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Faculdade de Farmácia

EFFECT OF NUTRITIONAL SUPPLEMENTS ON THE AQUAPORIN-MEDIATED OXIDATIVE STRESS

Duarte Alexandre Ferrão Lopes

Dissertação orientada pela Professora
Doutora Maria da Graça Soveral Rodrigues

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Ackowledgments

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Resumo

As aquaporinas (AQPs) são canais transmembranares que estão amplamente expressas em organismos vivos, sendo responsáveis pelo transporte de água e pequenos solutos, como o glicerol. Até à data, foram identificadas treze AQPs (0-12) em mamíferos que estão dividas em: aquaporinas ortodoxas (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 e AQP8), aquagliceroporinas (AQP3, AQP7, AQP9 e AQP10), dependendo da sua capacidade para transportar água ou água e glicerol. AQP11 e AQP12 estão inseridas no grupo de aquaporinas não-ortodoxas ou S-aquaporinas devido à sua localização subcelular e permeabilidade ainda incerta. Algumas aquaporinas têm a capacidade de transportar outros solutos como amónia (AQP8) e/ou peróxido de hidrogénio (hAQP1, hAQP3, rAQP5 e hAQP8), sendo que estas são chamadas de peroxiporinas.

O peróxido de hidrogénio (H_2O_2), é conhecido como uma espécie reativa de oxigénio que é capaz de provocar dano em proteínas, lípidos e ácidos nucleicos sendo bastante associado ao stress oxidativo. Contudo, recentemente o peróxido de hidrogénio tem vindo a ser identificado como tendo um papel essencial em várias funções vitais e vias de sinalização associadas à divisão, migração e sobrevivência celular. Estas espécies reativas de oxigénio estão constantemente a ser produzidas nas células devido aos processos metabólicos. Contudo podem também ser gerados pela exposição celular a radiação, xenobióticos ou metais pesados. O stress oxidativo ocorre quando a quantidade destas espécies reativas de oxigénio é bastante superior à capacidade de ação das defesas antioxidantes. Existem dois sistemas de defesa antioxidante bastante importante nas células. A enzima catalase e a glutationa, que funcionam como principais agentes de remoção de espécies reativas de oxigénio mantendo os níveis redox intracelular em níveis não-prejudiciais para a homeostasia celular.

Os polifenóis são caracterizados pela presença de grupos fenol na sua estrutura química. Os polifenóis são o maior grupo de fitoquímicos e existe em grande quantidade em plantas comestíveis. Uma dieta rica em polifenóis mostrou ser bastante benéfica para a saúde. Até agora, foram identificadas mais de 8000 estruturas fenólicas em vegetais, frutos, azeite e vinho. Podem ser divididos em vários subgrupos como: flavonoides, ácidos fenólicos, curcuminóides, estilbenos e taninos. Vários estudos propuseram que uma ingestão elevada de polifenóis pode ser associada à diminuição de vários tipos de doenças incluindo doenças cardiovasculares, várias formas de cancro e doenças neurodegenerativas. Recentemente foram descritos vários estudos sobre o efeito e a influência destes compostos nutricionais na expressão e atividade das AQPs. Polifenóis bioativos mostraram ter alguma influência na expressão, bem como nas

propriedades biofísicas das AQPs de mamífero. Devido à capacidade das AQPs para transportar peróxido de hidrogénio e aos efeitos benéficos dos polifenóis, decidimos investigar a permeação ao peróxido de hidrogénio pela aquaporina 3 humana e a aquaporina 5 humana, bem como, o efeito de compostos alguns compostos nutricionais (curcumina, naringenina e hesperidina) e de um extrato de uma planta chamada *Mahonia aquifolium* que tem vindo a mostrar efeitos antioxidantes, anti proliferativos, anti-inflamatórios e anticancerígenos, no stress oxidativo mediado pelas AQPs. Para este efeito, foi usado um sistema de expressão heteróloga de levedura *Saccharomyces cerevisiae* cujas aquaporinas endógenas foram silenciadas (aqy1 e aqy2). A aquaporina 3 e a aquaporina 5 de *Homo sapiens* foram previamente clonadas num vetor de expressão pUG35 que foi utilizado para transformar a estirpe de S. *cerevisiae* YSH 1770. Primeiramente, fomos determinar a função das aquaporinas através da técnica de interrupção brusca de fluxo "*stopped-flow*". Os ensaios foram realizados a pH 5 (pH ótimo das leveduras) e ao pH 7.4 (pH fisiológico em tecidos animais). Seguidamente fomos observar a sensibilidade das estirpes que expressam as aquaporinas através de dois ensaios: ensaio de fenótipo, onde as células foram sujeitas a um stress crónico (dois dias) com várias concentrações de H_2O_2, e um ensaio de formação de colónias, em que as células foram sujeitas a um stress agudo (uma hora) com uma concentração de H_2O_2. Posteriormente, com o objetivo de verificar se há efeito do H_2O_2 nos sistemas de defesa antioxidante das células, fomos verificar os níveis de atividade da catalase e o conteúdo total de GSH nas células em condições basais e após adição de H_2O_2. Por fim, fomos avaliar e quantificar a acumulação de ROS intracelular, bem como o efeito dos compostos acima mencionados através de um método que utiliza uma sonda específica para ROS (H_2DCFDA). Para este efeito, as células controlo que apenas possuem o vetor vazio (pUG35) e as células que expressam as aquaporinas 3 e 5 foram incubadas com várias concentrações de H_2O_2 e a variação de fluorescência devida à acumulação de ROS intracelular foi medida ao longo de uma hora.

Em seguida fomos investigar o efeito dos compostos nutricionais acima descritos no stress oxidativo mediado pelas aquaporinas. As células foram previamente incubadas com uma concentração conhecida de cada composto e tratadas com uma concentração escolhida no ensaio anterior. As variações de fluorescência foram acompanhadas ao longo de uma hora. Os nossos resultados mostraram que as ambas as aquaporinas estavam funcionais para o transporte de água (AQP5) e para o transporte de água e glicerol (AQP3). Uma vez que a AQP3 é regulada pelo pH, os resultados mostraram também que a pH 5 esta aquaporina está inativa, ou seja, não transporta água nem glicerol, e que a pH 7.4 se encontra aberta. Relativamente aos ensaios de fenótipo verificou-se uma alta sensibilidade por parte da estirpe com expressão de AQP5 ao

peróxido de hidrogénio a partir da concentração mais baixa, enquanto que, curiosamente a estirpe com expressão de AQP3 não apresentou qualquer sensibilidade. Nos ensaios de formação de colónias verificamos que a estirpe com expressão de AQP5 mostrou uma sensibilidade inicial ao H_2O_2 seguida de uma recuperação celular após uma hora de incubação. Relativamente à estirpe que expressa AQP3, verificamos que a pH 5 as células mostraram ter uma percentagem de sobrevivência ligeiramente maior que o controlo, enquanto que a pH 6.5 verificamos uma alta sensibilidade ao peróxido de hidrogénio após quinze minutos de incubação com uma ligeira recuperação após uma hora de incubação. Após verificar os níveis de atividade da enzima catalase e quantificar os níveis de glutationa total nas células, verificamos que não existiram variações significativas nestes níveis em condições basais nem após a incubação com uma concentração conhecida de peróxido de hidrogénio para as diferentes estirpes. Os resultados relativos à medição da acumulação de ROS intracelular mostraram que a estirpe com expressão de AQP3 apresentou níveis de acumulação bastante baixos a pH 5 e elevados níveis a pH 7.4 logo a partir de concentrações baixas de H_2O_2. Relativamente à estirpe que expressa a AQP5, os níveis de acumulação de ROS foram bastante similares para ambos os valores de pH embora estes níveis tenham sido significativamente inferiores aos observados para a AQP3 a pH 7.4. Por último, relativamente ao efeito dos compostos nutricionais no stress oxidativo mediado pelas aquaporinas verificamos que existe um efeito de remoção e diminuição dos níveis de ROS nas células. Em todas as estirpes os compostos naringenina e curcumina apresentaram os melhores resultados com importante destaque para a curcumina que apresentou um efeito de remoção bastante elevado. O extrato da planta *Mahonia aquifolium* apresentou os níveis mais baixos enquanto que o composto hesperidina, apesar de apresentar alguma função na diminuição dos valores de ROS na estirpe que expressa a AQP3, mostrou um efeito controverso na estirpe que expressa a AQP5.

Palavras-chave: aquaporinas, permeabilidade, stress oxidativo, espécies reativas de oxigénio, peróxido de hidrogénio, composto nutricionais.

Abstract

Aquaporins (AQPs) are transmembrane channel proteins widely expressed in living organisms responsible for the transport of water and small solutes such as glycerol. Until now, thirteen AQPs (0-12) were identified in mammals are grouped into orthodox AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) and aquaglyceroporins (AQP3, AQP7, AQP9, AQP10), depending on their ability to permeate water or water and glycerol. AQP11 and AQP12 are grouped as unorthodox or S-aquaporins due to their distinct evolutionary pathway and permeability still uncertain. Some AQPs are also able to transport other solutes such as ammonia (AQP8) and/or hydrogen peroxide (AQP1, AQP3, AQP5 and AQP8) being named peroxiporins.

The class of polyphenols is characterized by the presence of phenol units in their chemical structure. Polyphenols are the largest group of phytochemicals and they exist in eatable plants. Diets enriched in these polyphenols were found to exert many beneficial effects to human health. So far, more than 8000 phenolic structures have been identified in vegetables, fruits, olive oil and wine. They can be divided in many subfamilies such as flavonoids, phenolic acids, curcuminoids, stilbenes and tannins. Epidemiological studies propose that a high intake of polyphenols can be associated to a decrease of several types of diseases including cardiovascular diseases, specific forms of cancer, and neurodegenerative diseases. Several studies have reported the influence and effect of these nutritional compounds in AQPs expression and activity. Bioactive polyphenols have shown to have some influence in the expression as well as in biophysical properties of mammalian AQPs. Due to the ability of AQPs to permeate hydrogen peroxide and knowing the beneficial effects of polyphenols, we decided to investigate the permeation of hydrogen peroxide by human aquaporin 3 and human aquaporin 5, as well as the effect of specific nutritional compounds (curcumin, naringenin and hesperidin) and a plant extract of *Mahonia aquifolium* in the AQP-mediated oxidative stress. For this purpose, we used a yeast heterologous expression system of *Saccharomyces cerevisiae*, which is endogenous aquaporin-deleted (*aqy1* and *aqy2* knock-out). The *Homo sapiens* AQP3 and *Homo sapiens* AQP5 genes were previously cloned in an expression vector pUG35 and were used to transform the strain S. cerevisiae YSH 1770. Firstly, we determined the function of each aquaporin using the stopped-flow technique at pH 5 (yeast physiological pH) and at pH 7.4 (mammalian physiological pH), followed by assessment of the cells sensitivity to H_2O_2 using a phenotypic growth assay (chronic stress) and a colony forming unit assay (acute stress). Then, we decided to investigate the effect of H_2O_2 in the antioxidant defense system by measuring the

catalase levels and total GSH content in cells. Afterwards, the intracellular ROS accumulation and the effect of each compound were determined using a specific probe for ROS (H_2DCFDA). Our results showed a high sensitivity of hAQP5 expressing cells to H_2O_2 in the phenotypic growth assay but in the colony forming unit assay we observed an initial sensitivity followed by a cellular recuperation. Cells expressing hAQP3 showed higher intracellular ROS accumulation at pH 7.4 whereas cells expressing hAQP5 showed a similar accumulation at both pH 5 and pH 7.4. Curcumin and naringenin reduced ROS accumulation and showed to be the most efficient compounds against oxidative stress.

Keywords: aquaporins, permeability, oxidative stress, reactive oxygen species, hydrogen peroxide, nutritional compounds.

Abbreviations and Symbols

A	Area (cm^2)
AQP(s)	Aquaporins
GFP	Green Fluorescent Protein
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Glutathione disulfide
H_2O_2	Hydrogen peroxide
hAQP1	Human aquaporin 1
hAQP3	Human aquaporin 3
hAQP5	Human aquaporin 5
hAQP8	Human aquaporin 8
k	Rate constant
M. aquifolium	*Mahonia aquifolium*
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidases
OD	Optical density
osm_{out}	Internal osmolarity
Pf	Water permeability coefficient ($cm\ s^{-1}$)
Pgly	Glycerol permeability coefficient ($cm\ s^{-1}$)
R	Universal gas constant ($J\ mol^{-1}\ K^{-1}$)
rAQP5	Rat aquaporin 5
ROS	Reactive Oxygen Species
S. *cerevisiae*	*Saccharomyces cerevisiae*
V	Total cellular volume (cm^3)
YNB	Yeast Nitrogen Base

1. Introduction

1.1. Aquaporins

Water is the major component of cells and tissues throughout all forms of life and plays a crucial role due to its unique physical and chemical properties (Madeira, Moura et al., 2016). All the biochemical and physiological processes of an organism depend on the presence of water, which is the fundamental component for homeostasis maintenance. Due to the high-water permeability of the plasma membrane, cells are usually very sensitive to osmotic gradients that can cause volume changes (Moura, 2004). Aquaporins (AQPs) allow a bidirectional, fast, selective and regulated transport in response to osmotic gradients (Krane & Kishore, 2003) and for this reason, AQPs play a crucial role in the homeostasis maintenance and regulation.

The first studies on water transport began in the 1950s when Solomon and co-worker (Sidel & Solomon, 1957) verified the high permeability of red blood cells to water that could not be explain by a simple diffusion process (Benga, 2002). In 1970, Macey noted that the activation energy for water transport was low and the transport was strongly inhibited by mercurial compounds, such as mercury chloride. They demonstrated a reversible variation of plasma membrane water permeability and subsequent increase of activation energy in response to the application of this type of blocking agents (Farmer & Macey, 1970).

In 1987, Peter Agre and colleagues investigated the presence of the Rh antigen on red blood cells' membranes and obtained a secondary band on an SDS-PAGE gel with a molecular weight of about 28 kD (Agre, Saboori, Asimos, & Smith, 1987). Initially, this secondary band was considered as a product of antigen degradation, but they quickly concluded that in addition to being abundant in red blood cells it was also noted in renal tubules (Smith & Agre, 1991). In 1991, this protein sequence was discovered (Preston & Agre, 1991) and the analysis of its sequence revealed a strong homology with the MIP26 (*Major Intrinsic Protein* of *26kD*) protein bovine crystalline cell membrane already described in 1984 by Gorin (Gorin, Yancey, Cline, Revel, & Horwitz, 1984). These data suggested that this 28 kD protein belongs to the MIP transmembrane protein channels (Smith & Agre, 1991). At the time, this protein received the name CHIP28 (*Channel-forming Intrinsic Protein of 28 kD*). In 1992, the heterologous expression of CHIP28 in *Xenopus laevis* oocytes was very important for the discovery of aquaporins. By introducing a hypotonic osmotic shock, the expression of CHIP28 led to a significant increase in cell volume followed by oocyte rupture compared to the control oocytes (Preston, Carroll, Guggino, & Agre, 1992). The CHIP28 protein was then identified as a water channel. In 1993, CHIP28 was renamed to aquaporin-1 (AQP1). In 1999, Peter Agre reported

the first high-resolution image of AQP1 three-dimensional structure (Mitsuoka et al., 1999). A few years later, all the work developed by Peter Agre and co-workers in the discovery and characterization of AQP1 was recognized as the Chemistry Nobel Prize in 2003.

1.1.1. Structure of aquaporins

More than 300 different AQPs have been discovered so far in which thirteen isoforms have been identified (AQP0-AQP12) in humans. AQPs are integral, hydrophobic, transmembrane proteins that primarily facilitate the passive transport of water depending on the osmotic pressure on both sides of membrane. Subsequent studies showed that AQPs can transport water molecules but also other small, uncharged molecules such as glycerol, urea and hydrogen peroxide (Li & Wang, 2017).

The functional aquaporin unit is a homotetramer, and each monomer is a specific channel for water and/or other solutes. This homotetrameric assembly is crucial to the correct folding, stability, targeting to the plasma membrane and functionality of AQPs (Hachez & Chaumont, 2010).

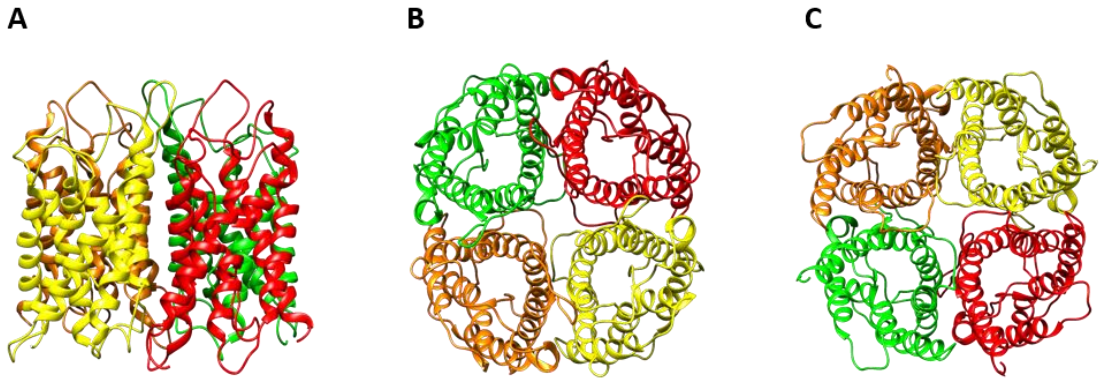

Figure 1.1 – High resolution tridimensional structure (3.7 Å) of human AQP1 (hAQP1). (A) lateral view; (B) top view; (C) bottom view.

In Figure 1.1, the functional unit of AQP1 is represented. It is formed by four monomers and each monomer forms an independent pore. It means that a functional aquaporin has four independent monomers. The junction of the four monomers forms a central pore, that is not able to transport water molecules but has been related to the transport of gases such as nitric oxide and oxygen in AQP4 (Wang & Tajkhorshid, 2010). Each monomer is constituted by six transmembrane α-helices connected by 5 loops, with the N- and C- termini domains sited in the cytoplasm, which are re-arranged three-dimensionally to form the pore. The loops B and E contain a highly conserved three amino acid zone embedding into the plasma membrane, known

as NPA (Asparagine-Proline-Alanin) motif, which is believed to be crucial for the selectivity of these proteins (Figure 1.2).

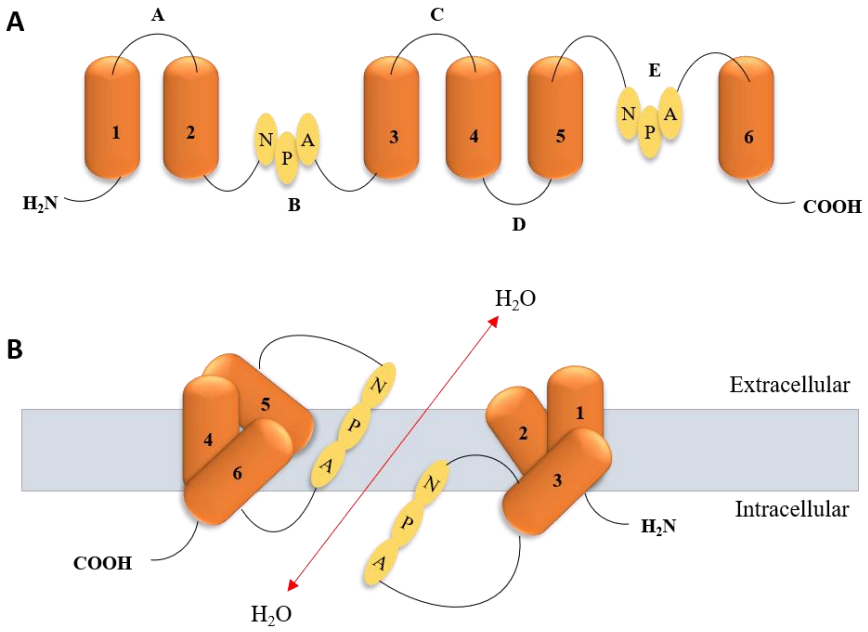

Figure 1.2 – (**A**) The AQP1 monomer is constituted by six transmembrane helices (1-6) connected by five loops (A-E); (**B**) The loops B and E form the selectivity filter, where are the NPA motifs (Asn-Pro-Ala). Adapted from (Zeuthen, 2001).

In addition to NPA motif (known as charge exclusion filter), each monomer has another selectivity filter responsible for size exclusion, the aromatic/arginine (ar/R) filter in the central region of the pore (Sui, Han et al., 2001), as shown in Figure 1.3.

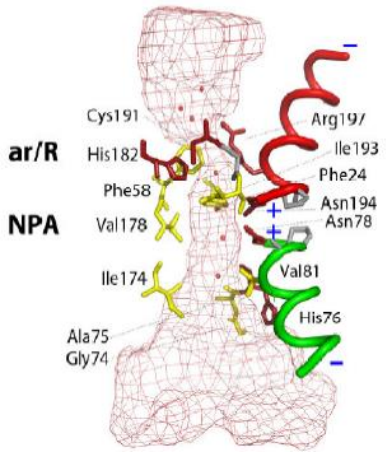

Figure 1.3 – Detailed view of bAQP1 pore where we can find the two selectivity filters: ar/R and NPA motif. In red are represented the hydrophilic residues (capable of establishing bonds with water molecules) and in yellow are represented the hydrophobic residues. Adapted from (Soveral, Trincão et al., 2011).

The overall AQP selectivity results from the combination of these two selectivity filters, NPA motifs and the ar/R filter. The presence of these particular hydrophilic and hydrophobic

residues makes the AQP pore able to exclude molecules due the charge characteristics or molecular size (Verkman, Anderson et al., 2014).

1.1.2. Selectivity of aquaporins

Aquaporins (AQPs) belong to a highly conserved group of membrane proteins called the major intrinsic proteins (Madeira et al., 2016). They can be divided in three subfamilies: (1) orthodox or classical aquaporins, which are primarily water selective; (2) aquaglyceroporins, which are permeable to glycerol and other small solutes in addition to water; (3) S-aquaporins, which also can be called super aquaporins or subcellular aquaporins. This subfamily is not only present in animals but in plants, fungi and bacteria and its permeability is still uncertain (Ishibashi, 2009).

There are 13 AQPs identified in mammalians (AQP0-AQP12) with similar structure and differing only in some amino acids located in crucial points which confer the subfamilies mentioned above. The initial ten AQPs (AQP0-AQP9) are relatively well described. The aquaglyceroporins AQP3, AQP7, AQP9 and AQP10 represent a subfamily that allows movement of water and other small solutes, such as glycerol, urea, hydrogen peroxide, some gases (ammonia, carbon dioxide and nitric oxide) and even some ions (Almasalmeh, Krenc et al., 2014, Verkman et al., 2014). Recently, AQP3 and AQP8 were reported as H_2O_2 transporters in mammalian cells, while the orthodox AQP1 is not able to permeate H_2O_2 across the membrane (Bienert, Moller et al., 2007, Miller, Dickinson et al., 2010). AQP9 was also disclosed as a peroxiporin in mammalian cells (Watanabe, Moniaga et al., 2016). In addition, a recent study from our group unveiled the ability of rat AQP5 to permeate H_2O_2 and reported rAQP5 involvement in cell oxidative stress (Rodrigues, Mosca et al., 2016)

Table 1.1 summarizes the functional characterization of each AQP, as well as its tissue distribution:

Table 1.1 - Tissue distribution and permeability characteristics of different aquaporins. Adapted from (Verkman, 2005).

Aquaporin	Permeability	Tissue expression
AQP0	H_2O	Eye lens fiber cells
AQP1	H_2O	Kidney tubules, endothelia, erythrocytes, choroid plexus, ciliary epithelium, intestinal lacteals, corneal endothelium
AQP2	H_2O	Kidney collecting duct
AQP3	H_2O, glycerol	Kidney collecting duct, epidermis, airway epithelium, conjunctiva, large airways, urinary bladder
AQP4	H_2O	Astroglia in brain and spinal cord, kidney collecting duct, glandular epithelia, airways, skeletal muscle, stomach, retina
AQP5	H_2O	Glandular epithelia, corneal epithelium, alveolar epithelium, gastrointestinal tract
AQP6	H_2O, anions	Kidney collecting duct intercalated cells
AQP7	H_2O, glycerol	Adipose tissue, testis, kidney proximal tubule
AQP8	H_2O	Liver, pancreas, intestine, salivary gland, testis, heart
AQP9	H_2O, small molecules	Liver, white blood cells, testis, brain
AQP10	H_2O, glycerol	Small intestine
AQP11	?	Kidney, liver
AQP12	?	Pancreatic acinar cells

1.1.3. Biological functions and associated diseases

Since a few years ago, there has been great progress in the understanding of the diverse roles of AQPs in human health and disease as well as in the physiology of other organisms. As mentioned before, AQPs are involved in the transport of water but also in permeation of small solutes such as glycerol, hydrogen peroxide, urea and possibly some gases and are responsible for the regulation of several cells functions, including water and energy homeostasis, cell migration, adhesion, proliferation and differentiation. AQPs play an essential role in physiologic and pathophysiologic processes such as osmoregulation, lipid metabolism, organogenesis and regeneration and vascular and cancer biology (Ishibashi, Kondo et al., 2011, Verkman, 2011).

Observation of the phenotype of AQPs knock-out mice have revealed a huge role in physiological functions and in the appearance and development of several pathologies (Ishibashi et al., 2011, Verkman, 2009), such as epilepsy, cerebral edema, glaucoma, cancer and obesity. Therefore, modulation of AQPs expression/function might be a novel strategy for clinical benefit in the treatment of numerous diseases, such as obesity, epilepsy, glaucoma, brain edema and cancer.

1.2. Oxidative stress

Oxidative stress may occur in several ways. One of the main possibilities is inadequate diet rich in antioxidants. Malnutrition may lead to inadequate dietary intake of α-tocopherol, ascorbic acid, sulfur-containing amino acids, which is needed for reduced glutathione synthesis (GSH), or riboflavin, which is needed to produce the flavin adenine dinucleotide (FAD) cofactor of glutathione reductase. A diet missing protein leads to inadequate synthesis of metal ion binding proteins (Golden, 1994) . Another possibility is an overproduction of O_2^- and H_2O_2 by exposure to drugs or toxins which are metabolized to produce free radicals, or by excessive activation of natural radical-producing systems such as phagocytes in chronic inflammatory diseases. Cells tolerate a soft oxidative stress by upregulating synthesis of antioxidant defense system to restore the balance. However, severe oxidative stress produces DNA damage, increases the intracellular free Ca^{2+} and iron, causes damage to proteins and lipid peroxidation that may lead to cell injury or death (Halliwell, 1996).

1.2.1. Hydrogen peroxide, a reactive oxygen species (ROS)

In biological systems, hydrogen peroxide (H_2O_2) is known as a reactive oxygen species (ROS), with the potential to damage proteins, lipids and nucleic acids. Recently, H_2O_2 has been identified to play a key role in several pathways associated to cell function and survival (Bienert, Schjoerring, & Jahn, 2006). H_2O_2 is produced in various metabolic processes of cells in aerobic organisms, like mitochondrial oxygen metabolism in electron transport chain, or can be formed by enzymes with the primary function of ROS generation, such as NADPH oxidases, xanthine oxidase and 5-lipoxygenase. This molecule is also involved in many regulatory cellular events, including the activation of transcription factors, cell proliferation and apoptosis. H_2O_2 derived from mitochondrial electron transport chain has been shown to play a role in hematopoietic cell differentiation and cell division. NADPH oxidase generated H_2O_2 can affect cardiac differentiation, vascularization and angiogenesis. H_2O_2 is also capable of modulating

several crucial signaling cascades including ERK, JNK, p38, MAPK and PI3K/Akt (Wittmann et al., 2012).

1.2.2. Aquaporins and hydrogen peroxide permeation

H_2O_2 is continuously produced intracellularly as a product of cellular metabolism but can be also generated in extracellular side of cell membrane by Nox proteins. Due to this natural extracellular H_2O_2 generation, the investigation of how cells seize H_2O_2 toward beneficial pathways have gained especial interest. For a long time, it was assumed that hydrogen peroxide diffusion through biological membranes was realized at a sufficient rate for metabolic purposes. However, more recently it was observed that H_2O_2 could cross the membrane through water channels, the aquaporins, which were then called peroxiporins. This discovery suggested a huge interest in the membrane transport of H_2O_2 field (Henzler & Steudle, 2000). The size and the electro-chemical properties of solutes are key features to the transport mediated by AQPs. There are some physicochemical similarities between H_2O_2 and water molecules and both can form hydrogen bonds. This characteristic is crucial to permeate the pore. The H_2O_2 molecule has a diameter of about 0.25-0.28 nm, which is lesser than the pore diameter of AQP1, a typical orthodox-aquaporin (Bienert et al., 2006). In 2000, Henzler and co-workers showed the first experimental evidences using a well-studied model for plants, the internodal cells of the algae *Chara corallina* (Henzler & Steudle, 2000). Briefly, they stimulated the H_2O_2 transport across membranes and estimated transport parameters. After testing the model, they obtained a H_2O_2 permeability coefficient like water. Moreover, they used mercuric chloride as control. Mercurial compounds were the first AQP-inhibitors referred in literature, however these reagents have not any therapeutic role due to its high toxicity. After the experiment with these compounds, Henzler and co-workers concluded that H_2O_2 was significantly reduced, which indicates H_2O_2 permeation through AQPs. Nowadays, some specific mammalian AQP-isoforms have been reported as channels for H_2O_2 permeation across plasma membranes (Bertolotti, Bestetti et al., 2013, Bienert & Chaumont, 2014, Bienert et al., 2007, Hara-Chikuma, Chikuma et al., 2012, Hara-Chikuma & Verkman, 2008). Recent studies showed that the aquaglyceroporin-3 (AQP3) and the orthodox aquaporin-8 (AQP8) are able to transport H_2O_2 across the plasma membrane in mammalian (Bienert et al., 2007, Miller et al., 2010). Briefly, they demonstrated that permeation of H_2O_2 is mediated by AQP3 using the genetically encoded fluorescent H_2O_2 sensor, Hyper. These transmembrane channel proteins can increase or reduce downstream signaling pathways dependent of H_2O_2. They used a human colon cancer adenocarcinoma cell line, where AQP3 and NOX proteins are naturally expressed to assess the

association between H_2O_2 production by NOX and the H_2O_2 transport by AQP3 and their influence in downstream signaling cascades H_2O_2 dependent, such as AKT signaling. Hara-Chikuma and co-workers (Hara-Chikuma et al., 2012) provived evidences that AQP3 has a role in H_2O_2 permeation in chemokime-dependent T lymphocyte migration during the immune response. AQP8 was also identified as a H_2O_2 transporter (Bienert et al., 2007). Bertolotti and co-workers used a HeLa cell model expressing a fluorescent H_2O_2 sensor, Hyper, and an AQP inhibitor, a mercurial compound. After application of H_2O_2, in the presence of mercurial compound, the fluorescence signal decreased. This fact indicated AQP-mediated H_2O_2 uptake. In addition, they silenced the AQP8 expression and also observed a decrease in fluorescence signal when cells were exposed to external AQP8 (Bertolotti et al., 2013). AQP1 has been reported as unable to permeate H2O2 (Bienert et al., 2007, Miller et al., 2010). Interestingly, a recent study suggested evidences that rat AQP1 was able to permeate H_2O_2 in rat aortic smooth muscle cells. They hypothesized that rAQP1 could have a role in H_2O_2 permeation in rASMCs (Al Ghouleh, Frazziano et al., 2013). AQP9 was also found to permeate H_2O_2 in mammalian cells. Overexpression of hAQP9 in Chinese hamster ovary K-1 potentiated the increase of cellular H_2O_2 after addition of exogenous H_2O_2. In contrast, AQP9 knockdown by siRNA in human hepatoma HepG2 cells reduced the uptake of extracellular H_2O_2.

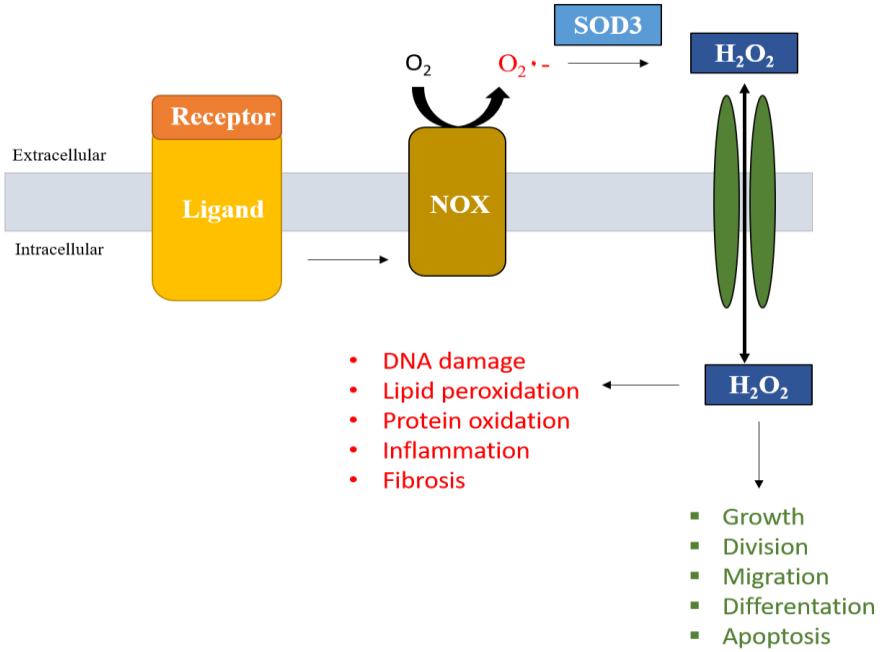

Figure 1.4 – Aquaporins as peroxiporins. NOX enzymes are activated upon ligand-receptor interaction, and extracellular SOD3 captures the superoxide, providing H_2O_2 for import by aquaporins. Green letters refer to physiological processes of H_2O_2 and red letters refers to pathophysiological processes of H_2O_2. Adapted from (Sies, Berndt et al., 2017).

1.2.3. Antioxidant defense system

Oxygen is a molecule with high reactivity and can be reduced forming several of reactive species, known as reactive oxygen species (ROS). The highly reactive species can be quite harmful to the cells, damaging cellular components including DNA, lipids and proteins (Storz, Christman et al., 1987). H_2O_2 and superoxide anions are highly toxic ROS and can induce the production of more reactive species, particularly in the presence of metal ions. As mentioned before, ROS are continuously being generated inside cells due to metabolic processes. However, ROS can also be generated by cell exposure to ionizing radiation, redox-cycling chemicals present in the environment or by exposure to heavy metals. Through these mechanisms, all the aerobic organisms are continuously exposed to ROS and the oxidative stress occurs when the amount of oxidant increases beyond the antioxidant capacity of the cells. For this reason, most of the organisms have developed means to protect their cellular components against oxidative stress.

Cells have enzymatic and non-enzymatic defense systems to protect their cellular constituents to maintain intracellular redox state and homeostasis. The enzyme catalase is part of the enzymatic defense system, which is in the cytoplasm and is responsible for the catalysis of H_2O_2 into water and oxygen, according to the reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$. It is a homotetrameric enzyme with the four monomers attached to an iron metal core, having a molecular mass between 225 kD and 250 kD. The iron atom (active center) is responsible for the reaction with H_2O_2. There are two types of catalase in Saccharomyces cerevisiae, catalase A and catalase T. The role of catalase A is thought to be related to the elimination of H_2O_2 from fatty acid oxidation (Jamieson, 1998), while catalase T is involved in the elimination of H_2O_2 which is generated by the action of several factors that lead to the occurrence of oxidative stress (Dorval & Hontela, 2003, Marchler, Schuller et al., 1993).

Glutathione (GSH), a tripeptide (ɣ-L-glutamyl-L-cystinylglycine), is a linear peptide consisting of three amino acids: glutamic acid, cysteine and glycine, being the thiol group of cysteine the active site responsible for its biochemical properties. The synthesis of glutathione is a process that occurs in cytosol. The tripeptide is a water-soluble antioxidant, recognized as the most important non-protein in living systems. GSH is involved in many cellular functions, DNA and protein synthesis, amino acid transport, enzymatic regulation and elimination of xenobiotics. However, its contribution against stressful situations make its role very important for cells (Anderson, 1998). The mechanism that evolves glutathione in response to oxidative stress is a process which occurs by chemical equilibrium without enzymatic catalysis or

involving the enzyme glutathione peroxidase (GPx). In this reaction, the GSH will reduce lipid peroxidation products into their respective alcohols, or in the case of hydrogen peroxide, will reduce it to H_2O according to the reaction: $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$.

In a situation of oxidative stress, much of the GSH is converted to GSSG to counter this unfavorable situation. Thus, the quotient between GSH and GSSG can be used as an indicator of the redox state of the cell (Hiroi, Ogihara et al., 2005, Wang, Gotoh et al., 2000). After the oxidation of GSH, it is crucial to replace its reduced form, i.e. it is necessary to recycle the glutathione so that the cell continues to have response mechanisms. This is a process that depends on the enzyme glutathione reductase, according to the following reaction: $NADPH + GSSG \xrightarrow{GR} NADP^+ + 2GSH$. The reaction occurs in the presence of NADPH which acts as reducing cossubstrate, reducing GSSG and consequent formation of GSH. Thus, the more reducing is the cellular environment more expressed is this enzyme. However, the NADPH availability is the limitant factor in this cycle, since this molecule is crucial in the process of reducing GSSG. The failure of any mechanisms associated with this cycles can compromise the cellular defense processes (Inoue, Matsuda et al., 1999).

1.3. Polyphenols

In recent years, fruits and vegetables have a considerable interest depending on type, number, and mode of action of the different components, called as phytochemicals. Plants are rich sources of functional dietary micronutrients, fibers and phytochemicals, such as ascorbic acid, carotenoids, curcuminoids, flavonoids, tannins, stilbenes and phenolic compounds. Individually, or in combination, these components may have beneficial effects for health since they demonstrate antioxidant activity *in vitro* (Liu, 2004, Percival, Talcott et al., 2006).

Polyphenols are a complex group of phytochemicals which are used by plants to defend themselves from pathogens, predators, and environmental stresses. They are also nutrients that may have an enormous effect on human health by changing genetic expression, maintaining gut health and controlling mitochondrial function. Nowadays, there are more than 8000 known polyphenols, and probably twice that number are not structurally analyzed (Scalbert, Johnson et al., 2005). It is known that polyphenols are powerful activators of human genes involved in the synthesis of antioxidant enzymes, modulation of anti-inflammatory pathways, and activation of anti-aging genes. There is enough data showing that a diet rich in polyphenols (vegetables, fruits, nuts, and whole grains) is associated with lower rate of chronic diseases and mortality (Bao, Han et al., 2013, Cassidy, Mukamal et al., 2013). Polyphenols can act as great

antioxidants by activating antioxidant genes such as Nrf2. Once these genes are activated, they generate increased expression of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (Erlank, Elmann et al., 2011, Hybertson, Gao et al., 2011, Scapagnini, Vasto et al., 2011).

Epidemiological studies propose that a high intake of polyphenols is related to a decrease of a range of illnesses including cardiovascular disease (Kuriyama, Shimazu et al., 2006), specific forms of cancer and neurodegenerative diseases (Checkoway, Powers et al., 2002). Polyphenols can be divided in four major groups: phenolic acids, flavonoids, tannins and stilbenes. Flavonoids have showed strong beneficial effects in human, animal and *in vitro* studies (Schroeter, Spencer et al., 2001). In cardiovascular health, flavonoids may alter lipid metabolism (Zern, Wood et al., 2005), inhibit low-density lipoprotein (LDL) oxidation (Jeong, Choi et al., 2005), reduce atherosclerotic lesion formation (Fuhrman, Volkova et al., 2005), decrease vascular cell adhesion molecule expression (Ludwig, Lorenz et al., 2004), improve endothelial function (Hallund, Bugel et al., 2006) and reduce blood pressure (Hodgson, 2006). This type of polyphenols has also shown beneficial cognitive effects and reverse specific age-related neurodegeneration (Joseph, Shukitt-Hale et al., 1999) and exhibit some anti-carcinogenic effects, including an ability to induce apoptosis in tumor cells (Fabiani, De Bartolomeo et al., 2002, Fini, Hotchkiss et al., 2008, Mantena, Baliga et al., 2006), inhibit cell proliferation (Corona, Deiana et al., 2009, Wang, Heideman et al., 2000) and prevent angiogenesis and tumor cells invasion (Piao, Mori et al., 2006).

1.3.1. Curcumin

Curcumin is a pigment from *Curcuma longa* L (turmeric) which is cultivated in tropical and subtropical regions and belongs to the family of curcuminoids. The major worldwide producer of turmeric is India, where it has been used as a homemade medicine for many diseases for a long time (Aggarwal, Kumar et al., 2003, Esatbeyoglu, Huebbe et al., 2012, Grynkiewicz & Slifirski, 2012, Wilken, Veena et al., 2011). Curcumin is a symmetric molecule, also known as diferuloyl methane. The chemical name of curcumin is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, with the chemical formula $C_{21}H_{20}O_6$ and molecular weight of 368.38. It has three chemical entities in its structure: two aromatic ring systems containing o-methoxy phenolic groups, connected by a seven-carbon linker consisting of an α, β-unsatured β-diketone moiety (Priyadarsini, 2014). Curcumin is one of the most promising natural products which has been intensively investigated by from both the biological and chemical point of view (Priyadarsini, 2014). Scientific research has been done over more than

four decades confirming the diverse pharmacological effects of curcumin and its ability to act as chemo preventive as well as a potential therapeutic agent against many chronic diseases. This compound, with almost two centuries of scientific history, is still being attractive for researchers from all over the world (Aggarwal et al., 2003, Esatbeyoglu et al., 2012, Grynkiewicz & Slifirski, 2012, Wilken et al., 2011). In 1815, curcumin was first isolated from turmeric and there were only a few reports till the 1970s on its chemical structure, synthesis, biochemical and antioxidant activity (Sharma, 1976, Vogel & Pelletier, 1815). In 1990s, Aggarwal and co-workers (Singh & Aggarwal, 1995) reported its potential anticancer effect and from that date curcumin research has grown rapidly, with more than 14000 citations on curcumin to date. Considering the many researches with curcumin in many fields because of its beneficial properties, it is important to evaluate its potential applications.

1.3.2. Naringenin

Naringenin belongs to the class of flavonoids called the flavanones. This class are very abundant in citrus fruits such as grapefruit (*Citrus paradisi*) and oranges (*Citrus sinensis*). The chemical name of naringenin is 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, and it has molecular weight of 272.26, with the chemical formula $C_{15}H_{12}O_5$. Naringenin is almost insoluble in water and is soluble in organic solvents such as ethanol or DMSO (Lisa, Nica et al., 1999). The role of naringin in the treatment of disease has been quite interesting, with interest as anticancer and antiatherogenic compound. Naringenin is associated with beneficial effects in osteoporosis, cancer and cardiovascular diseases (Patel, Singh et al., 2014). This compound is reported to have antiproliferative effects in different cancer cell lines, including colon, breast, and uterus cancer cell lines. Antioxidant capacity and radical scavenging activity of naringenin was studied *in vitro* and the results showed that naringenin exhibit a high antioxidant activity and is a high efficient hydroxyl and superoxide radical scavenger. Additionally, naringenin showed a significant effect in the protection against oxidative damage to lipids in dose-dependent manner (Cavia-Saiz, Busto et al., 2010).

1.3.3. Hesperidin

Hesperidin is isolated in substantial amounts from the discarded rinds of common orange *Citrus aurantium* L., *C. sinensis*, *C. unshiu* and other species of the genus *Citrus* (family Rutaceae) (Garg et al., 2001). Hesperidin is a flavanone glycoside, composed of an aglycone, hesperetin or methyl eriodyctiol and an attached disaccharide, rutinose. This compound has

molecular formula $C_{18}H_{34}O_{15}$ and a molecular weight of 610.57 Da. It has been reported to possess many biological effects including anti-inflammatory, antimicrobial, anticarcinogenic and antioxidant properties (Garg et al., 2001). For example, it has been found to reduce superoxide in electron transfer plus concerted proton transfer reaction *in vitro.* This antioxidant properties were also exhibited in liver homogenates for hydroperoxide-induced chemiluminescence (Fraga, Martino et al., 1987). Ahmadi A. and co-workers investigated the chemoprotective potential of hesperidin and they concluded that this compound have a potent chemoprotective effect against genotoxicity induced by cyclophosphamide in mouse bone marrow (Ahmadi, Hosseinimehr et al., 2008). A recent study showed a potential protective effect of hesperidin on RGC-5 cells against high glucose-induced oxidative stress. Hesperidin showed to increase the endogenous antioxidant defense mechanisms and the protection of mitochondrial function by modulating Bcl-2 family members, as well as by inhibiting caspases activation via a ROS-dependent p38 and JNK signaling pathways (Liu, Liou et al., 2017).

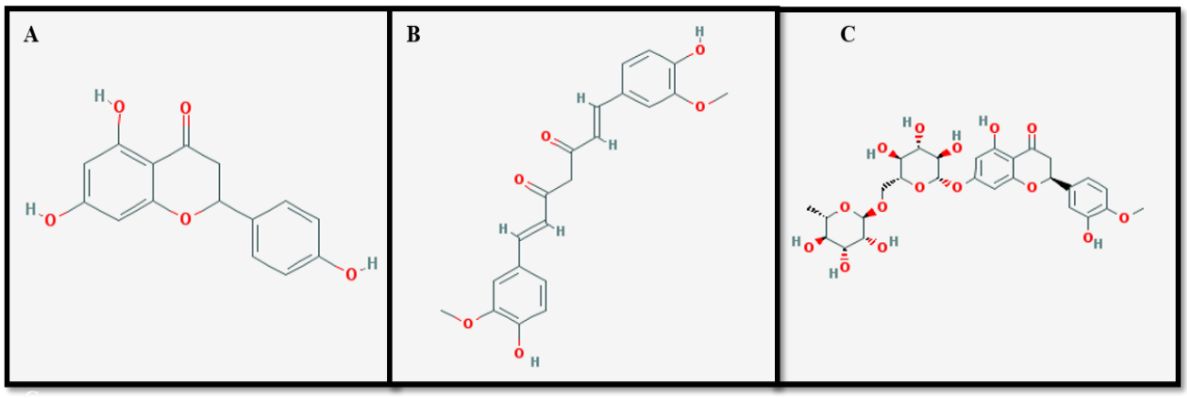

Figure 1.5 - Chemical structure of nutritional compounds used in this study. (A) Naringenin; (B) Curcumin; (C) Hesperidin.

1.3.4. *Mahonia aquifolium* extract

Mahonia is the second largest genus of the family Berberidaceae, which includes around 60 species and is distributed in Asia, America and Europe (Kost`alova, Kardosova et al., 2001). Plants of this genus have many uses in popular medicine as treatment for the psoriasis, dermatitis, fungal infections and in the Traditional Chinese Medicine for the treatment of tuberculosis, dysentery, pharyngolaryngitis, eczema and wounds (He & Mu, 2015). Many species of Mahonia were highly studied and showed to possess antibacterial, antifungal, anti-inflammatory, antioxidant, anti-proliferative and pro-apoptotic effects (He & Mu, 2015, Hu,

Yu et al., 2011, Kost`alova et al., 2001, Vollekova, Kost'alova et al., 2003, Wong, Hsiao et al., 2009, Zeng, Lao et al., 2003).

Mahonia aquifolium (Pursh) Nutt. *(M. aquifolium*) is an ornamental bush and is one of the most general plants of the genus *Mahonia* which contains important alkaloids, becoming one of the most extensively examined plants of this genus. It has been used in the American Traditional Medicine to treat different skin disorders (Kost`alova et al., 2001) and several researches concluded that M. *aquifolium* is has a great effect in patients with mild to moderate psoriasis through activities on different components of inflammation process and on keratinocyte (Augustin, Andrees et al., 1999, Galle, Muller-Jakic et al., 1994, Gulliver & Donsky, 2005, Muller, Ziereis et al., 1995). Traditional use of M. *aquifolium* to treat inflammatory conditions suggests that this plant may have a potential to be used in several fields, such as anti-cancer therapy, antioxidant activity, anti-inflammatory and anti-proliferative.

1.4. Aims and goals

AQPs are a family of transmembrane proteins that revealed to have a significant role in disease progression such as some types of cancer. The involvement of AQPs in disease may be due to their function as channel and/or because of their transported molecules effect on other processes associated with health and disease conditions such as oxidative stress. Hereupon, the role of AQPs as a H_2O_2 transporter might be investigated.

In recent years, fruits and vegetables have a considerable interest depending on type, number, and mode of action of the different components, so called as phytochemicals. Plants are rich sources of functional dietary micronutrients, fibers and phytochemicals, such as ascorbic acid, carotenoids, and phenolic compounds. Individually, or in combination, these components may have a potential to be used in several fields, such as anti-cancer therapy, antioxidant activity, anti-inflammatory and anti-proliferative.

Our group optimized a cell model for characterizing aquaporin activity and screening modulators using the yeast *Saccharomyces cerevisiae,* which is are silenced in endogenous aquaporins genes. Thus, human aquaporin 3 and human aquaporin 5 (referred as hAQP3 and hAQP5) were cloned in an expression vector pUG35 and used to transform the *S. cerevisiae* strains. The heterologous expression system can be used to assess mammalian aquaporin function using the stopped-flow assay and allows to investigate the role of AQPs in H_2O_2 permeation as well as to evaluate the effect of nutritional compounds on the oxidative stress mediated by aquaporins.

Thus, this work aims to investigate the role of human AQP3 and human AQP5 in H_2O_2 permeation and consequent involvement in cell oxidative stress response and evaluate the effect of nutritional compounds using the *S. cerevisiae* expression system.

To achieve the overall goal of this project some specific goals must be performed:

1. Characterization of the function of the different yeast strains using the stopped-flow technique;
2. Evaluation of the sensitivity to H_2O_2 of the different yeast strains using a phenotypic growth assay (chronic stress), and colony forming units assay (acute stress);
3. Evaluation of the cellular antioxidant defense system of control and AQP-transformed strains, by measuring the activity of catalase and GSH levels;
4. Measurement of intracellular ROS accumulation of the different AQP-transformed strains using a specific probe for ROS (H_2DCFDA).

5. Investigate the effect of different nutritional compounds on AQP-mediated oxidative stress, previously described to have some beneficial effects.

2. Materials and Methods

2.1. Plasmid and yeast strains

The yeast strain model used for the experimental assays in this work was previously established by our group. All the strains were transformed from the parental *Saccharomyces cerevisiae (S. cerevisiae)* YSH1770, which is endogenous aquaporin-defective (aqy1 and aqy2 knock-out). The *Homo sapiens* AQP3 (hAQP3) and *Homo sapiens* AQP5 (hAQP5) genes were previously cloned in an expression vector pUG35 (Vector *shuttle S. cerevisiae-Escherichia. coli*, Ampr, URA3) thereby forming the recombinant plasmids pUG35-hAQP3 and pUG35-hAQP5. These plasmids were used to transform the strain S. *cerevisiae* YSH 1770. This expression vector allows the cloning of any gene in frame with the reporter gene yEGFP (yeast-enhanced Green Flourescece Protein) enabling the cell localization of the protein fused with yEGFP. All strains and plasmids used in this work are showed and described in the table 2.1.

Table 2.1 - Description of the plasmids/strains used in this work.

Plasmids/strains	Genotype
Plasmids	
pUG35	Vector *shuttle S. cerevisiae-E. coli*, Ampr, URA3
pUG35-hAQP3	*Homo Sapiens* AQP3 cloned in pUG35
pUG35-hAQP5	*Homo Sapiens* AQP5 cloned in pUG35
S. cerevisiae strains	
YSH 1770	Double mutant strain *aqy1 aqy2* (MATα *leu2*::*hisG tpr1*::*hisG his3*::*hisG ura352 apy*1D::KanMX *aqy2*D::KanMX)
YSH1770/pUG35	YSH 1770 transformed with empty expression vector pUG35
YSH1770/pUG35-hAQP3	YSH 1770 transformed with gene *Homo Sapiens* AQP3 cloned in pUG35
YSH1770/pUG35-hAQP5	YSH1770 transformed with gene *Homo Sapiens* AQP5 cloned in pUG35

2.2. Yeast growth conditions

All the culture medium used for cell growth were sterilized after their preparation using an autoclave (JP Selecta and/or AJC Uniclave 88) for 20 minutes.

For the growth and maintenance of the strains used in this work was used yeast nitrogen base (YNB) with 2% glucose (w/v) and supplemented with the required concentrations of leucine, histidine and tryptophan (table 2.2, annex 6.1) and grown with agitation at 28°C. The cells were stored on solid media (plates and/or angled tubes) at 4°C using the same medium with 2% (w/v) agar. Cell growth was monitored by measuring optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultraspec 2100 Pro).

2.3. Functional assays – *Stopped-flow*

The stopped-flow technique is because the intensity of scattered light or fluorescence is proportional to the cell volume of a cell suspension. In this case, cells loaded with a volume-sensitive dye equilibrated in an isotonic buffer are rapidly mixed with solutions with different osmolarities, creating an osmotic gradient between the exterior and the interior of the cells. The osmotic gradient causes an outward or inward movement of water causing a change in cell volume until a new osmotic equilibrium is reached. This volume change is reflected in a variation of the fluorescence which is recorded over time.

Briefly, two solutions are placed in two syringes. The first syringe contains the yeast cell suspension previously equilibrated in a solution with osmolarity $(osm_{out})_0$ = 1400 mOsM and the second syringe contains the shock solution with a different osmolarity. Yeast strains were initially grown overnight in liquid YNB medium with 2% glucose (w/v) and supplemented with leucine, histidine and tryptophan at 28°C with orbital shaking up to OD_{600} = 2. Cells were then harvested by centrifugation at 3829 g, 4°C for 10 minutes (Allegra® 6 Series Centrifuges, Beckman Coulter®, Brea, CA, USA). Pellet was washed three times with sorbitol solution 1.4M (with osmolarity osm_{out} = 1400mOsM), which was prepared in K^+-citrate buffer 50mM (pH 5 or pH 7.4) and resuspended in 3 mL of the same solution per gram of pellet.

For the experiments, resuspended cells were incubated during 20 minutes at 30ºC with 10 µL of non-fluorescent permeable precursor, cFDA (5-(6-carboxyfluorescein diacetate) 100 µM, which is intracellularly hydrolyzed yielding the impermeable form (CF) (λ_{ex} = 492 nm; λ_{em} = 517 nm). Cells were then diluted 1:10 with sorbitol solution 1.4 M and immediately used for the experiments.

Experiments were performed on a HI-TECH Specific PQ/SF-53 apparatus, which has a 2 ms dead time, temperature controlled, interfaced with an IBM PC/AT compatible 80386 microcomputer. Water permeability coefficient was calculated through the equation: $Pf = k\ (V0\ /A)\ [1/Vw\ osmout)\ \infty]$ (equation 1) where k is the rate constant, V_0 is the initial volume before the osmotic shock, V_w is the water molar volume (18 cm^3 mol^{-1}), $(osm_{out})_{\infty}$ is the final medium osmolarity and A is the cellular area. Glycerol permeability coefficient was calculated through the equation: $Pgly = m\ (V0/A)$ (equation 2), where m is the slope, V_0 is the initial volume before the osmotic shock and A is the cellular area.

2.4. Growth curves

To evaluate the growth rate of *S. cerevisiae* control (pUG35) and AQP-transformed strains, we performed growth curves. Yeast strains were grown overnight in liquid YNB medium with 2% glucose (w/v) and supplemented with leucine, histidine and tryptophan at 28°C with orbital shaking. All strains were normalized at OD_{600} = 0.2 and optical density was measured every hour for 2 hours, corresponding to lag phase. Optical density was then measured every 60 minutes for 8 hours, corresponding to exponential phase, until stationary phase was reached. To evaluate the effect of H_2O_2 in cell growth, we added a known amount of H_2O_2 to a final concentration of 1mM in the growth medium. Optical density was measured every 2 hours for 10 hours corresponding to lag phase. When cells reached the exponential phase, the optical density was measured every hour for 16 hours until stationary phase. The optical density was measured using a spectrophotometer (Ultraspec 2100 Pro). Growth rate (k) was calculated through the slope obtained of linear regression of exponential phase.

2.5. Phenotypic growth assays

The phenotypic growth assay was chosen as an assessment of H_2O_2 sensibility of AQP-transformed strains compared to control and it was performed according to (Sabir, Leandro et al., 2014). Briefly, serial dilutions of yeast strains were made, and cell sensitivity of the different isoforms was compared under different concentrations of H_2O_2. This assay was performed on agar plates containing growth media YNB with 2% glucose (w/v) (pH 5 and pH 6.5) and the required amino acids (leucine, histidine and tryptophan). The plates were prepared with different concentrations of H_2O_2 (0.25, 0.5, 0.75, 1, 1.5 and 2 mM) and without H_2O_2 (control plates) and were kept in the dark to avoid H_2O_2 degradation by light. Yeast strains were initially grown overnight in liquid YNB medium with 2% (w/v) with leucine, histidine and tryptophan

at 28°C with orbital shaking until OD_{600} = 1 (~ 3 x 10^7 cells/mL). To ensure the same number of cells of each AQP-transformed strain was tested, volumes were corrected according to the required cell number. After normalization for the same number of cells in all the different strains, cells were centrifuged at 3829 g, at 4°C for 10 minutes (Allegra® 6 Series Centrifuges, Beckman Coulter®, Brea, CA, USA) and pellet was used immediately for the experiment. Serial 10-fold dilutions up to 10^{-7} of the initial concentrated culture were made with sterile distilled water in a 96-well plate and a 3 µL aliquot from each dilution was spotted using a replica platter device on the plates previously prepared. The plates were incubated at 28°C and the differences in growth phenotypes of yeast strains were recorded after 2 days of incubation.

2.6. Colony forming unit assay

Colony forming unit (CFU) is a method based on the acute stress suffered by cells in the presence of H_2O_2. It was performed in agar plates containing YPD medium, a complete medium for yeast growth to avoid additional factors that can contribute to fluctuations in resistance, such as variations in the amount of amino acids, and evaluate only the effect of H_2O_2 (table 2.3, annex 6.1). Yeast strains were initially grown overnight in liquid YNB medium with 2% (w/v) with leucine, histidine and tryptophan at 28°C with orbital shaking until OD_{600} = 1. Firstly, 100 µL of untreated cells were taken from each culture and diluted up to 10^{-5}. Then, 100 µL of 10^{-5} dilution was plated on agar plates with YPD medium. Cells were then treated with 1 mM H_2O_2 for 60 minutes, at 28°C with orbital shaking. After incubation, 100 µL aliquot was taken from each condition, diluted up to 10^{-5} and 100 µL was plated on agar plates with YPD medium. Finally, agar plates were incubated for 2 days at 28°C and colonies were counted. For each strain and condition, triplicates were made. The results were treated normalizing to the percentage of control (cells not treated with hydrogen peroxide).

2.7. Antioxidant measurements

2.7.1. Preparation of cell lysates for colorimetric assays

Firstly, a yeast pre-inoculum (10 mL) was prepared in YNB medium supplemented with leucine, histidine and tryptophan and incubated overnight at 28°C with orbital shaking. In the next day, was prepared an inoculum (50 mL) from pre-inoculum and it was incubated overnight at 28°C with orbital shaking. Then, 25 mL of inoculum was transferred for 50 ml tubes and cells were harvested by centrifugation at 3829 g for 10 minutes (Allegra® 6 Series Centrifuges,

Beckman Coulter®, Brea, CA, USA). The supernatant was discarded, and pellet was transferred to 2 mL eppendorfs. Pellet was centrifuged at 634 g for 10 minutes (Micro 1207, VWR, Pennsylvania, USA) to obtain a dry pellet and stored at -80°C.

For antioxidant measurements, dry pellet was dissolved in 500 µL phosphate buffered saline (PBS) and disrupted mechanically by vigorous agitation with glass beads for 10 times cycles of 1-minute vortex and 1 minute on ice. After disruption, cell lysates were cleared by centrifugation at 12000 g for 10 minutes (Micro 1207, VWR, Pennsylvania, USA). The supernatant was transferred to 1,5 mL eppendorfs and used immediately.

Prior to performing the assays, protein concentration of cell lysates was determined according to Bradford assay kit (Pierce® BCA protein assay kit, ThermoFisher, USA), using bovine serum albumin (BSA) as a standard.

2.7.2. Determination of catalase activity

Catalase activity was measured by modified method of Goth (Goth, 1991). This method is based on the measurement of H_2O_2 degradation in oxygen and water in cell lysate, which occurs mostly by catalase activity. The remain hydrogen peroxide and molybdate ions forms a yellowish complex. Firstly, phosphate buffer 60 mM pH 7.4 and ammonium molybdate 32.4 mM were prepared. For H_2O_2 quantification in samples, a H_2O_2 calibration curve was prepared according to the table 2.4 (Annex 6.2).

Then, 100 µL of each standard and 20µL of each sample (1:10 diluted cell lysate) were added to 96-well plate (duplicates) and 100 µL of 65 mM H_2O_2 were added to each sample. Absorbance was measured immediately at 405 nm using a microplate reader (Zenyth 3100, Anthos Labtec Instruments, Salzburg, Austria). After 5 minutes of incubation at room temperature (RT), 100 µL of ammonium molybdate 32.4 mM (stop solution) was added to each well. After 1 minute of incubation at RT, absorbance was measured again at 405 nm (Zenyth 3100, Anthos Labtec Instruments, Salzburg, Austria). For catalase activity calculations, we ploted a linear regression of Abs_{405nm} vs. [H_2O_2] standards and calculated the amount of H_2O_2 which was not consumed by catalase. Catalase activity (U) was calculated through the equation: $Activity\ (U) = (Quantity\ (\mu mol))/\ time(\text{min})$ (equation 3). One unit of catalase activity is defined as the amount of enzyme needed for degradation of 1 µmol of H_2O_2/min at 25 °C. Specific catalase activity was expressed as units of catalase per milligram of proteins in cell lysate ($U \cdot mg^{-1}$) and it was calculated through the equation: $Specific\ activity\ (U/mg\ protein) = (Activity\ (U))/(mg\ protein)$ (equation 4).

2.7.3. Determination of GSH levels

Intracellular GSH content was measured by modification of the protocol described by Tietze (Tietze, 1969). Samples were diluted to 0,03 mg/mL in final volume 400 µL in PBS from the initial protein quantification. A reduced glutathione (GSH) calibration curve in HCl was prepared according to the table 2.5 (Annex 6.2).

Then, 150 µL of each standard and sample was added to 96-well plate (duplicates). Reaction was started by addition of 75µL freshly prepared reaction mix (proportion ratio 1:1:1): 5.4 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent), 0.4U GSH reductase and 0.9 mM NADPH in phosphate buffer 0.1M and absorbance was measured immediately in a plate reader at 405 nm (blank) (Zenyth 3100, Anthos Labtec Instruments, Salzburg, Austria). The reaction was monitored spectrophotometrically after 3 and 5 minutes in a microplate reader (Zenyth 3100, Anthos Labtec Instruments, Salzburg, Austria) at 405 nm. The Ellman's reagent (DTNB) is a colorimetric compound added to the reaction which allows us to quantify the total GSH level. The GSH levels of our samples were calculated through the linear regression of Abs_{405} nm vs [GSH] standards.

2.8. Intracellular ROS measurement

The non-fluorescent probe 2',7, -dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to evaluate H_2O_2 influx. Briefly, H_2DCFDA (Invitrogen, ThermoFisher, Massachusetts, USA) probe is a chemically reduced form of fluorescein and is used as an indicator of ROS production/accumulation in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted in a highly fluorescent 2'7'-dichlorofluorescein (DCF) and its unable to cross the membrane.

Yeast strains were grown overnight in liquid YNB medium with 2% (w/v) glucose with leucine, histidine and tryptophan at 28°C with orbital shaking until OD_{600} = 1. Medium was discarded by centrifugation at 3829 g for 10 minutes (Allegra® 6 Series Centrifuges, Beckman Coulter®, Brea, CA, USA) and the pellet was washed three times with phosphate buffer 0.1 M pH 5 and pH 7.4 and resuspended in the same buffer to a final $OD_{600\ nm}$ = 1.4 in a final volume of 2 mL. Then, 5 µL of H_2DCFDA was added to the cell suspension in a final concentration of 5µM and incubated for 45 minutes at 30°C. After incubation, cells were washed one time with phosphate buffer 0.1 M. Finally, 100 µL of cells were added to a black 96-well plate and treated with several concentrations of H_2O_2 (0.5 – 50mM). Fluorescence intensity was measured in a microplate (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) after 1, 5, 10, 15, 30, 45

and 60 minutes of incubation with H_2O_2 at an excitation/emission of 485/520 nm. The empty vector pUG35 and hAQP-transformed strains non-treated with H_2O_2 were used as control. Cells treated with the H_2O_2 concentrations mentioned above were normalized to control cells and the intracellular ROS accumulation was calculated through the slopes for each concentration along the time.

2.9. Evaluation of effect of nutritional compounds

To evaluate the effect of anti-oxidant compounds in the control (pUG35) and AQP-transformed strains, the procedure was identical to the one explained in material and methods 2.8. Stock solutions of each compound were previously dissolved in dimethyl sulfoxide (DMSO). Control and AQP-transformed strains were incubated 1 hour with a known amount of each compound to a final concentration of: 50 µM naringenin; 50 µM curcumin; 50 µg/mL *Mahonia aquifolim* ethanol extract and 50 µM hesperidin, whose were previously described to have anti-oxidant, anti-inflammatory and anti-proliferative effects. Finally, cells were treated with 10 mM of H_2O_2. Fluorescence intensity was measured in a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) after 1, 5, 10, 15, 30, 45 and 60 minutes of incubation with H_2O_2 at an excitation/emission of 485/520 nm. The empty vector pUG35 and hAQP-transformed strains treated with 10 mM H_2O_2 were used as control. Cells treated with 10 mM of H_2O_2 as well as with the concentrations of each compound mentioned above, were normalized to control cells and the effect of any compound in the intracellular ROS accumulation was calculated through the slopes for each concentration along the time.

3. Results and Discussion

3.1. Detection of AQP cellular localization using GFP as reporter

Green Fluorescent Protein (GFP) was discovered by Shimomura et al. as a complementary protein to aequorin, which is the chemiluminescent protein from *Aequorin* jellyfish. GFP has been the most effective protein in biology thanks to its varied applications. Due to its ability to emit fluorescence in the visible region without requiring cofactors, it has been used as a marker of gene expression levels. Also, this protein has been used as a fusion protein in order to monitor the subcellular localization of proteins of interest, *in vivo* (Tsien, 1998). However, its genetic sequence was modified to improve the protein performance. Cormack and colleagues built a synthetic GFP gene. They optimized codons for translation in *Candida albicans* and incorporated two mutations in the chromophores region increasing the fluorescence emitted by the protein. Thus, an improved GFP was built, known as yEGFP (yeast-enhanced GFP) which can be used as a reporter gene in yeasts, including S. *cerevisiae*, more efficiently (Cormack, Valdivia et al., 1996). For this work, the cloning of the aquaporin coding sequences to create a fusion with the yEGFP gene contained in the expression vector allows a quick observation of aquaporins cell location by fluorescence microscopy. Before proceeding to the assays, it was important to verify the membrane location of the aquaporins. In Figure 3.1, we can observe in YSH 1770 cells transformed with the empty pUG35 plasmid, that the GFP protein is in fact expressed but is homogeneously distributed in the cell cytoplasm. This is because GFP in these cells is not fused to any aquaporin, thus not having membrane signaling.

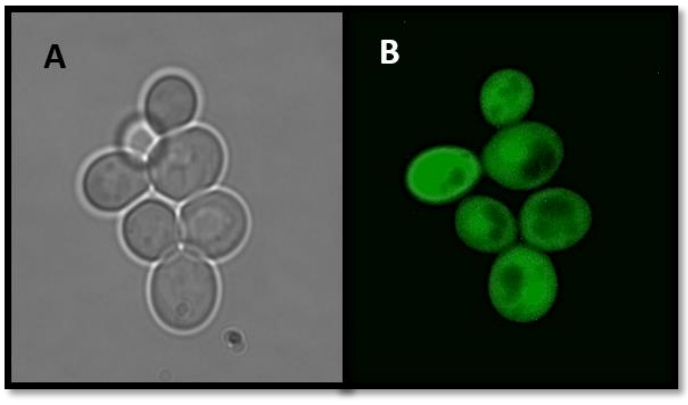

Figure 3.1 – Representative images obtained by visible-light (A) and fluorescence microscopy (B) of YSH 1770 S. cerevisiae cells transformed with the empty plasmid pUG35.

Comparing with cells transformed with the GFP protein-containing plasmid fused on the C-terminal side of the aquaporin sequence, a much narrower and localized cell distribution

is found in the plasma membrane, although expression is also seen in intracellular membranes (Huh, Falvo et al., 2003), possibly at a stage prior to its transport to the plasma membrane (Figure 3.2).

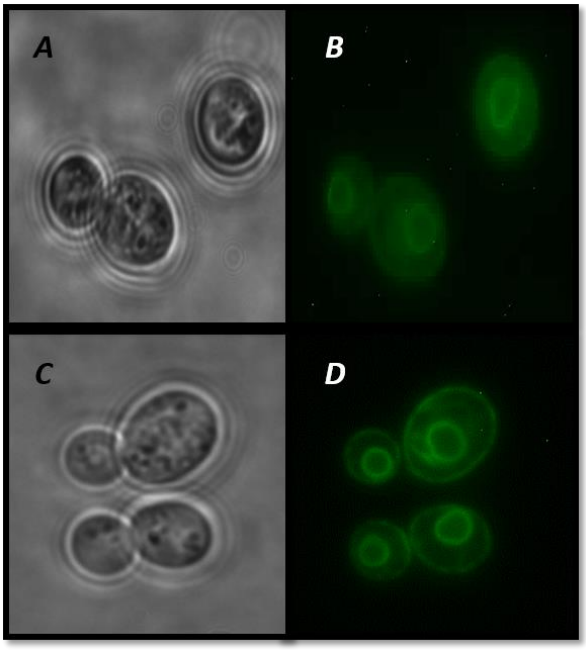

Figure 3.2 – Representative images obtained by visible-light (A and C) and fluorescence microscopy (B and D) of YSH 1770 S. cerevisiae cells transformed with the recombinant plasmid pUG35-hAQP3 (B) and pUG35-hAQP5 (D).

3.2. Functional assays – *Stopped-flow*

To evaluate the AQP-transformed strains functionality in YSH 1770 S. *cerevisiae*, stopped-flow spectroscopy was performed. Aquaporin functional studies using heterologous expression of aquaporins in an aqy-null strain of S. cerevisiae, have been described previously by our group. To perform these assays cells were previously transformed with either the empty plasmid pUG35 (control cells) or the plasmid containing the human AQP3 or the human AQP5 gene (mentioned as hAQP3 and hAQP5, respectively).

The stopped-flow technique allows monitoring the volume change of cells subjected to hypo and hyperosmotic stress. When cells are exposed to a hyperosmotic shock with impermeant solutes, water outflows inducing cell shrinkage. However, if the osmotic shock is performed using a permeable solute as glycerol, cells first shrink due to the water outflow and afterwards swell again due to glycerol entrance. Thus, water and glycerol permeation by cells are evaluated according to cell swelling or shrinkage monitored by 90° light scattering detected in the stopped-flow apparatus. In this study, cells were pre-loaded with carboxifluorescein-

diacetate (cFDA) that is membrane permeable and non-fluorescent, which being intracellularly hydrolyzed yields the non-permeable fluorescent product that stays trapped inside the cell. Thus, the variation of fluorescence intensity after an osmotic shock reflects the cell volume changes. Firstly, to evaluate the effect of external pH on the orthodox AQP5 activity of hAQP5-transformed strain, both control and hAQP5 cells were incubated at two different pH values. Since the physiological pH of yeast's natural environment is acidic (3.5-6.5) (Orij, Postmus et al., 2009), and conditions at which cells show optimal growth and expression and trafficking mechanisms are expected to be completely active, the external pH 5.0 value was chosen to test water permeability. Because a mammalian aquaporin is being expressed, the physiological pH 7.4 value was also used for water permeability measurements. As shown in Figure 3.3 (panel A), cells expressing hAQP5 show a much faster volume change after a hyperosmotic shock for both pH values. In Figure 3.3 (panel B), we can observe the water permeability coefficient Pf of control at pH 5.0 and at pH 7.4 ((0.260 ± 0.007) x 10^{-3} cm s^{-1} and (0.270 ± 0.013) x 10^{-3} cm s^{-1}, respectively) and hAQP5 expressing cells at pH 5.0 and at pH 7.4 ((5.530 ± 0.790) x 10^{-3} cm s^{-1} and (5.340 ± 0.590) x 10^{-3} cm s^{-1}, respectively). The water permeability coefficient was 20-fold (data not shown) higher for hAQP5 cells at both pH 5.0 and pH 7.4 comparing with control cells, suggesting an increase in membrane permeability conferred by hAQP5 expression. These data can also show that there is no difference in water permeability coefficient for cells expressing hAQP5 at pH 5.0 and pH 7.4, indicating that the acidic external pH does not affect hAQP5 activity.

For cells expressing hAQP3, the procedure was the same used for hAQP5. It is known from the literature that AQP3 is regulated by pH (de Almeida, Martins et al., 2016). Both control and hAQP3 expressing cells were incubated at two different pHs. (pH 5.0 and pH 7.4) to induce closed (pH 5.0) and open (pH 7.4) hAQP3-conformations. As shown in Figure 3.3 (panel C), cells expressing hAQP3 show a much faster volume change after a hyperosmotic shock when incubated at pH 7.4, while cells expressing hAQP3 at pH 5.0 show a behavior similar to control cells. Figure 3.3 (panel D) shows the water permeability coefficient Pf of control cells at pH 5.0 and at pH 7.4 ((0.260 ± 0.007) x 10^{-3} cm s^{-1} and (0.270 ± 0.013) x 10^{-3} cm s^{-1}, respectively) and hAQP3 expressing cells at pH 5.0 and at pH 7.4 ((0.266 ± 0.053) x 10^{-3} cm s^{-1} and (0.860 ± 0.049) x 10^{-3} cm s^{-1}, respectively). The water permeability coefficient was 3-fold higher for cells expressing hAQP3 at pH 7.4.

As an hAQP3 is an aquaglyceroporin, is crucial to evaluate its ability to transportglycerol across the cell membrane to validate its functionality. For this experiment, both control (pH 5.0 and pH 7.4) and cells expressing hAQP3 (pH 5.0 and 7.4) were confronted

with a hyperosmotic shock with a glycerol solution. Figure 3.3 (panel E) demonstrate the glycerol signals from stopped-flow. We can observe that at pH 5.0 cells expressing hAQP3 show no permeation to glycerol, however cells expressing hAQP3 at pH 7.4 have a significant increase in the glycerol permeability. The glycerol permeability coefficient Pgly is shown in Figure 3.3 (panel F) for control at pH 5.0 and at pH 7.4 ((0.456 ± 0.054) x 10^{-7} cm s^{-1} and (0.660 ± 0.076) x 10^{-7} cm s^{-1}, respectively) and for hAQP3 expressing cells at pH 5.0 and at pH 7.4 ((0.613 ± 0.048) x 10^{-7} cm s^{-1} and (5.642 ± 0.593) x 10^{-7} cm s^{-1}, respectively). These data confirm the closed and open states of AQP3 at different pH values (de Almeida et al., 2016).

Concluding, both hAQP3 and hAQP5-transformed strains were functional and available to proceed for the next experiments.

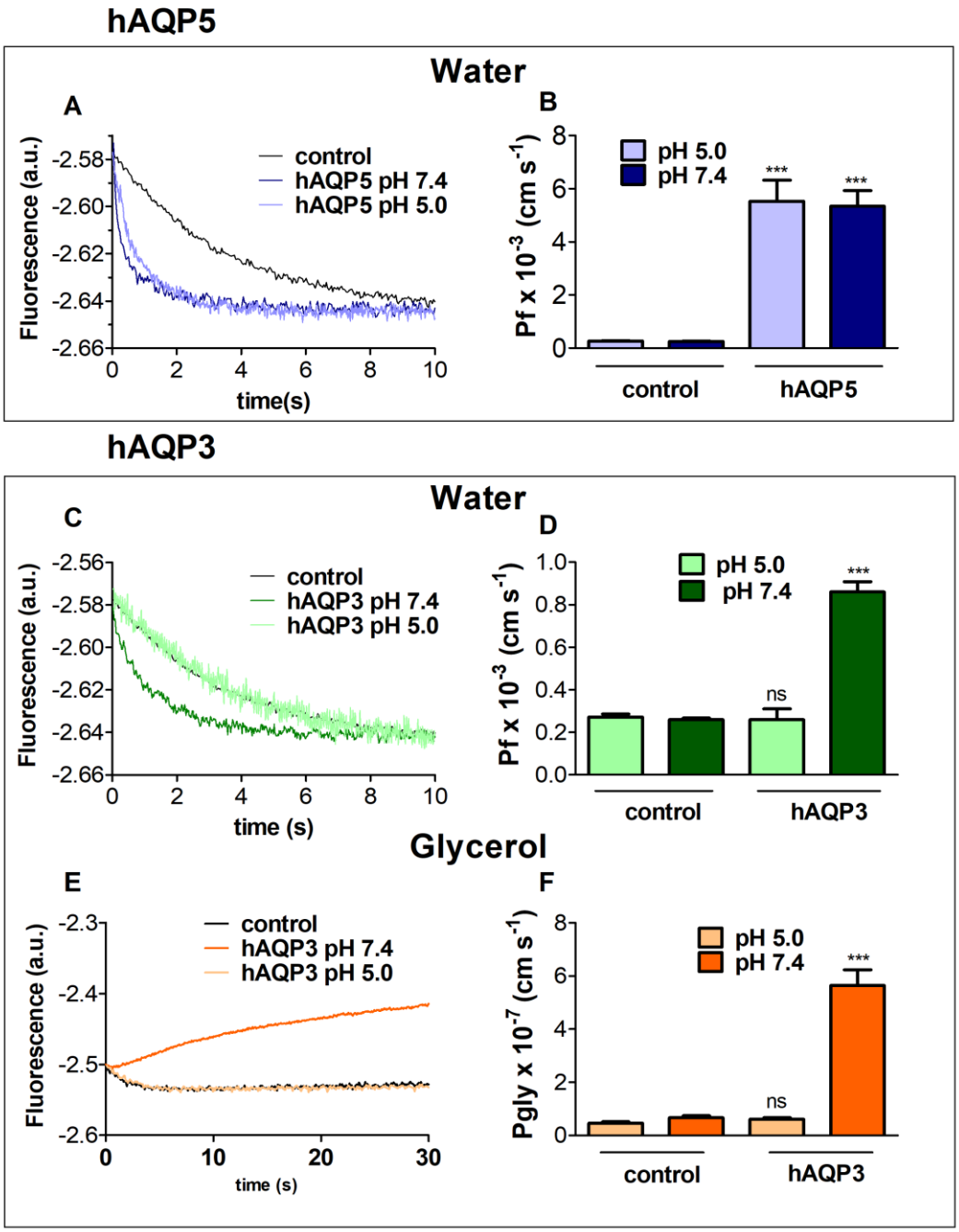

Figure 3.3 – Functional assay. (**A**) Representative time course of the relative cell volume (V/V_0) changes after a hyperosmotic shock with sorbitol solution 2.1M inducing cells shrinkage for control and cells expressing hAQP5 (pH 5 and7.4); (**B**) Water permeability coefficients of control pH 5.0, control pH 7.4, cells expressing hAQP5 pH 5.0 and cells expressing hAQP5 pH 7.4; (**C**) Representative time course of the relative cell volume (V/V_0) changes after a hyperosmotic shock with sorbitol solution 2.1M inducing cells shrinkage for control and cells expressing hAQP3 (pH 5 and7.4); (**D**) Water permeability coefficients of control pH 5.0, control pH 7.4, cells expressing hAQP3 pH 5.0 and cells expressing hAQP3 pH 7.4; (**E**) Representative time course of the relative cell volume (V/V_0) changes after a hyperosmotic shock with glycerol solution 2.1M inducing cells shrinkage for control and cells expressing hAQP3 (pH 5 and7.4). (**F**) Glycerol permeability coefficients of control pH 5.0, control pH 7.4, cells expressing hAQP3 pH 5.0 and cells expressing hAQP3 pH 7.4.

3.3. Growth curves

After performing functional studies to validate water and glycerol permeability by the different AQP-isoforms, we decided to investigate if the growth rate of yeast strains is affected by the AQPs expression as well as by the effect of H_2O_2 on cellular viability. For this, growth curves were established. In accordance with previous studies, AQP3 maximal permeability for both water and glycerol is between pH 6.5 and 7.5 (de Almeida et al., 2016). Thus, we decided to use pH 6.5 not to drastically affect the physiological pH of yeast's natural environment and metabolism. Briefly, cells were grown in YNB with 2% (w/v) glucose supplemented with the required amino acids (leucine, histidine, tryptophan) in the presence and absence of 1 mM of H_2O_2. Figure 3.4 shows a growth curve of hAQP3-transformed strain before and after addition 1 mM to the growth liquid medium. As depicted, there is no difference between control and hAQP3 cells in basal conditions. After addition of 1 mM of H_2O_2 there is a huge increase in the latency phase for both control and hAQP3 cells.

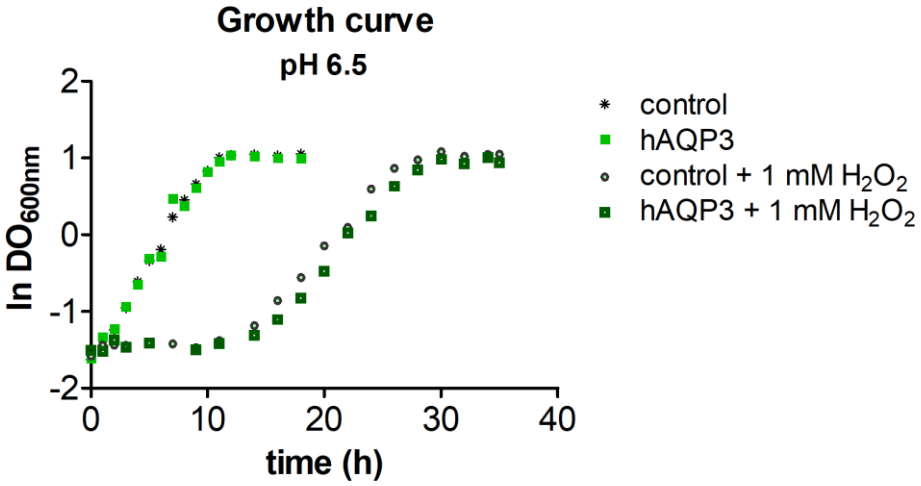

Figure 3.4 - Representative growth curve of control (pUG35) and hAQP3-transformed strains at pH 6.5. (∗) and (■) represents the growth curve of control and hAQP3 in normal growth. (○) and (□) represents the growth curve of control and hAQP3 after addition of 1 mM H_2O_2 to the growth medium. Data are representative of two independent experiments.

In Figure 3.5 is represented the growth rate obtained for control and hAQP3 cells cultured in YNB with 2% (w/v) glucose supplemented with the required amino acids. From the results of pH 5, we can observe that the growth rate of control and hAQP3 cells are very identical ($k = 0.2240 \pm 0.0014\ h^{-1}$ and $k = 0.2334 \pm 0.0011\ h^{-1}$, respectively).

After addition of 1 mM of H_2O_2 to the growth medium we can observe a great decrease in the growth rate for control and hAQP3 ($k = 0.1229 \pm 0.0006\ h^{-1}$ and $k = 0.1235 \pm 0.0011\ h^{-1}$, respectively), although the values are similar (panel A). On the other hand, at pH 6.5 after addition of 1 mM of H_2O_2 to the growth medium, cells expressing hAQP3 have a decrease in

the growth rate comparing to control cells (k = 0.1955 ± 0.0008 h^{-1} and k = 0.1684 ± 0.0011 h^{-1}). These results suggest that hAQP3 is open at 6.5 being responsible for the H_2O_2 uptake leading to changes in cell metabolism, which can affect cell viability and growing.

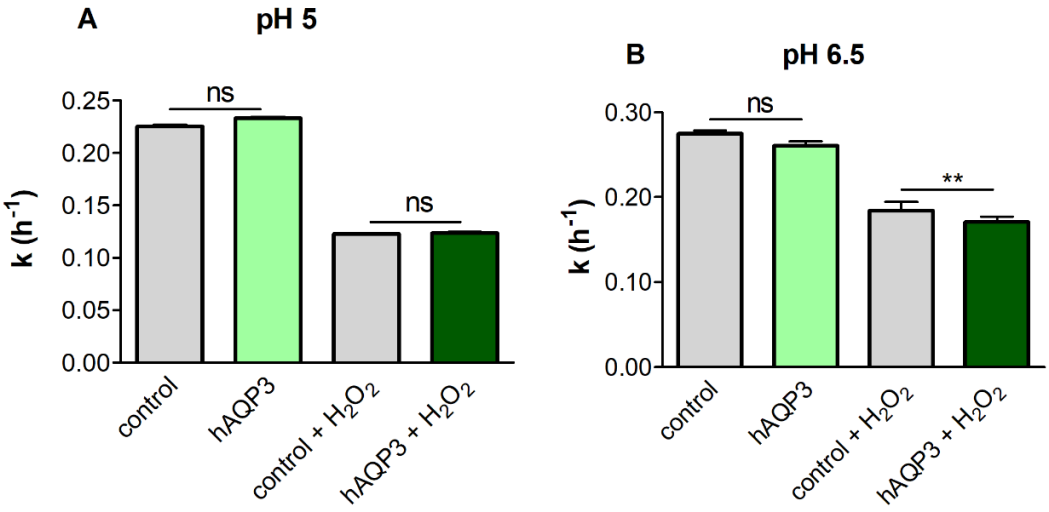

Figure 3.5 - Growth rate of control and hAQP3. (**A**) Growth rate of control (k = 0.2240 ± 0.0014 h^{-1}) and hAQP3 (k = 0.2334 ± 0.0011 h^{-1}); control + H_2O_2 (k = 0.1229 ± 0.0006 h^{-1}) and hAQP3 + H_2O_2 (k = 0.1235 ± 0.0011 h^{-1}) in growth medium at pH 5. (**B**) Growth rate of control (k = 0.2603 ± 0.00075 h^{-1}) and hAQP3 (k = 0.2515 ± 0.00005 h^{-1}); control + H_2O_2 (k = 0.1955 ± 0.0008 h^{-1}) and hAQP3 + H_2O_2 (k = 0.1684 ± 0.0011 h^{-1}) in growth medium at pH 6.5. ns $p > 0.05$; **$p < 0.01$.

3.4. Phenotypic growth assay

The phenotypic growth assay allows a rapid and simple comparative evaluation of the H_2O_2 effect in cell growth and viability of the AQP-transformed strains in the presence of several H_2O_2 concentrations. In addition, this assay can indirectly evidence AQPs as candidates for the transport of H_2O_2 across plasma membrane. The high concentrations of H_2O_2 can lead to metabolic disorders, which may induce reduction or increased growth of yeast cells. Yeast cells exposed to a chronic oxidative stress and the eventual H_2O_2 permeability of the expressed AQP-isoforms may lead to growth inhibition or cell death due to the increased H_2O_2 influx into cells.

Growth sensitivity can indicate a putative role of AQP-isoforms in H_2O_2 transport, since an increase in H_2O_2 level into yeast cells can lead to disruptions in cellular metabolism. The H_2O_2 is a ROS, and due to their reactive nature, ROS can damage proteins, lipids and nucleic acids. In addition, they can initiate several signaling pathways leading to oxidative stress. For this reason, H_2O_2 plays a significant role in cellular processes associated to cell death and growth conditions.

Figure 3.6 shows the results of growth and cell survival of the two AQP-transformed strains recorded after two days of incubation. In general, it was observed an impaired growth in all yeast strains at higher H_2O_2 concentrations. At pH 5, the hAQP3-transformed strain shows an identical behavior compared to control strain for the lowest concentrations (0.25 and 0.75 mM) but for other concentrations (0.5 and 1 to 1.5 mM) we can observe a better survival of cells expressing hAQP3 comparing to control cells this is in accordance with previous results, which demonstrated that hAQP3 is not able to transport at pH 5. However, according to the literature, AQP3 is described as a H_2O_2 transporter (Bienert & Chaumont, 2014; Miller et al., 2010) and it was expected to observe increased sensitivity to H_2O_2 for hAQP3-transformed strains at pH 6.5. Curiously, hAQP3-expressing strain showed tolerance to H_2O_2 and was able to grew similar to the control strain (0.25 to 0.75 mM) but for 1 and 1.5 mM cells expressing hAQP3 were able to survive better than control. According to a work previously developed by our group, rAQP5 showed ability to transport H_2O_2 (Rodrigues et al., 2016). In this work, cells expressing hAQP5 showed higher sensitivity to H_2O_2 at pH 5 and pH 6.5 since the lowest H_2O_2 concentration (0.25 mM). Based on this result, we can consider hAQP5 as a good H_2O_2 transporter.

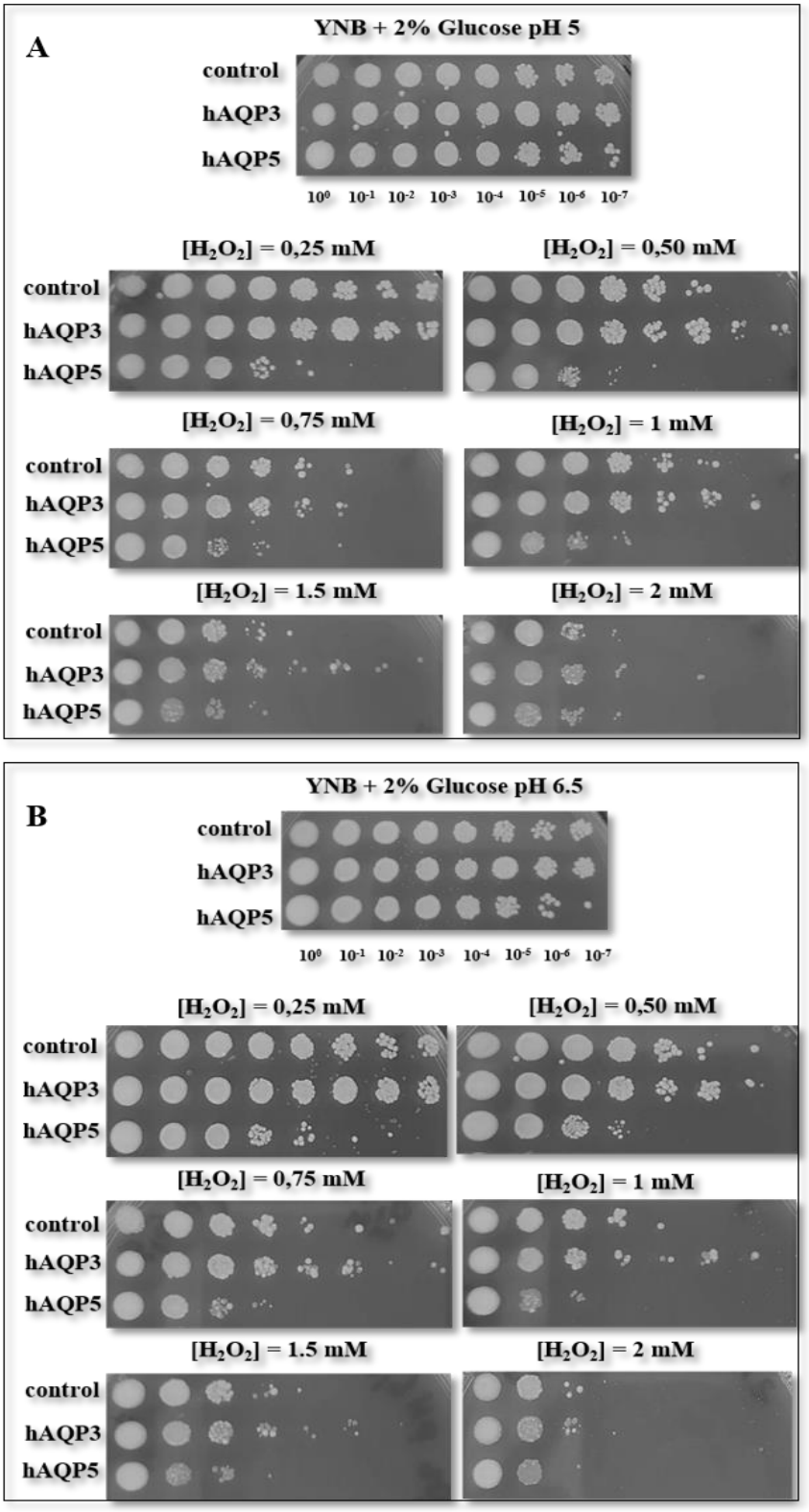

Figure 3.6 - Phenotypic growth assay of S. cerevisiae strains expressing hAQP3 and hAQP5 on solid YNB + 2% glucose medium supplemented with amino acids (leucine, histidine, tryptophan) at pH 5 (**A**) and pH 6.5 **(B)**. Different yeast strains are spotted from the top to the bottom in 10-fold serial dilution (from the left to the right column). AQP-transformed strains are compared to the control (pUG35). Several H_2O_2 were tested (0 to 2 mM). The results were recorded after two days of incubation. Data are representative of two independent experiments

3.5. Colony forming unit assay

After investigating the effect of H_2O_2 in cells exposed to a chronic oxidative stress (two days of incubation), we decided to evaluate the effect of H_2O_2 after a brief period. Cells suspended in liquid medium at different pH values (5.0 and 7.4) were treated with 1 mM H_2O_2 and, after incubation for different time intervals (15 and 60 min), the survivability and capacity of cells to form colonies was evaluated. In Figure 3.7 (panel A) are represented the results of both control and AQP-transformed strains at pH 5.0. Cells expressing hAQP3 at pH 5.0 appear to have a higher percentage of survival after 15 and 60 minutes than control. Interestingly, hAQP5 cells are more sensitive to H_2O_2 than control after 15 min but after a longer period of incubation (60 min) hAQP5 cells survive better and are significantly more resistant to the oxidative stress ($p < 0.001$). For cells incubated at pH 6.5 (panel B) we can observe a different comportment for hAQP3-transformed strain. After 15 minutes of incubation cells survivability is much lower than control probability indicating H_2O_2 uptake by the hAQP3 isoform inducing cell injury and/or death ($p < 0.05$), but after 60 minutes there is an increase of cell survival. Cells expressing hAQP5 have an identical behavior, showing more sensivity to H_2O_2 after 15 minutes. This is effect is reversed after 60 minutes, when cells show to survive significantly better than control ($p < 0.05$) (Figure 3.7)

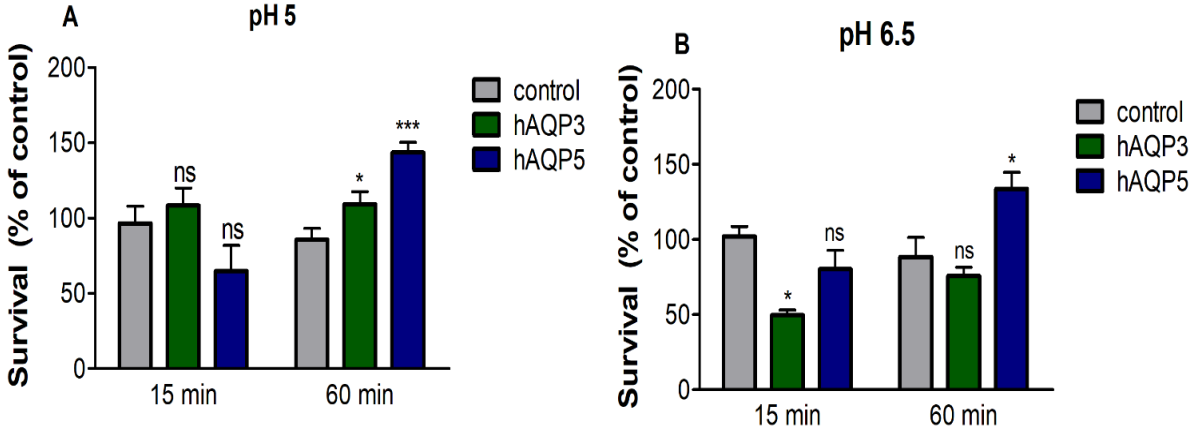

Figure 3.7 - Colony forming units. Time course of cell survival of AQP-transformed yeast strains compared to control in liquid medium after treatment with 1 mM H_2O_2. (A) Growth medium at pH 5; (B) Growth medium at pH 6.5. Percentage survival is expressed relative to untreated controls. Values are mean ± SD of triplicates. Data are representative of four independent experiments. ns, non significant, $p > 0.05$; *$p < 0.05$; *** $p < 0.001$.

3.6. Antioxidant defense system

Catalase and glutathione are known as very important antioxidant defense mechanisms against ROS and free radicals, protecting lipids, DNA and proteins form oxidative modification (Hadwan & Ali, 2017). For this reason, is very important to investigate its levels in our yeast model. Briefly, cells were grown in liquid medium at different pH values (5.0 and 6.5), in basal conditions and incubated with H_2O_2 for 1 hour, and then were harvested by centrifugation Pellet was disrupted mechanically by agitation with glass beads and cleared by centrifugation. Prior to performing the assays, protein concentration of cell lysates was determined according to Bradford using bovine serum albumin as a standard. Figure 3.8 represents the results of catalase activity and total intracellular GSH content.

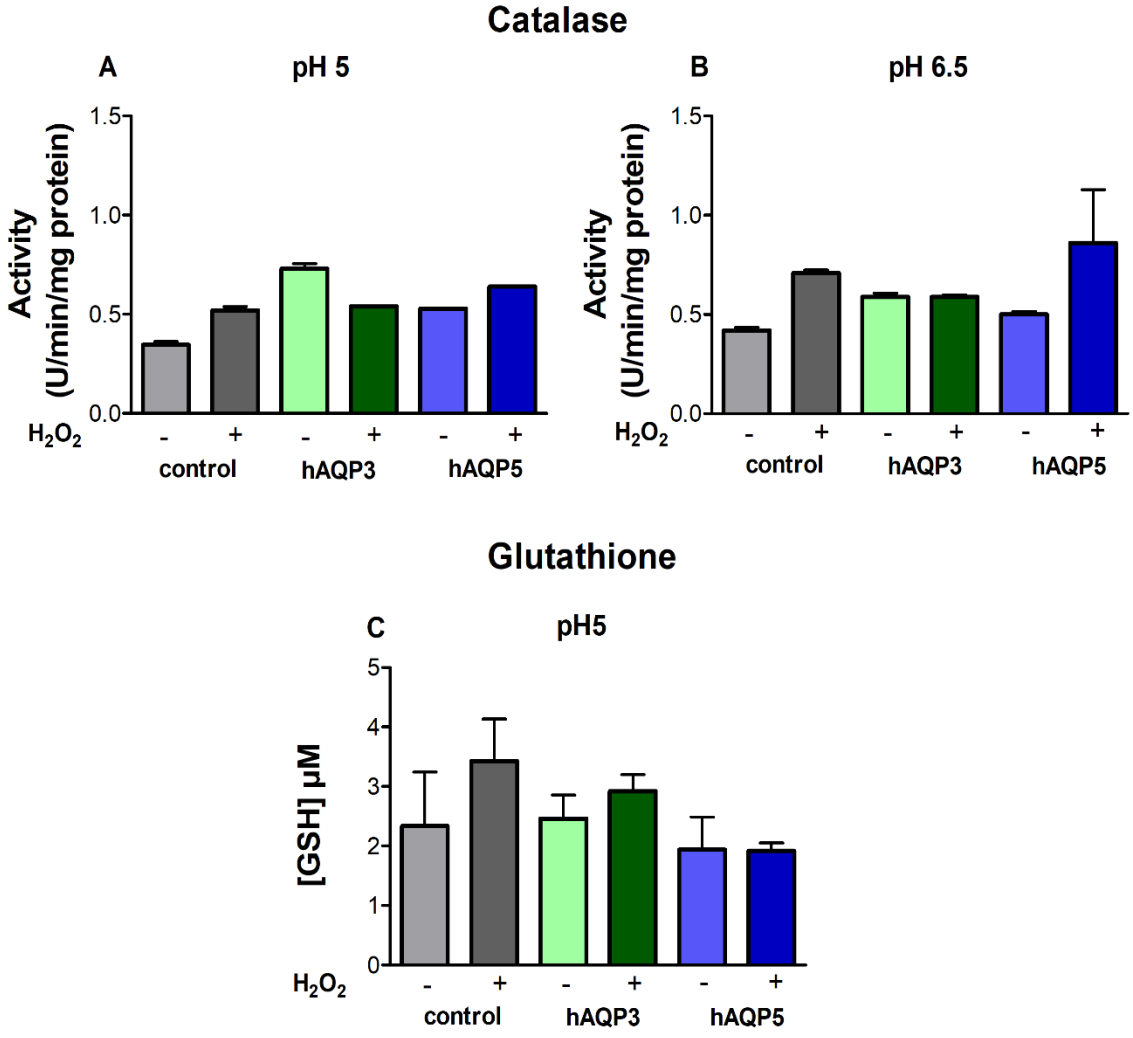

Figure 3.8 - Antioxidant defense system. (A) Catalase activity at pH 5 and (B) Catalase activity at pH 6.5. (C) Total intracellular GSH content of yeast strains. (-) represent cells before H2O2 addition and (+) represent cells after H2O2 addition. Data are representative of two independent experiments. Values are means ± SD of duplicates.

To confirm if the addition of H_2O_2 is affecting the antioxidant defense system of cells we determined the catalase activity and the GSH levels in basal conditions (before addition of H_2O_2) and after addition of 1 mM of H_2O_2. An increase in these two scavengers after addition of a certain amount of H_2O_2 could explain the high resistance of hAQP3 in the phenotypic growth assay and the recovery of cells expressing hAQP5 after one hour in the colony forming unit assay. However, we can observe in the Figure 3.8 that there are no significant differences in control, hAQP3 and hAQP5 strains before and after addition of H_2O_2 indicating that these scavengers are not contributing for the results observed previously.

3.7. Intracellular ROS measurement

To verify if the disappearance of extracellular H_2O_2 was due to cellular uptake through hAQP3 and hAQP5 isoforms rather than extracellular degradation, the intracellular levels of ROS were measured after acute stress with several concentrations of H_2O_2. For this experiment cells were previously incubated with a non-fluorescent probe, H_2DCFDA, which becomes fluorescent and membrane-impermeable after the acetate groups are removed by intracellular esterases and oxidation occurs within the cell.

Both groups of control and hAQP-transformed strains were also incubated at different pH values (5.0 and 7.4) to verify if the uptake of H_2O_2 by the AQPs could be affected by pH. As shown in Figure 3.9, control cells are responding to oxidative stress induced by H_2O_2, which may be explained by basal H_2O_2 membrane lipid diffusion. In Figure 3.9, panel A, it was expected to observe no difference between control and hAQP3-transformed cells because AQP3 is closed at pH 5.0 as shown in previous results. Curiously, we observed ROS accumulation for hAQP3-transformed strain at pH 5.0 higher than control cells. However, at pH 7.4 hAQP3-transformed strain shows a much larger increase in ROS accumulation since the lowest H_2O_2 concentration (0.5 mM) confirming the fact that AQP3 is open at pH 7.4 and probably the H_2O_2 uptake is much faster explaining the large amount of intracellular ROS (Figure 3.9 panel B). As expected, a higher intracellular level of ROS was detected for hAQP5 cells comparing to control cells. In this case we can also observe that the levels of intracellular ROS are similar for both pH values (5.0 and 7.4) confirming the fact that AQP5 acts as a H_2O_2 transporter independently of pH (Figure 3.9 panel C and D).

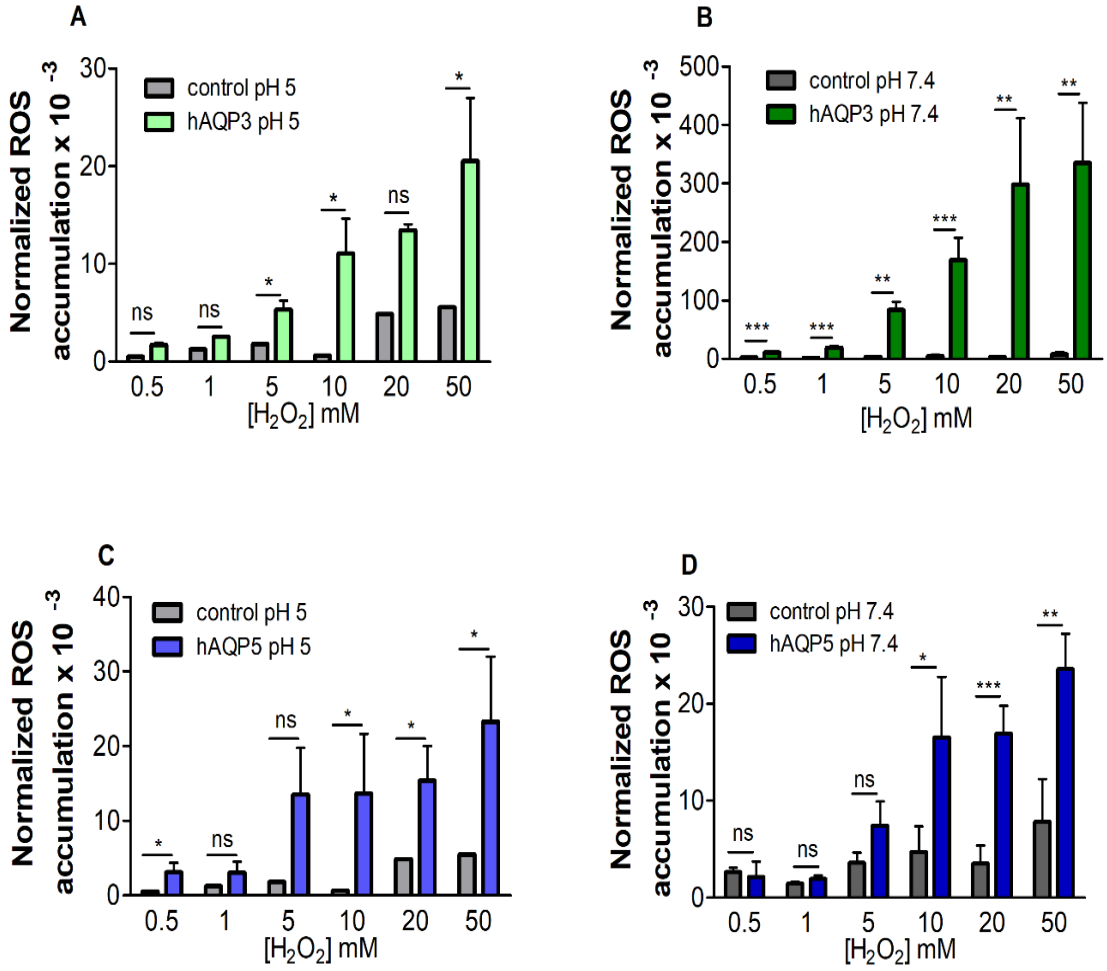

Figure 3.9 - Intracellular ROS accumulation after acute stress induction with several H_2O_2 concentrations (0.5 - 50 mM). (A) hAQP3-transformed strain pH 5 vs. control pH 5. (B) hAQP3-transformed strain vs. control pH 7.4. (C) hAQP5-transformed strain vs. control pH 5. (D) hAQP5-transformed strain pH 7.4 vs. control pH 7.4. Data are representative of five independent experiments. ns, non-significant, $p > 0.05$; *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.8. Effect of nutritional compounds on AQP-mediated oxidative stress

The effect of nutritional compounds was evaluated by the same method used in the previous experiment. Control cells and cells expressing AQPs were incubated with 50µM of each compound for one hour before fluorescence measurements. Immediately after the addition of H_2O_2 the fluorescence was measured for one hour. In the Figure 3.10 we can observe the results of the nutritional compounds in AQP-mediated oxidative stress induced with H_2O_2.

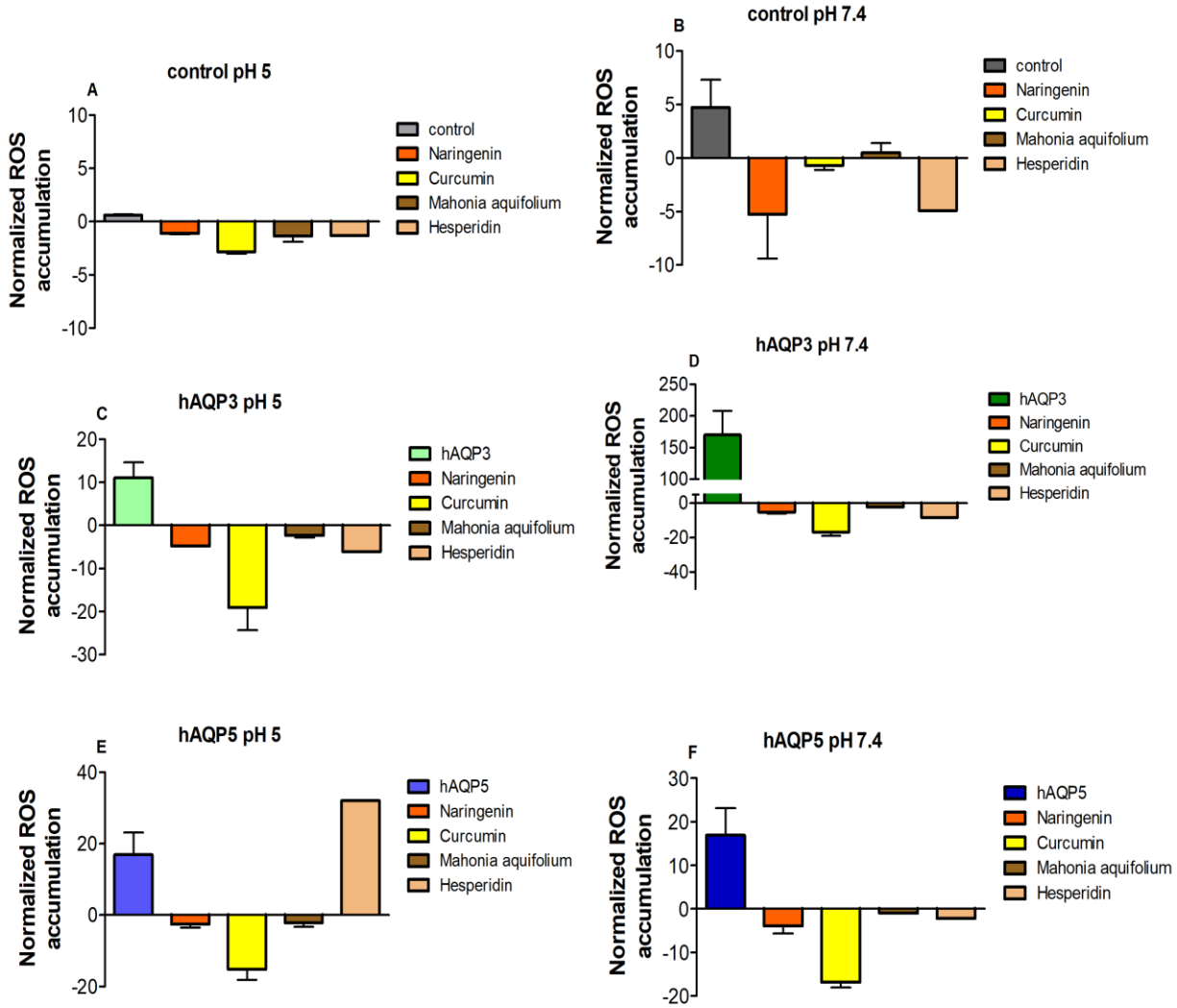

Figure 3.10 – Effect of antioxidant compounds in AQP-mediated oxidative stress. (**A**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the control pUG35 at pH 5. (**B**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the control pUG35 at pH 7.4. (**C**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the hAQP3-transformed strain at pH 5. (**D**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the hAQP3-transformed strain at pH 7.4; (**E**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the hAQP5-transformed strain at pH 5. (**F**) Effect of naringenin, curcumin, *mahonia aquifolium* ethanol extract and hesperidin in the hAQP5-transformed strain at pH 7.4.

Despite these results are still preliminary, we can see in the Figure 3.10 a positive effect of the nutritional compounds tested in this work. In general, we can observe that these natural compounds are acting as ROS scavengers in all the scenarios presented in the Figure. Curcumin is described in the literature as having many beneficial abilities, such as anti-inflammatory, anti-proliferative, anti-angiogenic and mainly antioxidant effects (Tsao, 2010). From the results, curcumin is found to act as the best ROS scavenger in all the AQP-transformed strains.

Recently, naringenin showed capacity to facilitate H_2O_2 elimination through AQPs in HeLa cells (Pellavio, Rui et al., 2017). This evidence increases the idea of the role of AQPs as physiologic modulators of H_2O_2 diffusion as well as the role of the flavonoid naringenin as ROS eliminator. Hesperidin is a flavanone isolated from many fruits and it's highly expressed in the *Citrus* species. As curcumin and naringenin, this flavonoid showed to have anti-inflammatory, anticancer and antioxidant activity (Ahmadi & Shadboorestan, 2016, Ahmadi, Shadboorestan et al., 2015). According to the results, hesperidin seems to be effective as ROS eliminator, however these results are still uncertain and should be confirmed. Finally, we decided to test an extract of M. *aquifolium,* a plant that showed many beneficial effects such as antibacterial, antifungal, anti-inflammatory, anti-proliferative and antioxidant (He & Mu, 2015, Kost`alova et al., 2001, Vollekova et al., 2003, Zeng et al., 2003) and is widely used in Traditional Chinese Medicine for the treatment of many diseases. From the results, we can infer that this plant has some ROS scavenger activity, although in comparison with the polyphenols, its activity is not so well defined.

4. Conclusions

To achieve to overall goal of this project we used a heterologous expression system of S. *cerevisiae* previously developed by our group. The project was divided in three crucial tasks: 1) evaluation of subcellular location and function of both AQP-transformed strains (hAQP3 and hAQP5); 2) evaluation the hAQP-transformed strains sensitivity to H_2O_2 and of the overall antioxidant defense system; 3) measurement of intracellular ROS accumulation by each hAQP-transformed strain and evaluation of the effect of nutritional compounds in the oxidative stress mediated by AQPs.

Due to fusion with the fluorescent protein GFP, we assessed the subcellular location of each strain using fluorescence microscopy. We observed that in the empty plasmid pUG35, the GFP is expressed but resides in the cell cytoplasm. This is because GFP is not fused with any aquaporin, thus not having membrane signaling. On the other hand, we observed that in hAQP3 and hAQP5-transformed strains, where the GFP protein is fused on the C-terminal side of the aquaporin sequence, the subcellular location was in the plasma membrane as well as in intracellular membranes. Then, the function of each strain was assessed and for this purpose the stopped-flow technique was used. The cells were subjected to a hyperosmotic shock with sorbitol solution (water permeability) and glycerol solution (glycerol permeability). We observed that hAQP5 showed a Pf value much higher than control cells indicating that it is functional for water permeability at pH 5 and pH 7.4 and, in case of the aquaglyceroporin hAQP3, we observed that Pf and Pgly values were higher than control cells, indicating that AQP3 is closed at pH 5, but functional for water and glycerol permeability at the physiological pH 7.4 (de Almeida et al., 2016).

The second part of this work consisted in the evaluation of the hAQP-transformed strains sensitivity to H_2O_2. Firstly, we performed the phenotypic growth assay (Sabir et al., 2014) where cells were subjected to a chronic stress for two days at different pH values (5 and 6.5), with several concentrations of H_2O_2. We observed that hAQP5 was sensitive to H_2O_2 since the lowest concentration tested at both pH values. Interestingly, hAQP3 showed tolerance to H_2O_2 at pH 6.5 although we were expected to detect sensitivity once hAQP3 is active at pH 6.5 and hAQP3-expressing strain showed to be affected by the presence of H_2O_2 in the growth curves.

The next step consisted in submitting cells to an acute stress with H_2O_2 for one hour through the colony forming unit assay. In the case of hAQP3, cell sensitivity to H_2O_2 was not observed at pH 5, however at pH 6.5 a significant sensitivity was observed after fifteen minutes of incubation, followed by small cell recuperation. These results can suggest that a prolonged exposure to H_2O_2 may activate signaling pathways responsible for cell survival and could be

related with the results obtained in the phenotypic growth assay. In case of hAQP5 we observed a huge sensitivity after fifteen minutes of incubation, but cells seemed to have a good recovery after sixty minutes at both pH values. Finally, we measured the catalase activity levels and total GSH content in basal conditions and after addition of H_2O_2 to achieve if the addition of H_2O_2 was influencing the antioxidant defense system. We observed that, either catalase activity levels or GSH total content showed no significant differences in basal conditions and after addition of H_2O_2. These data suggest that these ROS scavengers are not influenced by the presence of H_2O_2 and not contributing for the previous results.

In the last part of this work, the intracellular ROS accumulation and the effect of nutritional compounds (curcumin, naringenin, hesperidin and M. *aquifolium extract*) was evaluated using a specific probe for ROS, H_2DCFDA. From the results, we observed a small accumulation of ROS in cells expressing hAQP3 at pH 5. On the other hand, cells expressing hAQP3 at pH 7.4 showed a substantial increase in intracellular ROS accumulation, confirming the fact that hAQP3 is open at the physiological pH 7.4 contributing for a faster and much higher ROS accumulation in cells. In the case of cells expressing hAQP5, the results showed an increase on intracellular ROS after incubation with H_2O_2 with similar levels at both pH values. The values of ROS accumulation in hAQP5 were not as high as the values observed for hAQP3 at pH 7.4, which probably means that AQP5 is less expressed in the plasma membrane or the affinity for H_2O_2 transport is smaller. In all experiments we also observed a small response of control cells to oxidative stress that can be explained by the basal lipid bilayer H_2O_2 diffusion.

In summary, the main goals of this work were successfully achieved, and our results validate the beneficial effects of some nutritional compounds in the oxidative stress mediated by aquaporins:

1- we validated the function of each hAQP-transformed strain, where cells expressing hAQP5 showed to be functional at pH 5 and pH 7.4 and cells expressing hAQP3 showed to be functional at pH 7.4 for water and glycerol and closed at pH 5;
2- we assessed the effect of H_2O_2 on cell growing and viability, where cells expressing hAQP5 showed to have a great sensitivity to H_2O_2 at both pH values and cells expressing AQP3 showed behavior identical to control cells.
3- we found no differences in catalase activity and GSH content levels in basal conditions and after addition of H_2O_2, which may indicate there are no differences between strains is the basal antioxidant defense systems and that it is not affected by H_2O_2;

4- we measured the ROS accumulation in all strains used in this work and verified that hAQP3 at pH 7.4 showed the higher ROS accumulation and hAQP5 was able to accumulate ROS at both pH values but in less quantity comparing to AQP3 at pH 7.4;
5- we assessed the effect of polyphenols in the aquaporin-mediated oxidative stress and verified that some of them could have a potential antioxidant effect acting as ROS scavengers.

In conclusion, we confirmed that the transport of water and glycerol of hAQP3 is regulated by pH and H_2O_2 transport is also regulated by pH. hAQP5 can transport H_2O_2 and this transport is not pH-dependent and curcumin and naringenin stand out as the more promising tested nutritional compounds against AQP-mediated oxidative stress.

More studies are required to further confirm these results, such as: performing the phenotypic growth assays and the colony forming unit assays where the different cells will be incubated with each compound before the experiment; evaluation of catalase activity and GSH total content in the presence of each compound to investigate if these nutritional supplements could by themselves affect the antioxidant defense system in basal conditions as well as in the presence of H_2O_2. Despite the positive effect of the nutritional compounds, more experiments should be performed to obtain better and reproducible results validating the beneficial effects reported in the literature for these polyphenols.

5. References

Aggarwal BB, Kumar A, Bharti AC (2003) Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 23: 363-98

Ahmadi A, Hosseinimehr SJ, Naghshvar F, Hajir E, Ghahremani M (2008) Chemoprotective effects of hesperidin against genotoxicity induced by cyclophosphamide in mice bone marrow cells. Arch Pharm Res 31: 794-7

Ahmadi A, Shadboorestan A (2016) Oxidative stress and cancer; the role of hesperidin, a citrus natural bioflavonoid, as a cancer chemoprotective agent. Nutr Cancer 68: 29-39

Ahmadi A, Shadboorestan A, Nabavi SF, Setzer WN, Nabavi SM (2015) The Role of Hesperidin in Cell Signal Transduction Pathway for the Prevention or Treatment of Cancer. Curr Med Chem 22: 3462-71

Al Ghouleh I, Frazziano G, Rodriguez AI, Csanyi G, Maniar S, St Croix CM, Kelley EE, Egana LA, Song GJ, Bisello A, Lee YJ, Pagano PJ (2013) Aquaporin 1, Nox1, and Ask1 mediate oxidant-induced smooth muscle cell hypertrophy. Cardiovasc Res 97: 134-42

Almasalmeh A, Krenc D, Wu B, Beitz E (2014) Structural determinants of the hydrogen peroxide permeability of aquaporins. FEBS J 281: 647-56

Anderson ME (1998) Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111-112: 1-14

Augustin M, Andrees U, Grimme H, Schopf E, Simon J (1999) Effects of Mahonia aquifolium ointment on the expression of adhesion, proliferation, and activation markers in the skin of patients with psoriasis. Forsch Komplementarmed 6 Suppl 2: 19-21

Bao Y, Han J, Hu FB, Giovannucci EL, Stampfer MJ, Willett WC, Fuchs CS (2013) Association of nut consumption with total and cause-specific mortality. N Engl J Med 369: 2001-11

Bertolotti M, Bestetti S, Garcia-Manteiga JM, Medrano-Fernandez I, Dal Mas A, Malosio ML, Sitia R (2013) Tyrosine kinase signal modulation: a matter of H2O2 membrane permeability? Antioxid Redox Signal 19: 1447-51

Bienert GP, Chaumont F (2014) Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim Biophys Acta 1840: 1596-604

Bienert GP, Moller AL, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 282: 1183-92

Cassidy A, Mukamal KJ, Liu L, Franz M, Eliassen AH, Rimm EB (2013) High anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. Circulation 127: 188-96

Cavia-Saiz M, Busto MD, Pilar-Izquierdo MC, Ortega N, Perez-Mateos M, Muniz P (2010) Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. J Sci Food Agric 90: 1238-44

Checkoway H, Powers K, Smith-Weller T, Franklin GM, Longstreth WT, Jr., Swanson PD (2002) Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. Am J Epidemiol 155: 732-8

Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173: 33-8

Corona G, Deiana M, Incani A, Vauzour D, Dessi MA, Spencer JP (2009) Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. Mol Nutr Food Res 53: 897-903

de Almeida A, Martins AP, Mosca AF, Wijma HJ, Prista C, Soveral G, Casini A (2016) Exploring the gating mechanisms of aquaporin-3: new clues for the design of inhibitors? Mol Biosyst 12: 1564-73

Dorval J, Hontela A (2003) Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (Oncorhynchus mykiss). Toxicol Appl Pharmacol 192: 191-200

Erlank H, Elmann A, Kohen R, Kanner J (2011) Polyphenols activate Nrf2 in astrocytes via H2O2, semiquinones, and quinones. Free Radic Biol Med 51: 2319-27

Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G (2012) Curcumin--from molecule to biological function. Angew Chem Int Ed Engl 51: 5308-32

Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G (2002) Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur J Cancer Prev 11: 351-8

Fini L, Hotchkiss E, Fogliano V, Graziani G, Romano M, De Vol EB, Qin H, Selgrad M, Boland CR, Ricciardiello L (2008) Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. Carcinogenesis 29: 139-46

Fraga CG, Martino VS, Ferraro GE, Coussio JD, Boveris A (1987) Flavonoids as antioxidants evaluated by in vitro and in situ liver chemiluminescence. Biochem Pharmacol 36: 717-20

Fuhrman B, Volkova N, Coleman R, Aviram M (2005) Grape powder polyphenols attenuate atherosclerosis development in apolipoprotein E deficient (E0) mice and reduce macrophage atherogenicity. J Nutr 135: 722-8

Galle K, Muller-Jakic B, Proebstle A, Jurcic K, Bladt S, Wagner H (1994) Analytical and pharmacological studies on Mahonia aquifolium. Phytomedicine 1: 59-62

Golden M (1994) Free radicals and the aetiology of Kwashiorkor. The Biochemist: 12-17

Goth L (1991) A simple method for determination of serum catalase activity and revision of reference range. Clin Chim Acta 196: 143-51

Grynkiewicz G, Slifirski P (2012) Curcumin and curcuminoids in quest for medicinal status. Acta Biochim Pol 59: 201-12

Gulliver WP, Donsky HJ (2005) A report on three recent clinical trials using Mahonia aquifolium 10% topical cream and a review of the worldwide clinical experience with Mahonia aquifolium for the treatment of plaque psoriasis. Am J Ther 12: 398-406

Hadwan MH, Ali SK (2017) New spectrophotometric assay for assessments of catalase activity in biological samples. Anal Biochem 542: 29-33

Halliwell B (1996) Antioxidants in human health and disease. Annu Rev Nutr 16: 33-50

Hallund J, Bugel S, Tholstrup T, Ferrari M, Talbot D, Hall WL, Reimann M, Williams CM, Wiinberg N (2006) Soya isoflavone-enriched cereal bars affect markers of endothelial function in postmenopausal women. Br J Nutr 95: 1120-6

Hara-Chikuma M, Chikuma S, Sugiyama Y, Kabashima K, Verkman AS, Inoue S, Miyachi Y (2012) Chemokine-dependent T cell migration requires aquaporin-3-mediated hydrogen peroxide uptake. J Exp Med 209: 1743-52

Hara-Chikuma M, Verkman AS (2008) Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. Mol Cell Biol 28: 326-32

He, Mu (2015) The medical uses of the genus Mahonia in traditional Chinese medicine: An ethnopharmacological, phytochemical and pharmacological review. J Etnhopharmacol 175

Hiroi M, Ogihara T, Hirano K, Hasegawa M, Morinobu T, Tamai H, Niki E (2005) Regulation of apoptosis by glutathione redox state in PC12 cells exposed simultaneously to iron and ascorbic acid. Free Radic Biol Med 38: 1057-72

Hodgson JM (2006) Effects of tea and tea flavonoids on endothelial function and blood pressure: a brief review. Clin Exp Pharmacol Physiol 33: 838-41

Hu W, Yu L, Wang MH (2011) Antioxidant and antiproliferative properties of water extract from Mahonia bealei (Fort.) Carr. leaves. Food Chem Toxicol 49: 799-806

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003) Global analysis of protein localization in budding yeast. Nature 425: 686-91

Hybertson BM, Gao B, Bose SK, McCord JM (2011) Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. Mol Aspects Med 32: 234-46

Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A (1999) Genetic analysis of glutathione peroxidase in oxidative stress response of Saccharomyces cerevisiae. J Biol Chem 274: 27002-9

Ishibashi K (2009) New members of mammalian aquaporins: AQP10-AQP12. Handb Exp Pharmacol: 251-62

Ishibashi K, Kondo S, Hara S, Morishita Y (2011) The evolutionary aspects of aquaporin family. Am J Physiol Regul Integr Comp Physiol 300: R566-76

Jamieson DJ (1998) Oxidative stress responses of the yeast Saccharomyces cerevisiae. Yeast 14: 1511-27

Jeong YJ, Choi YJ, Kwon HM, Kang SW, Park HS, Lee M, Kang YH (2005) Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids. Br J Nutr 93: 581-91

Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ, Bickford PC (1999) Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 19: 8114-21

Kost`alova D, Kardosova A, Hajnicka V (2001) Effect of Mahonia aquifolium stem bark crude extract and one of its polysaccharid components on production of IL8. Fisioterapia 72: 802-806

Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, Tsubono Y, Tsuji I (2006) Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. JAMA 296: 1255-65

Lisa J, Nica M, Murray W (1999) Antiatherogenic Properties of Naringenin, a Citrus Flavonoid. Cardiovascular Drug Reviews 17: 160-178

Liu RH (2004) Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr 134: 3479S-3485S

Liu WY, Liou SS, Hong TY, Liu IM (2017) Protective Effects of Hesperidin (Citrus Flavonone) on High Glucose Induced Oxidative Stress and Apoptosis in a Cellular Model for Diabetic Retinopathy. Nutrients 9

Ludwig A, Lorenz M, Grimbo N, Steinle F, Meiners S, Bartsch C, Stangl K, Baumann G, Stangl V (2004) The tea flavonoid epigallocatechin-3-gallate reduces cytokine-induced VCAM-1 expression and monocyte adhesion to endothelial cells. Biochem Biophys Res Commun 316: 659-65

Madeira A, Moura TF, Soveral G (2016) Detecting Aquaporin Function and Regulation. Front Chem 4: 3

Mantena SK, Baliga MS, Katiyar SK (2006) Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. Carcinogenesis 27: 1682-91

Marchler G, Schuller C, Adam G, Ruis H (1993) A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J 12: 1997-2003

Miller EW, Dickinson BC, Chang CJ (2010) Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proc Natl Acad Sci U S A 107: 15681-6

Moura TF (2004) Cristalografia e Função de Canais Membranares. 27-37

Muller K, Ziereis K, Gawlik I (1995) The antipsoriatic Mahonia aquifolium and its active constituents; II. Antiproliferative activity against cell growth of human keratinocytes. Planta Med 61: 74-5

Orij R, Postmus J, Ter Beek A, Brul S, Smits GJ (2009) In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in Saccharomyces cerevisiae reveals a relation between intracellular pH and growth. Microbiology 155: 268-78

Patel K, Singh GK, Patel DK (2014) A review on pharmacological and analytical aspects of naringenin. Chin J Integr Med

Pellavio G, Rui M, Caliogna L, Martino E, Gastaldi G, Collina S, Laforenza U (2017) Regulation of Aquaporin Functional Properties Mediated by the Antioxidant Effects of Natural Compounds. Int J Mol Sci 18

Percival SS, Talcott ST, Chin ST, Mallak AC, Lounds-Singleton A, Pettit-Moore J (2006) Neoplastic transformation of BALB/3T3 cells and cell cycle of HL-60 cells are inhibited by mango (Mangifera indica L.) juice and mango juice extracts. J Nutr 136: 1300-4

Piao M, Mori D, Satoh T, Sugita Y, Tokunaga O (2006) Inhibition of endothelial cell proliferation, in vitro angiogenesis, and the down-regulation of cell adhesion-related genes by genistein. Combined with a cDNA microarray analysis. Endothelium 13: 249-66

Priyadarsini KI (2014) The chemistry of curcumin: from extraction to therapeutic agent. Molecules 19: 20091-112

Rodrigues C, Mosca AF, Martins AP, Nobre T, Prista C, Antunes F, Cipak Gasparovic A, Soveral G (2016) Rat Aquaporin-5 Is pH-Gated Induced by Phosphorylation and Is Implicated in Oxidative Stress. Int J Mol Sci 17

Sabir F, Leandro MJ, Martins AP, Loureiro-Dias MC, Moura TF, Soveral G, Prista C (2014) Exploring three PIPs and three TIPs of grapevine for transport of water and atypical substrates through heterologous expression in aqy-null yeast. PLoS One 9: e102087

Scalbert A, Johnson IT, Saltmarsh M (2005) Polyphenols: antioxidants and beyond. Am J Clin Nutr 81: 215S-217S

Scapagnini G, Vasto S, Abraham NG, Caruso C, Zella D, Fabio G (2011) Modulation of Nrf2/ARE pathway by food polyphenols: a nutritional neuroprotective strategy for cognitive and neurodegenerative disorders. Mol Neurobiol 44: 192-201

Schroeter H, Spencer JP, Rice-Evans C, Williams RJ (2001) Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. Biochem J 358: 547-57

Sharma OP (1976) Antioxidant activity of curcumin and related compounds. Biochem Pharmacol 25: 1811-2

Sies H, Berndt C, Jones DP (2017) Oxidative Stress. Annu Rev Biochem 86: 715-748

Singh S, Aggarwal BB (1995) Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 270: 24995-5000

Soveral G, Trincão J, Moura TF (2011) Aquaporin water channels. Canal BQ - Revista da Sociedade Portuguesa de Bioquímica 8: 36-43

Storz G, Christman MF, Sies H, Ames BN (1987) Spontaneous mutagenesis and oxidative damage to DNA in Salmonella typhimurium. Proc Natl Acad Sci U S A 84: 8917-21

Sui H, Han BG, Lee JK, Walian P, Jap BK (2001) Structural basis of water-specific transport through the AQP1 water channel. Nature 414: 872-8

Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27: 502-22

Tsao R (2010) Chemistry and biochemistry of dietary polyphenols. Nutrients 2: 1231-46

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67: 509-44

Verkman AS (2005) More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 118: 3225-32

Verkman AS (2009) Knock-out models reveal new aquaporin functions. Handb Exp Pharmacol: 359-81

Verkman AS (2011) Aquaporins at a glance. J Cell Sci 124: 2107-12

Verkman AS, Anderson MO, Papadopoulos MC (2014) Aquaporins: important but elusive drug targets. Nat Rev Drug Discov 13: 259-77

Vogel HA, Pelletier J (1815) Curcumin - biological and medicinal properties. J Pharma 2: 50

Vollekova A, Kost'alova D, Kettmann V, Toth J (2003) Antifungal activity of Mahonia aquifolium extract and its major protoberberine alkaloids. Phytother Res 17: 834-7

Wang T, Gotoh Y, Jennings MH, Rhoads CA, Aw TY (2000) Lipid hydroperoxide-induced apoptosis in human colonic CaCo-2 cells is associated with an early loss of cellular redox balance. The FASEB Journal 14: 1567-1576

Wang W, Heideman L, Chung CS, Pelling JC, Koehler KJ, Birt DF (2000) Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol Carcinog 28: 102-10

Watanabe S, Moniaga CS, Nielsen S, Hara-Chikuma M (2016) Aquaporin-9 facilitates membrane transport of hydrogen peroxide in mammalian cells. Biochem Biophys Res Commun 471: 191-7

Wilken R, Veena MS, Wang MB, Srivatsan ES (2011) Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer 10: 12

Wong BS, Hsiao YC, Lin TW, Chen KS, Chen PN, Kuo WH, Chu SC, Hsieh YS (2009) The in vitro and in vivo apoptotic effects of Mahonia oiwakensis on human lung cancer cells. Chem Biol Interact 180: 165-74

Zeng X, Lao B, Dong X, Sun X, Dong Y, Sheng G, Fu J (2003) [Study on anti-influenza effect of alkaloids from roots of Mahonia bealei in vitro]. Zhong Yao Cai 26: 29-30

Zern TL, Wood RJ, Greene C, West KL, Liu Y, Aggarwal D, Shachter NS, Fernandez ML (2005) Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. J Nutr 135: 1911-7

Zeuthen T (2001) How water molecules pass through aquaporins. Trends in Biochemical Sciences 26: 77-79

6. Annexes

a. Media compositions

Table 2.2 - Composition of YNB culture medium

YNB medium

Compound	Concentration
Yeast Nitrogen Base	0,67% (w/v)
Glucose	2% (w/v)
Leucine	40 mg/ml
Histidine	20 mg/ml
Tryptophan	15 mg/ml
*Agar**	2% (w/v)

Table 2.3 - Composition of YPD medium

YPD medium

Compound	Concentration
Yeast extract	0,5% (w/v)
Peptone	1% (w/v)
Glucose	2% (w/v)
*Agar**	2% (w/v)

*For solid media

b. Standard curves for colorimetric assays

Table 2.4 - Standard curve for H_2O_2 determination

Sample	**Concentration mM**
1	0
2	4.375
3	8.75
4	17.5
5	35
6	45
7	55
8	65
9	75

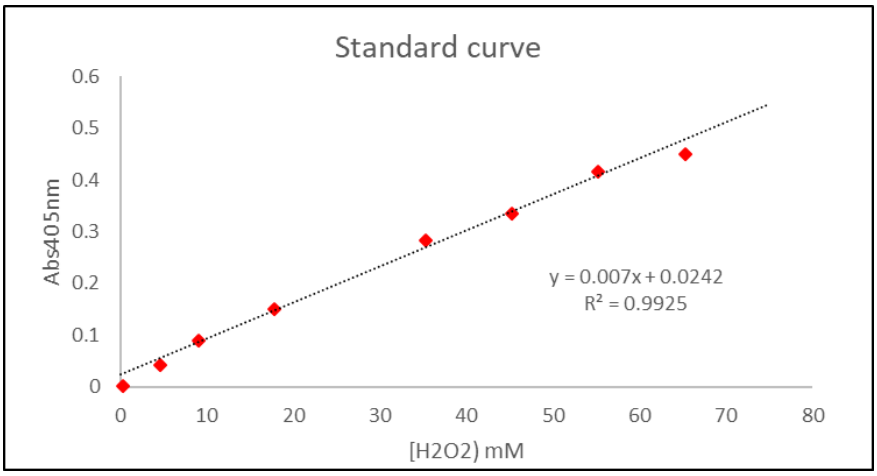

Figure 6.1 – Standard curve for protein quantification for catalase activity determination.

Table 2.5 - Standard curve for GSH quantification.

Sample	Concentration µM
1	0
2	0.1
3	0.25
4	0.5
5	1
6	2
7	5
8	10
9	15
10	20
11	100

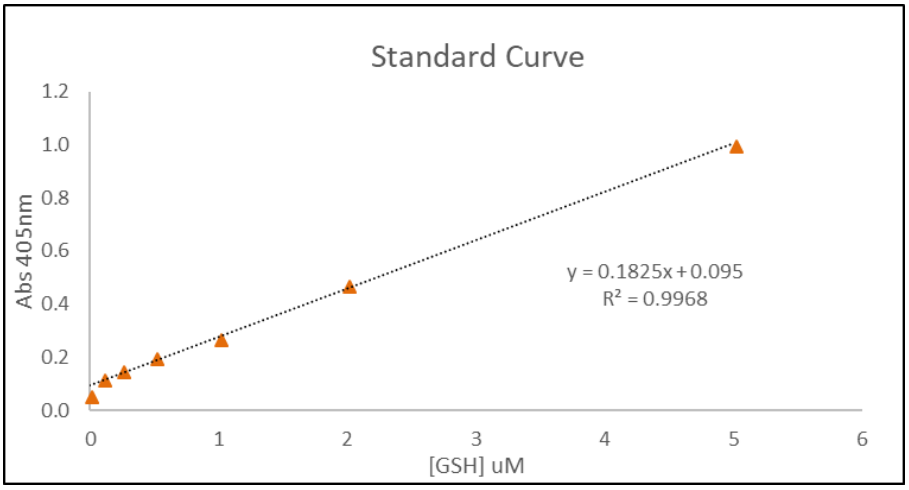

Figure 6.2 – Standard curve for protein quantification for total GSH content determination.