after submitted to *A. unedo* fruit PDF have an improvement of viability, demonstrating a protective effect (Fig. 18). Adaptive stress response towards H_2O_2 is performed by a complex network of several different regulons. For example, several genes appear to be regulated directly by the transcription factor Yap1p, whereas others are regulated by other mechanisms [11, 28, 42].

The fact of *Δsod1* present a similar behaviour to BY4741 in presence of PDFs, is according with that fact of *Δsod1* mutant possessing different mechanism of defense against oxidative damage and in absence of SOD1 gene cells can still respond and maintain activity of mitochondrial enzymes. The improvements observed in metabolism of BY4741 after induction of oxidative stress and PDFs incubation, which is not observed in *Δsod1*, could be explained by the fact of BY4741 containing all oxidative stress related defenses enabling a better metabolic response [9, 31]. The fact that BY4741 and *Δyap1* presents improvement more notable than in *Δsod1* by this methodology it can be indicated that the mechanisms of defense related with Sod1p are not equally affected by these compounds and that exist different stimuli and pathways to control oxidative stress. To understand the main reasons of these differential changes and confirm the influence of the oxidative species in cellular mechanisms, important markers were evaluated such as ROS and GSH/GSSG. The CellTiter-Blue viability assay results (Fig. 18) together with the results of spot assays (Fig. 17) indicated that oxidative stress is evolved in cellular dysfunctions that lead to mitochondrial disruption and cell death by several pathways.

The CellTiter-Blue viability assay results suggest the mitochondria as one of the most important organelles involved in oxidative stress. Based on these arise the necessity of understand

more directly the role of mitochondria, an important evaluation to perform in further studies with more targeted methodologies.

4.4. Evaluation of cellular oxidative stress markers

4.4.1. Reactive Oxygen Species

Research in the yeast *S. cerevisiae* has provided considerable information about oxidative stress and the mechanisms employed by cells in response to increased of ROS [8]. Cells use an elaborate enzymatic machinery consisting of superoxide dismutases, reductases, catalases, peroxiredoxins, glutaredoxins, and glutathione transferases to maintain redox balance. ROS generation is the final step in many cell degeneration pathways and act as a non-specific rather than specific damage mechanism. This regulation can be mentioned as an additive mechanism of neuroprotection by polyphenols, claiming a therapeutic effect for polyphenols via ROS regulation as the only mechanism that seems to be overdrawn. ROS production is counteracted by an intricate antioxidant defense system that includes antioxidant enzymes, scavengers molecules but also the activation of signalling pathways that mediates cell growth and differentiation [7, 10, 16, 34].

In this task ROS production was measured by H2DCF (2,7-dichlorofluorescein) in the *S. cerevisiae* strains BY4741, *Δyap1* and *Δsod1*. This methodology is important to determine how oxidative compounds, such as H_2O_2 or menadione, modify cellular molecules and activate defense mechanisms in the cells. ROS evaluation also allows examining if PDFs act through ROS scavenging mechanisms.

The increase of ROS are represented in relative fluorescence units (R.F.U.) determined based on the ROS production during an incubation time (*t*), normalized for the number of cells per well and for initial fluorescence read. This allows the removal of the differences related with the initial number of cells per well and temporal differences between the H_2O_2 addition and the first fluorescence measurement. To infer the results of ROS increase, the fluorescence values were measured during 1 DT according with the strain at each 10 min and only were used values that represented linearity.

Contrary to what could be expected for all strains, was not observed any differences on ROS production after cells being incubated with the PDFs followed by oxidative stress, demonstrating that scavenging of ROS are not the mechanism by which PDFs protect cells (Fig. 19). The treatment described in section 4.3.2 was also used to test ROS production. The results obtained for this assay indicates that *Δyap1* have a similar performance to BY4741, both representing the higher values for ROS production when cells are incubated with H_2O_2. For the tested PDFs *Δyap1* is the strain that represent a higher difference between production of ROS when cells are incubated in the presence or absence of H_2O_2, which indicates that this is the strain with more difficulty to eliminate the ROS produced, an expected result based on its absence of Yap1p transcriptional factor (Fig. 19).

The *A. unedo* fruit PDF is the only condition in this results that present a significant alteration on *Δsod1* cells. When *Δsod1* cells are incubated in presence of *A. unedo* fruit and subsequently submitted to oxidative stress no difference is observed compared to cells only incubated with the PDFs. However *A. unedo* fruit PDF seems capable of induce a slightly increase on ROS production,

demonstrated by the difference between cells only gowth in presence of *A. unedo* PDFs and cells growth in normal conditions (Fig. 19). This could suggest that cells prepare themself to respond to H_2O_2 injury by several mechanisms, such as the increase of ROS function or by genes activation and signalling pathways.

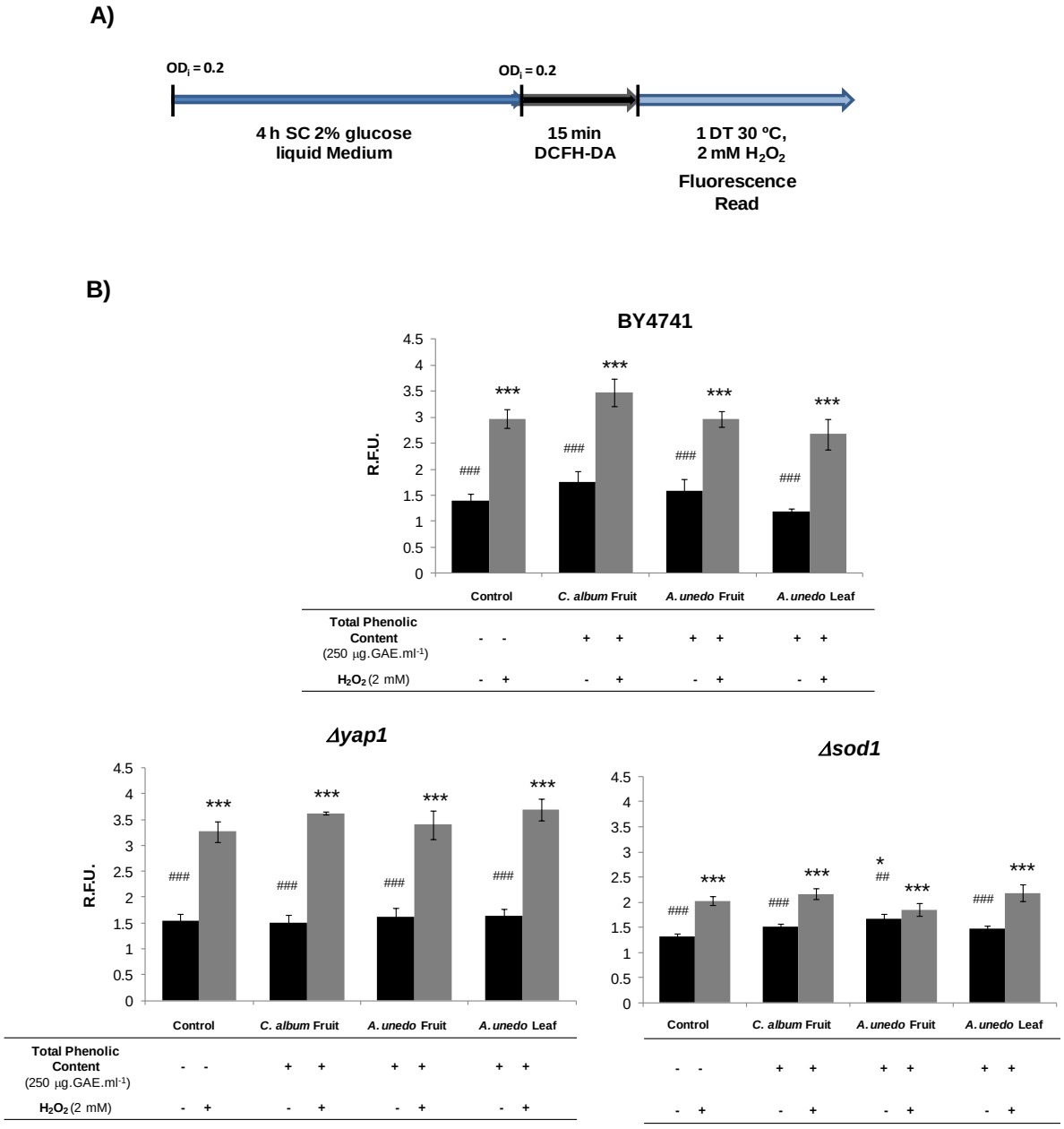

Figure 19. Evaluation of ROS levels in *S. cerevisiae* strains (BY4741, *Δyap1* and *Δsod1*) growth in the presence of PDFs and subjected to an oxidative stress. A) Schematic representation of tested conditions in this experiment. B) Results are expressed in relative fluorescence units normalized for number of cells and initial fluorescence (R.F.U) representing the increase of ROS produced during time of exposure to oxidative stress. Strains were grown in SC liquid medium with glucose supplemented with 0 and 250 µg GAE. mL^{-1} of *C. album* fruit, *A. unedo* fruit and leaf PDFs for 4h at 30 ºC, with constant shaking. Cells were harvested and grown in presence of 0 mM (Grey bars) and 2 mM (Black bars) of H_2O_2 for 1 DT according with the strain (2 h for BY4741 and 3 h for *Δyap1* and *Δsod1*) at 30 ºC. ROS produced were quantified by Diclhrofluorescein assay. Values represent mean ± SD of three independent biological replicates. *, ** and *** represent statistically significance for a control of cells without PDFs and H_2O_2, for a p<0.05, p<0.01 and p<0.001 respectively. #, ## and ### represent statistically significance for a control of cells without PDFs and in presence of 2 mM of H_2O_2, for a p<0.05, p<0.01 and p<0.001 respectively.

These results together with the results obtained by spot assays (Fig. 17) and CellTiter-Blue viability assay (Fig. 18) suggest that *A. unedo* fruit PDF increase ROS production as a preconditioning mechanism to improve cellular viability.

Natural phytochemicals can exert their antioxidant effects through different mechanisms and interaction with transcriptional regulators of several pathways or modulating the activity of enzymes involved in ROS. These capacities are attributed to the phenolics compounds, such as anthocyanins or other flavonoids [16, 33, 58, 92].

Detoxification of ROS can be can be mediated by increasing activity of antioxidant enzymes. H_2O_2 can be readly converted to H_2O in mitochondria by the activity of several antioxidant enzymes such as Cat1p, Sod2p and Gpxp, which is oxidize GSH into GSSG [93]. ROS act as a signaling molecule in the early events of autophagy and is essential for the induction of this process. Systems such as ubiquitin-proteossome and autophagy lysosome pathways are responsible for the recognition and degradation of non-functional cytosolic components. When both systems are overwhelmed apoptotic mechanisms are activated. Mitochondrial turnover is dependent on autopahgy as a strategy of cell to prolong life span [12, 23].

The obtained results for ROS quantification together with the results of CellTiter-Blue viability assay reinforce the idea that mitochondria could play an importante role in oxidative stress. Also based on these results is observed that *C. album* fruit and *A. unedo* leaf PDFs besides to present an increase on viability in some circumstances but not able to reduce the ROS production. Our results suggest that on contrary to historical idea of being scavengers of ROS, these phytochemicals fractions are acting through other cellular mechanisms to confer cell protection.

4.4.2. Glutathione

The increase in GSSG at the expense of GSH in response to H_2O_2 is consistent with the role of GSH both as a radical scavenger and as an electron donor for glutathione peroxidase [10, 47]. GSH may account for 0.5 to 1% of the dry weight in the yeast *S. cerevisiae*, depending on the growth conditions and accounts for more than 95% of the amount of non-protein thiols [31].

Since direct scavenging of ROS is not one of the mechanisms by each PDFs induce improvements on cell metabolism. First GSH was evaluated for all strains only when cells are injury by the presence of H_2O_2, to understand the response of this redox system to oxidative stress (Fig. 20). When just quantified the GSH, was observed that the WT strain do not show differences between control and stressed cells. These results are in agreement with some studies showing that due to the fact of WT strain pocesses all antioxidant enzymes and all mechanism for dealing with oxidative damage it is capable of keep GSH to normal levels even in presence of H_2O_2 [31].

The *Δyap1* and *Δsod1* strains when submitted to an oxidative damage induced by H_2O_2 present alterations on GSH levels (Fig. 20). For these reasons, in order to have an idea of the balance in cells of the redox status in presence of PDFs and detect small differences we decided to quantify both forms GSH and GSSG (Fig. 21) only quantified for *Δyap1* and *Δsod1* strains after treatments. Cells were incubated for 4 h in presence of PDFs and subsequently submitted to oxidative stress as described in section 3.4.3.

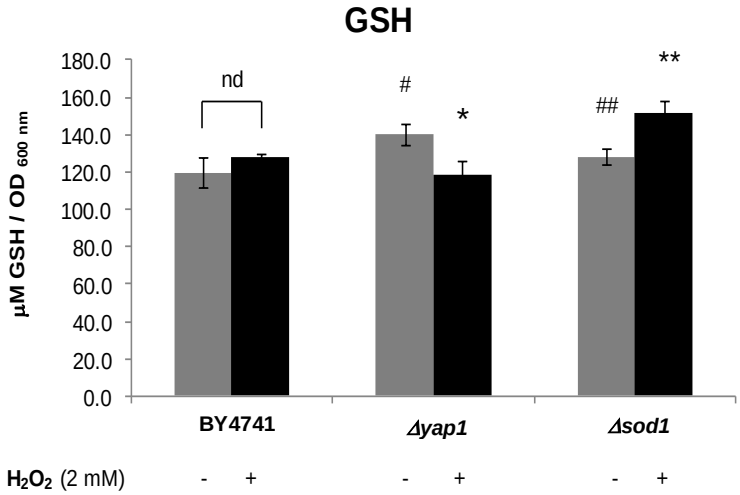

Figure 20. GSH determination of *S. cerevisiae* strains (BY4741, *Δyap1* and *Δsod1*) growth in presence of an oxidative stress. Strains were grown in SC liquid medium with glucose for 4h at 30 ºC with constant shaking. Cells were harvested and grown in presence of 0 mM (Grey bars) and 2 mM (Black bars) of H_2O_2 for 1 DT according with the strain (2 h for BY4741 and 3 h for *Δyap1* and *Δsod1*) at 30 ºC. GSH was quantified by a HPLC method and results are expressed in µM GSH normalized for number of cells (µM GSH/$OD_{600\ nm}$). Values represent mean ± SD of three independent biological replicates. *, ** and *** or. #, ## and ### representing statistically significance, for a p<0.05, p<0.01 and p<0.001 respectively.

When in the presence of oxidant molecules the GSH is oxidized into GSSG and the cell redox homeostasis is restored. For this cycle when the normal values of GSH are altered by the redox state of cell, the GSSG is also altered. Due to the fact that this ratio represents the influence of oxidant molecules on redox state of cell, it is consider a marker of how cell leads with oxidative injury. These mechanisms are also changed and show different behaviour according with the origin of oxidative compounds, such as H_2O_2 or menadione. Besides GSH/GSSG ratio is indirectly regulated by Yap1p transcriptional factor [37, 38, 45, 92].

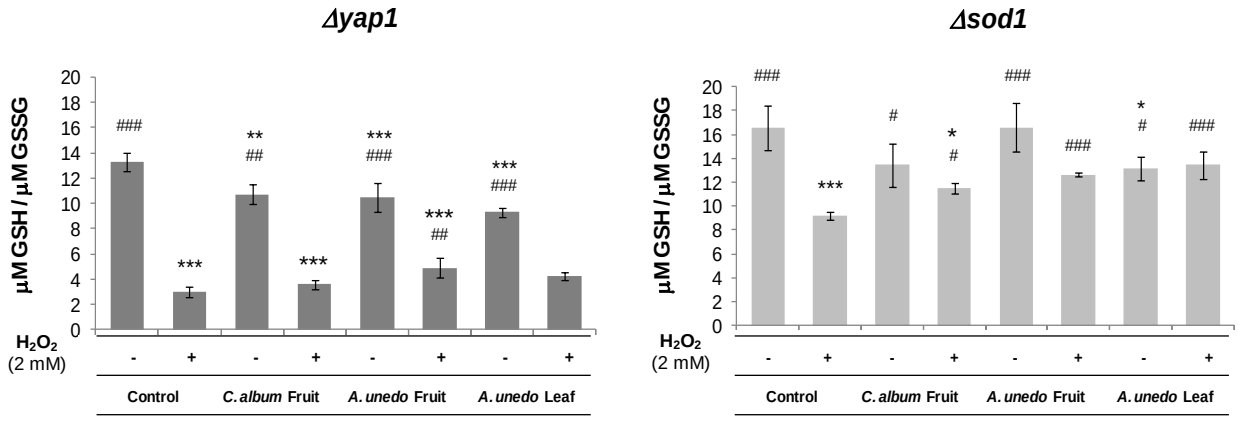

Figure 21. GSH/GSSG determination for *Δyap1* and *Δsod1 S. cerevisiae* strains growth in presence of PDFs and subsequently submitted to an oxidative stress. Strains were grown in SC liquid medium with glucose supplemented with 0 and 250 µg GAE. mL^{-1} of *C. album* fruit, *A. unedo* fruit and leaf PDFs for 4h at 30 ºC with constant shaking. Cells were harvested and grown in presence of 0 mM or 2 mM of H_2O_2 for 1 DT according with the strain (2 h for BY4741 and 3 h for *Δyap1* and *Δsod1*) at 30 ºC. GSH and GSSG values were quantified by HPLC and results are expressed in µM GSH / µM GSSG. Values represent mean ± SD of three independent biological replicates. *, ** and *** represent statistically significance for a control of cells without PDFs and H_2O_2, for a p<0.05, p<0.01 and p<0.001 respectively. #, ## and ### represent statistically significance for a control of cells without PDFs and in presence of 2 mM of H_2O_2, for a p<0.05, p<0.01 and p<0.001 respectively.

The results demonstrate that *Δyap1,* in all situations of oxidative stress induction a very low ratio than when in absence of oxidative stress (Fig. 21). The decrease on ratio values indicates that GSH is oxidized into GSSG. The *Δyap1* results (Fig. 21) illustrate that this mutant even when incubated with the PDFs in absence of H_2O_2 undergoes metabolic alterations that lead to GSH oxidation. This is demonstrated by the significant lower values of the GSH/GSSG for all tested PDFs. Phytochemicals presents in PDFs are compounds that cells do not possess in normal situations and that have electrophilic properties, which can act by similar mechanisms of action of ROS, activating the gene expression of antioxidant enzymes, signalling pathways, growth/differentiation pathways and adaptation proteins. When *Δyap1* cells are incubated with PDFs it seems that cells suffer a metabolic adaptation that could later contribute to a better cell performance after injury, by a preconditioning mechanism. For the other hand the same effect is not so significant for the metabolic answer of *Δsod1*, since that the GSH/GSSG parameter only presence a decrease for cells incubated in presence of *A. unedo* leaf with less significance.

The ratio values for both strains incubated with PDFs and consequently submitted to oxidative stress are above of the GSH/GSSG values of cells only incubated in presence of PDFs, however *Δsod1* cells incubated only with PDFs present higher GSH/GSSG values than cells only incubated with H_2O_2, indicating an amelioration of cell metabolism. *A. unedo* fruit PDF show values significantly higher than cells only incubated with H_2O_2, for *Δyap1* and *Δsod1*, suggesting that this fraction is pobably involved in redox metabolism and can improve cell state of both strains.

The results obtain for GSH/GSSG (Fig. 21) together with the results obtained for CellTiter-Blue viability assay (Fig. 18) suggests that both *A. unedo* fruit and leaf PDFs improve the viability of *Δyap1* cells that seems associated to a decrease on GSH/GSSG ratio. On the other hand when *Δyap1* cells are submitted to an oxidative stress after being incubated in presence of *A. unedo* fruit PDF is observed an increase of GSH/GSSG ratio, which also reflects in an increase of viability (Fig. 17 and 18) compared with cells only incubated in presence of H_2O_2.

When *Δsod1* is incubated in the presence of *C. album* fruit or *A. unedo* leaf PDFs and submitted to oxidative stress is observed a higher ratio than control of cells only in the presence of H_2O_2, which in turn is also equal to the ratio of cell just growth in the presence of this PDFs. This observation indicates that these fractions are also capable of ameliorate the *Δsod1* phenotype. These processes to improve cell redox homeostasis can be performed by several mechanisms.

For *Δsod1* cells in general is observed an increase of ratio GSH/GSSG for cells incubated in presence of PDFs and then submitted to oxidative stress comparing with the control cells only incubated with H_2O_2 (Fig. 21), however though the results obtained by CellTiter-Blue viability assay (Fig. 17) is not clear by each mechanisms these PDFs influence cells viabilty. Only *A. unedo* fruit PDF is capable of restore the viability of *Δsod1* cells submitted to oxidative stress to the level of cells growth only in presence of this PDF.

The GSH/GSSG is very important for cells and beside its cytosolic function, this ratio it can be also found in mitochondrial matrix. GSH is required for Sod1p activation when under the influence of oxidant compounds capable of generat O^{2-}. These results demonstrate that *Δsod1* seem less affected

by oxidative stress than *Δyap1*, as a result of higher levels of GSH/GSSG ratio when *Δsod1* cells are incubated with H_2O_2, comparing to *Δyap1* (Fig. 21). *Δsod1* cells demonstrate a decrease of GSH/GSSG ratio after being submitted to oxidative stress even after growth in presence of PDFs, when compared with the cells in normal conditions. Nevertheless the difference in the ratio relating to control cells is not as huge as for *Δyap1*. A possible explanation for this result can be the fact that Yap1p regulates Sod1p enzyme and its absence makes *Δyap1* cells more susceptible to oxidative stress induced by H_2O_2. The *Δyap1* is also affected in other several pathways involved in oxidative stress, such as G-6-PDH. Moreover in the absence of SOD1 gene, the cell still possesses the principal transcriptional factors that activate other molecules such as Grxp, Gpxp, Trxp and Trr1p to maintain the redox state, became *Δyap1* cells more resistence to oxidative stress.

Besides GSH other molecules and enzymes are involved in redox homeostasis and GSH/GSSG ratio mantainance, such as glutaredoxins and thioredoxins, important protein sources of sulfhydryl groups in the cell and are structurally and functionally conserved. Both GSH and TRx can function as electron donors for thiol-dependent peroxidases. Even the YAP1 gene is regulated by thioredoxin but not by glutathione. Trxp could be another important components to maintain mitochondrial activity intact because it is known that oxidative stress and the decrease in glutathione content leads to the oxidation of Trxp *in vivo* [16, 28, 32, 94].

Peroxiredoxins (Prxp) are ubiquitous thiol-specific proteins that have multiple functions for stress protection as antioxidants, molecular chaperones and in the regulation of signal transduction. The mitochondrial thioredoxin system is important for the detoxification of ROS through the activity of Prxp [28, 32]. All the results in this study could be an indicator about the biological role of PDFs in mitochondrial homeostasis.

5. Final considerations and future perspectives

This study was important to select the plant species with protective potential in a general model of degeneration for further studies in a yeast model of Parkinson's Disease, already implemented in DSB laboratory. The evaluation of PDFs with protective potential in yeast model of PD will allow understanding the influence of these compounds in the molecular mechanisms related with this disease in particularly. Nevertheless in the end of this study many questions remain to be answer.

The four strains of the yeast model of degeneration, represented different sensibilities to oxidative stress induced by H_2O_2, being *Δyap1* the most affected strain. The H_2O_2 sensibilities of all strains tested by different exposures of cells to oxidative stress demonstrated to be dependent of the type of exposure (chronic or acute).

The IVD process is important to access the group of compounds that mimics what can be recovered after all physic and chemical transformations that plants constituents suffer during human gastrointestinal digestion [52, 57, 76]. Since that IVD process only mimics the gastrointestinal digestion, to understand the total bioavailabilty of phenolic compounds will be interesting use a more complete model that also mimics the microflora action and the metabolization by the cells.

In this study according with the chemical characterization, the PDFs from leaves seems to be me more promising to protect cells from degeneration since these PDFs present the higher antioxidant potential and the higher levels of total phenolic compounds. However spot assays results demonstrated that in general PDFs from fruits have more protective potential than leaves, since that from the two chosen plants for this study only *A. unedo* leaf PDF showed protective potential.

Despite of *A. unedo* fruit and leaf PDFs do not denoted to be the most promising extract based on chemical characteristics, the results obtained in this study indicate that the phytochemicals present in these PDFs are capable of improve the *Δyap1* cells viability in presence and absence of H_2O_2. These raise the question of how the H_2O_2 and phytochemicals activate the enzymatic defense and cell survival by distinct signals and what is the biological role of phytochemicals in cell mechanisms of degeneration. On the other hand *Δsod1* improvements on viability are only observed for *A. unedo* fruit PDF when cells are growth in presence of H_2O_2 suggesting that a mechanism of protection through Yap1p activation can be proposed.

According with the results of CellTiter-Blue viability assay, ROS and Glutathione quantification is inferred that *Δyap1* is the strain that suffer more significant metabolic alterations. The results also corroborate that *A. unedo* PDFs from fruits and leaves are capable of diminish the oxidative state of cell by decreasing the GSSG levels. However was also possible to observe that PDFs from fruits and leaves of *A. unedo* present different mechanisms of action in *Δyap1* cell probably due to the different phytochemical composition. *A. unedo* PDFs possibly present multitarget capacity and potential for pharmaceutical applications in order to prevent degenerative and age-related disorders development.

Peroxide is a promiscuous oxidizing agent that acts to damage many different macromolecules in the cell. Yap1p transcriptional factor can be activated by several different molecules including H_2O_2, thiol oxidants and metals. Oxidation of Yap1p cysteines indicates that its activity is regulated by post-translational mechanisms and raises the question of whether Yap1p oxidation occurs directly by the hydroperoxides or whether there is an intermediary molecule that allows its oxidation [28, 29, 42, 95]. Our results demonstrate that the absence of Yap1p transcriptional factor became cells much sensitive to H_2O_2 even in presence of PDFs, while cells without Sod1p were less affected. These reinforce the importance of this transcriptional factor in oxidative stress response. Moreover another transcriptional factor is evolved in oxidative stress, the Snk7p. The fact of these two transcriptional factors have similar roles in cell redox state suggests that in presence of H_2O_2 and in the absence of Yap1p, the Snk7p could be responsible for maintaining cells homeostasis [37, 92]. Several studies associate both Yap1p and Snk7p transcription factors to the regulation of gene expression induced by oxidative stress such as GSH1, GPX2, GLR1, TRX2 and TRR1, but are differentially activated in presence of diverse oxidant molecules, inducing different cellular responses by the presence of H_2O_2, diamide or menadione [28, 35, 37, 41].

For these reasons could be interesting evaluate the involvement of other oxidants in the mechanism of degeneration and the importance of this second transcriptional factor in defense mechanism induced by PDFs.

Plant-derived polyphenols have been shown to be especially important in biological chemistry due to their capacity as modulators involved in ROS detoxification [15, 48, 53, 55, 78]. The Yap1p is a

DNA binding protein of the AP-1 family that was identified as a functional homologue of the mammalian AP-1, the Nrf2p transcriptional factor. The similarity of the cystein thiol reactivity of both Yap1p and Nrf2p makes from *S. cerevisiae* a good model to investigate the mechanisms of action involved in cell defense against oxidative stress. Besides, Nrf2p regulates the chemical stress response pathways being trigger by ROS such as H_2O_2 and phytochemicals as a defense to oxidative stress [96, 97]. The fact of *Δyap1* strain presents cell viability improvements in presence of PDFs probably indicate that the compounds present in these extracts, such as polyphenols are capable of regulate the expression of gene associated to cell death/survival and cell signaling through Yap1p.

Moreover, after ROS determination we conclude that ROS scavenging is not the mechanism by which cells improve their metabolism in presence of PDFs, however the mechanisms inherent to cellular improvements by this phytochemicals is not totally clear. Besides ROS early mechanisms of defense based on signaling pathways and gene activation, the autophagy-lysosome pathway has been recently implicated as one of the most important mechanisms in development of degenerative diseases. The reduction of mitochondrial oxidative damage could slow or even prevent the progression of degeneration. It is now considered that constitutive basal autophagic activity is a quality control process that selectively disposes aberrant protein aggregates and damaged organelles for degradation [12, 24, 93].

Differences in cellular response based on GSH/GSSG ratio of *Δyap1* and *Δsod1* indicate that PDFs affect differentially the two mutants, which can indicate the possibility of the phytochemicals besides to keep redox homeostasis by glutathione systems are also probably capable of activate other mechanisms such as glutaredoxins and thioredoxins. Further studies could provide more insights about how these mechanisms interplay in cell degeneration processes.

The cross-talk between autophagy, redox signaling and mitochondrial dysfunction is not totally clear yet. In conclusion, to understand the impact of natural phytochemicals in degenerative diseases some key molecules involved in all this steps of metabolism must be evaluated with more targeted methodologies.

6. References

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