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Ana Cristina Pontes de Carvalho **Hormetic induction of antioxidant defenses for promotion of healthy aging**

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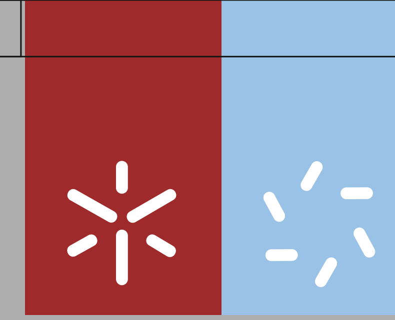

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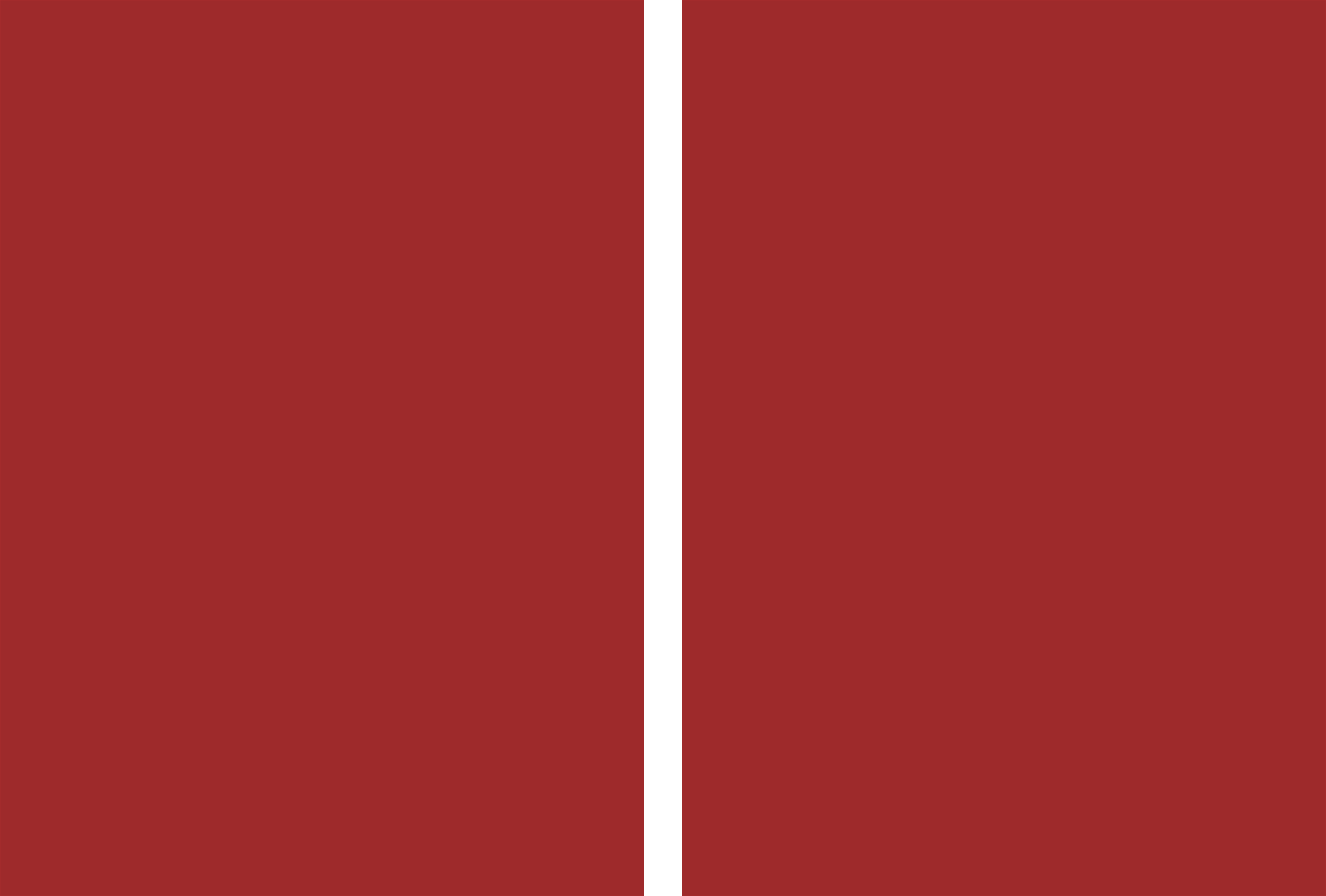

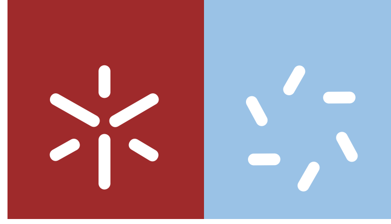

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Hormetic induction of antioxidant defenses for promotion of healthy aging

Tese de Doutoramento
Doutoramento em Biologia Molecular e Ambiental
Especialidade em Biologia Celular e Saúde

Trabalho efetuado sob a orientação da
Professora Doutora Andreia Ferreira de Castro Gomes
e do
Doutor Cristóvão Fernando Macedo Lima

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DE ACORDO COM A LEGISLAÇÃO EM VIGOR, NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA TESE

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"To accomplish great things we must not only act, but also dream; not only plan, but also believe." – Anatole France

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ABSTRACT

People are living longer but often suffer from multiple diseases related to aging which has important social and economic implications. Consumption of fruits and vegetables has been associated with reduced risk of age-related diseases but the scientific basis supporting the strategy to improve health through diet is largely missing. The main objective of this doctoral thesis was to clarify if the hormetic induction of cellular stress responses by phytochemicals, in particular the Nrf2/ARE signaling pathway, can provide anti-aging effects and therefore explain the health beneficial effects of consumption of fruits and vegetables.

In chapter 2, the extensive available literature on curcumin was reviewed. The ancestral use of turmeric in traditional medicine and the extensive research over the last decades indicate that curcumin is an interesting candidate for interventions to achieve healthy aging. Currently, investigations are being conducted to overcome the limitations of the use of curcumin in aging interventions.

In chapter 3, the ability of the phenolic diterpenes carnosic acid (CA) and carnosol (CS) to induce antioxidant defenses in normal human fibroblasts was evaluated and related with anti-aging effects. CA and CS induced antioxidant defenses in human fibroblasts associated with Nrf2 signaling. The stress response elicited by CS conferred a cytoprotective action against a following oxidant challenge with *tert*-butyl hydroperoxide (*t*-BOOH), confirming its hormetic effect. CS also protected against H_2O_2-induced premature senescence and ameliorated several features in cells undergoing replicative senescence *in vitro*.

In chapter 4, the ability of extracts of *Hypericum perforatum* cells (control HP) and of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens* (elicited HP) to protect against oxidative stress induced in HepG2 cells was evaluated. The methanolic extract of elicited HP, in contrast to the one from control HP, significantly inhibited *t*-BOOH-induced cell death, GSH depletion and DNA damage, in pre- and co- incubation regimes. The elicited HP extract significantly induced antioxidant defenses, whereas the extract of control HP did not, and that induction was associated with Nrf2 signaling.

Overall, these findings support that phytochemicals can be viewed as a mean to promote healthy aging. In particular, the hormetic induction of stress responses by CS in normal human cells supports its further development for nutraceutical interventions during aging.

RESUMO

As pessoas vivem mais tempo mas muitas vezes sofrem de múltiplas doenças associadas ao envelhecimento, o que tem importantes implicações sociais e económicas. O consumo de frutas e vegetais tem sido associado a um risco reduzido de doenças relacionadas com o envelhecimento, mas a base científica para a estratégia de melhorar a saúde através da dieta ainda não está devidamente fundamentada. O objetivo principal desta tese de doutoramento foi esclarecer se a indução hormética de respostas celulares de stress por fitoquímicos, em particular através da via de sinalização Nrf2/ARE, tem efeitos anti-envelhecimento e, nesse sentido, poderá explicar os efeitos benéficos para a saúde do consumo de frutas e vegetais.

No capítulo 2, a extensa literatura disponível sobre curcumina foi revista. O uso ancestral de turmérico na medicina tradicional e a extensa investigação nas últimas décadas indicam que a curcumina é um candidato interessante para estratégias que visam alcançar um envelhecimento saudável. Atualmente estão a ser conduzidos estudos para superar as limitações do uso de curcumina em intervenções no envelhecimento.

No capítulo 3, avaliou-se a capacidade dos diterpenos fenólicos ácido carnósico (CA) e carnosol (CS) de induzir defesas antioxidantes em fibroblastos humanos normais, o que foi relacionado com efeitos anti-envelhecimento. Ambos os compostos induziram defesas antioxidantes associadas à ativação da sinalização Nrf2. A resposta ao stress provocada pelo CS conferiu uma ação citoprotetora contra um estímulo tóxico posterior induzido pelo *terc*-butil hidroperóxido (*t*-BOOH), confirmando o seu efeito hormético. O CS protegeu igualmente contra a senescência prematura induzida por H_2O_2 e melhorou várias características das células em senescência replicativa *in vitro*.

No capítulo 4, avaliou-se a capacidade de extratos de células de *Hypericum perforatum* (HP) e de células de *Hypericum perforatum* elicitadas com *Agrobacterium tumefaciens* (HP+AT) em proteger contra o stress oxidativo induzido em células HepG2. O extrato HP+AT inibiu significativamente a morte celular, a depleção de GSH e os danos no DNA induzida pelo *t*-BOOH, nos regimes de pré- e co-incubação. O extrato HP+AT induziu defesas antioxidantes associadas à ativação da sinalização Nrf2.

Em conjunto, estes dados sustentam a visão de que os fitoquímicos podem ser considerados um meio para promover um envelhecimento saudável. Em particular, a indução hormética de respostas de stress pelo CS sustentam a importância de estudos posteriores que visem a sua utilização em intervenções no envelhecimento.

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ABBREVIATIONS

AKT – Protein kinase B (PKB)

AMPK – AMP-activated protein kinase

ANOVA – Analysis of variance

ARE – Antioxidant response element

ASF-2 – Normal diploid adult human skin fibroblasts

AT – *Agrobacterium tumefaciens*

ATCC – American Type Culture Collection

BTB – Broad complex, Tramtrack and Bric-a-brac

bZip – Basic leucine zipper

CA – Carnosic acid

CNC – Cap'n'collar transcription factor family

CPD – Cumulative population doubling

CR – Caloric restriction

CRISPR/Cas9 – CRISPR associated protein 9

CS – Carnosol

Cul3 – Cullin3

DCF – 2',7'-dichlorofluorescin

DCFH$_2$-DA – 2',7'-dichlorodihydrofluorescein diacetate

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNMT – DNA methyltransferase

EGCG – Epigallocatechin gallate

ERK – Extracellular signal–regulated kinase

FBS – Fetal bovine serum

FDA – U.S. Food and Drug Administration

FGF2 – Fibroblast growth factor 2

FTH – Ferritin heavy chain

FTL – Ferritin light chain

G6PD – Glucose-6-phosphate dehydrogenase

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GCLC – Gutamate–cysteine ligase catalytic

GCLM – Glutamate–cysteine ligase modifier

GPX2 – Glutathione peroxidase 2

GSH – Glutathione

GSR – Glutathione reductase

GSSG – Glutathione disulfide

GST – Glutathione S-transferases

H_2O_2 – Hydrogen peroxide

H3K9 – Histone H3 lysine 9

HDAC – Histone deacetylase

HepG2 – Human hepatocellular carcinoma cells

HMOX1 or HO-1 – Heme oxygenase 1

HP – *Hypericum perforatum*

HP1α – Heterochromatin protein 1α

HRP – Horseradish peroxidase

HSF – Heat shock transcription factors

HSP – Heat shock proteins

hTERT – Human telomerase reverse transcriptase

IDH1 – Isocitrate dehydrogenase 1

IFN-γ – Interferon-γ

IGF1 – Insulin-like growth factor 1

IIS – Insulin and IGF-1 signaling

IL – Interleukin

ITP – Interventions Testing Program

IVR – Intervening region

JNK – c-Jun N-terminal kinase

Keap1 – Kelch-like ECH-associated protein 1

LDH – Lactate dehydrogenase

MAPK – Mitogen-activated protein kinase

MARS – Maintenance and repair mechanisms systems

MCP – Monocyte chemoattractant protein

ME1 – Malic enzyme 1

MEM – Minimum essential medium eagle

MFRTA – Mitochondrial free radical theory of aging

MGMT – O^6-methylguanine-DNA methyltransferase

MIP – Macrophage inflammatory protein

mtDNA – Mitochondrial DNA

mTOR – Mechanistic target of rapamycin

mTORC1 – mTOR complex 1

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC – N-acetylcysteine

nDNA – Nuclear DNA

Neh – Nrf2-ECH homologies

NQO1 – Quinone oxidoreductase 1

Nrf2 – Nuclear factor erythroid 2-related factor 2

PGC-1α – PPAR-γ coactivator-1 α

PHGDH – Phosphoglycerate dehydrogenase PHGDH),

PI3K – Phosphatidylinositol 3-kinase

PPAR-γ – Peroxisome proliferator-activated receptor γ

PRDX1 – Peroxiredoxin 1

Rbx1 – RING box protein 1

ROS – Reactive oxygen species

SAHF – Senescence-associated heterochromatic foci

SAMP8 – Senescence-accelerated mouse prone 8

SASP – Senescence-associated secretory phenotype

SA-β-gal – Senescence-associated β-galactosidase

SEN – Senescent phenotype

SIRT – Sirtuin

SOD – Superoxide dismutase

TAME – Targeting Aging with Metformin

***t*-BOOH or *tert*-BOOH** – *tert*-Butyl Hydroperoxide

THC – Tetrahydrocurcumin

TNF-α – Tumor necrosis factor α

TXN – Thioredoxin

TXNRD1 – Thioredoxin reductase 1

UCP2 – Uncoupling protein 2

XCT – Cystine/glutamate transporter

CHAPTER 1

General introduction

CHAPTER 1: General introduction

1. Aging

People have always sought eternal life and everlasting youth and, thus, the attempts to control aging have been part of human culture since early civilizations (de Magalhães, 2014; da Costa *et al.*, 2016). However, one thing that characterizes anti-aging endeavors is their public perception as something simultaneously seductive and desirable yet also transgressive, suspicious, and even dangerous. Aging research was considered an obscure and unappreciated field and the attempts to control human aging were regarded as a questionable pursuit due to: perceptions that their efforts were associated with charlatanic medical practices; that anti-aging was a "forbidden science" ethically and scientifically; and that the field was scientifically devoid of rigor and scientific innovation. Nevertheless, aging research has recently flourished and gained substantial legitimacy within the scientific community and public acceptance (Fishman *et al.*, 2008). Currently, aging research attracts the attention of high-impact journals, such as Cell, Nature, and Science, resulting in a significant increase in publications using the term "aging" (Martin, 2011). Leading scientists in aging have different opinions in respect to the pursuit of an increasingly longer life, but they all agree that it is necessary to discover and develop safe interventions to delay the onset of multiple age-related diseases and prolong healthy lifespan (healthspan) (Warner *et al.*, 2005; Rae *et al.*, 2010; Longo *et al.*, 2015).

1.1. Population aging

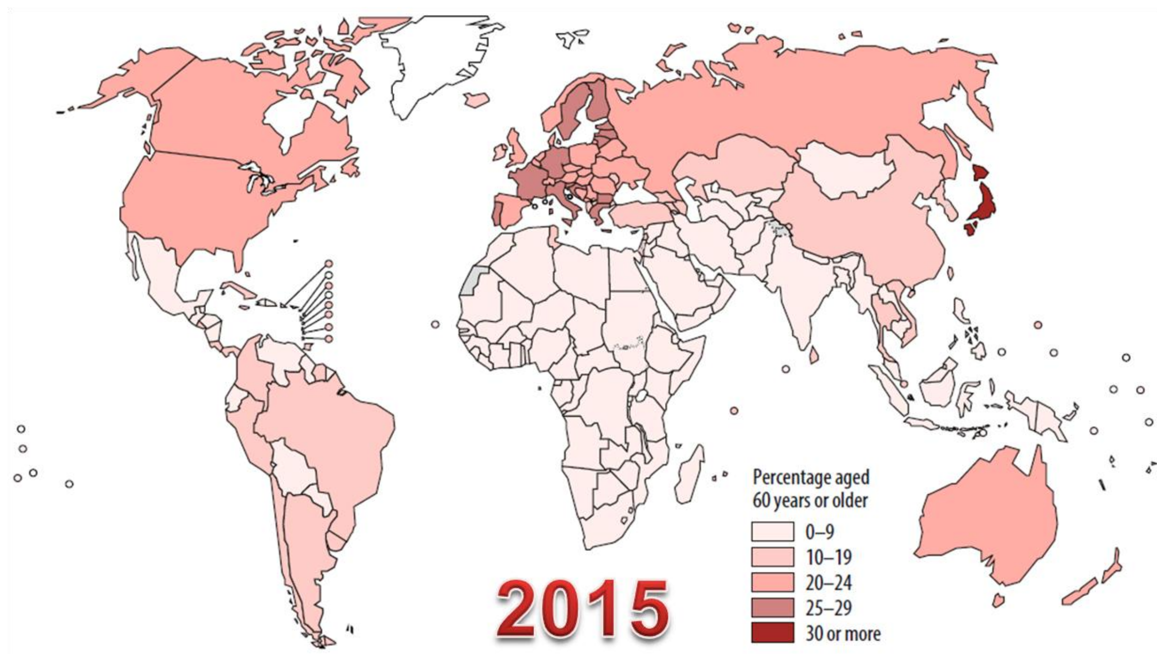
Population aging is one of the most important trends of the 21st century and it is generating serious social, economic and cultural challenges to individuals, families, societies and the global community (United Nations Population Fund and HelpAge International, 2012). It can be defined as the process by which older people become a proportionally larger share of the total population, as a result of declining fertility rates and increasing life expectancy. Greater longevity has been achieved by a combination of improved nutrition, sanitation, medical advances, health care, education and economic well-being, and it is considered one of humanity's greatest achievements (United Nations Population Fund and HelpAge International, 2012; United Nations, 2013; World Health Organization, 2015). Indeed, life expectancy increased 5 years between 2000 and 2015,

and nowadays, for the first time in history, most people can expect to live 60 years or more (World Health Organization, 2016). In accordance with the global trend, life expectancy at birth in Portugal increased from 76,4 to 80,6 between 2000 and 2015 (https://www.pordata.pt). This unprecedented phenomenon is occurring in all regions and in countries at various levels of socioeconomic development. In low- and middle-income countries, life expectancy has been increasing mainly due to large reductions in mortality at younger ages and from infectious diseases. In high-income countries, continuing increases in life expectancy are now mainly due to declining mortality at older ages (United Nations Population Fund and HelpAge International, 2012; United Nations, 2013; World Health Organization, 2015).

Virtually all countries in the world will experience a profound demographic shift in the next decades. In 2015, Japan was the only country where the proportion of people aged 60 years or older exceeded 30% but, in the upcoming decades, many countries (including Portugal) will have a similar proportion of older people (Fig. 1). Between 2015 and 2050, the proportion of the world's population over 60 years is expected to nearly double from 12% to 22% (World Health Organization, 2015).

The opportunities that a socially and economically active, secure and healthy aging population can bring to society are countless (United Nations Population Fund and HelpAge International, 2012). However, the continuous rise in life expectancy has significantly contributed to a higher incidence of chronic diseases in several countries, regardless of income level (United Nations Population Fund and HelpAge International, 2012; United Nations, 2013). Moreover, given that aging is an important risk factor for developing many of the major chronic diseases, including cardiovascular diseases, cancer, chronic respiratory diseases, and diabetes, it is clear that a better understanding of the molecular mechanisms of aging will impact on the prevention, progression, and prognosis of disease and disability (Niccoli and Partridge, 2012; Kirkland, 2013; National Institute on Aging, 2016).

A



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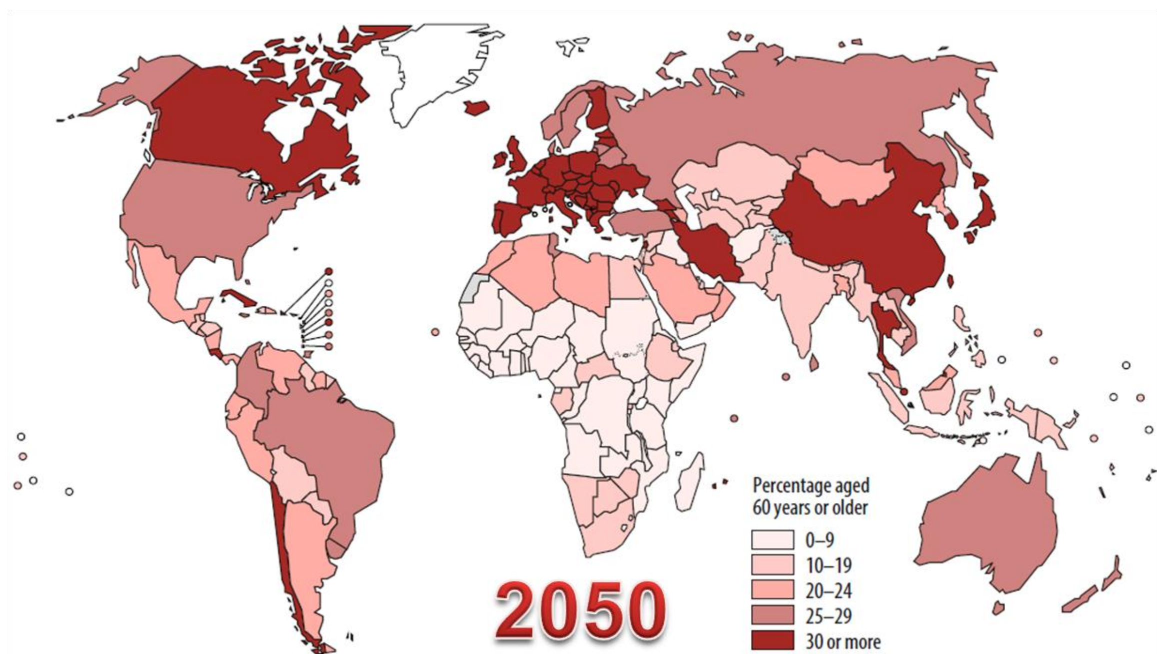

Fig. 1. Proportion of people aged 60 years or older by country in 2015 (A) and projections for 2050 (B). Adapted from (World Health Organization, 2015).

1.2. Biological aging

Aging is a complex and multifactorial biological process that is characterized by a progressive and generalized impairment of function of the organisms, which renders them more vulnerable to disease and ultimately to death. This process appears to be determined by several genetic, epigenetic, and environmental factors but, due to the complexity and multiplicity of mechanisms involved in this process, the full understanding of the aging process is far from being achieved. However, in the last decades, new analytical techniques has allowed a better knowledge of the pathways underlying this process and a deeper understanding of the molecular basis of aging with the ambition of extending human healthspan (Gems and Partridge, 2013; Kennedy *et al.*, 2014).

1.2.1. Theories of aging

Over the years, several theories have been formulated to attempt to explain the aging process. Those theories can be divided into two main categories: programmed and damage/error theories. The programmed theories hypothesize that aging is regulated by biological clocks operating throughout lifespan. This regulation would depend on changes in gene expression that affect the systems responsible for maintenance of homeostasis and for activation of defense responses. In contrast, the damage or error theories consider that aging is caused by environmental insults to living organisms that induce a progressive accumulation of damage at various levels (Weinert and Timiras, 2003; Jin, 2010). None of these theories has been able to explain all facets of aging but, to achieve a better understanding of this phenomenon, it is necessary to consider all the possible factors proposed to have an impact on aging. Thus, some of the most important aging theories are briefly reviewed below.

1.2.1.1. Programmed theories

(1) Programmed longevity theory postulates that aging is the result of the sequential switching of gene expression, with senescence being characterized as the time when age-associated pathologies are manifested. Indeed, the hypothesis that genetic instability is the precipitating factor for aging has withstood many tests and continues to be reaffirmed (Davidovic *et al.*, 2010).

(2) Endocrine theory: proposes that the pace of aging is controlled by biological clocks that act through hormones. Several studies corroborate that aging is

hormonally regulated and that the evolutionarily conserved insulin/ insulin-like growth factor 1 (IGF1) signaling (IIS) pathway plays a key role in the hormonal regulation of aging (van Heemst, 2010).

(3) Immunological theory implies that the immune system is programmed to decline over time, which leads to an increased vulnerability to infectious disease and ultimately to death. Multiple mechanisms contribute to this age-related decline of the immune system, including defects in the haematopoietic bone marrow and defects in peripheral lymphocyte migration, maturation and function. In addition, thymic atrophy is thought to be one of the major contributing factors to the loss of immune function with increasing age (Gruver *et al.*, 2007).

(4) Telomere theory states that the repetitive nucleotide sequences at the ends of chromosomes called telomeres, become progressively shorter with successive cell divisions, resulting in critically short telomeres, altered telomere structure, and eventual replicative senescence. Certain cell types such as stem cells, germ cells, and T lymphocytes express the telomerase enzyme that will either maintain telomere length or delay telomere attrition. However, telomerase activity has been found to be absent in most normal somatic cells and present in cancerous and immortalized cell lines. Moreover, telomere shortening has been implicated in several age-related pathologies (Blackburn *et al.*, 2015).

1.2.1.2. Damage/error theories

(1) Wear and tear theory: vital components of cells and tissues wear out after time, leading to dysfunction, disease and death. This hypothesis is intuitive by comparison with daily observation of inanimate objects, such as cars and clothes, which wear out and become less functional with time (Jin, 2010).

(2) Rate of living theory: energy consumption limits longevity, i.e., a greater metabolic rate determines a shorter lifespan. This theory seems to withstand when smaller species with faster metabolisms (e.g. mice) are compared with larger species with slower metabolisms (e.g. tortoises) (Conti *et al.*, 2006; Jin, 2010).

(3) Cross-linking theory: accumulation of cross-linked molecules damages cells and tissues, resulting in aging. This theory is also referred to as the glycation theory because one of the main ways cross-linking occurs is through a process called glycation. Reducing sugars (e.g. glucose, fructose) react non-enzymatically

with the free amino groups of proteins to form advanced glycation end products, which play an important role in the structural and functional alterations of proteins that occur during aging (Jin, 2010; Gkogkolou and Böhm, 2012).

(4) Free radical theory: Free radicals and oxidants, commonly called reactive oxygen species (ROS), are highly reactive molecules that can damage various cellular components such as nucleic acids, proteins, and lipids. The free radical theory of aging simply argues that aging is caused by accumulation of damage inflicted by ROS. Recent research supports that ROS are key players in the aging process and therefore this subject will be further developed in a forthcoming section (Jin, 2010; Kirkwood and Kowald, 2012).

(5) Somatic mutation theory: genetic mutations occur and accumulate with increasing age, causing the deterioration and malfunction of somatic cells. DNA damage occurs continuously in cells of living organisms and results from either endogenous sources (e.g. hydrolysis, oxidation, alkylation, and mismatch of DNA bases) or exogenous sources (e.g. ionizing radiation, ultraviolet radiation, and various chemicals agents). DNA damage that is not properly repaired can lead to genomic instability, which significantly affects the aging process (Hakem, 2008; Jin, 2010).

1.2.2. Hallmarks of aging

Each theory listed above may explain one or more aspects of aging, but none of them manages to encompass the entire aging phenomena (Jin, 2010). This strict division in too divergent and sometimes exclusive theories is a quite outdated point of view and just a comprehensive and unifying approach can help us to really understand the aging process.

López-Otín and colleagues (2013) attempted to identify and categorize the cellular and molecular hallmarks of aging. They proposed nine candidate hallmarks that contribute to the aging process and together determine the aging phenotype. Each hallmark should ideally fulfill the following criteria: (1) it should manifest during normal aging; (2) its experimental aggravation should accelerate aging; and (3) its experimental amelioration should retard aging and, consequently, increase healthspan. This set of ideal requisites is met to varying degrees by the proposed hallmarks of aging that are: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated

nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Fig. 2) (López-Otín *et al.*, 2013).

The most interesting aspect about this approach for understanding aging is the fact that it considers the hallmarks to be extensively interconnected, implying that amelioration of one particular hallmark may impact on others. The authors classified the hallmarks into three different categories: primary, antagonistic, and integrative hallmarks (Fig. 2). The primary hallmarks – genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis – are considered to be the primary causes of cellular damage. The antagonistic hallmarks – deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence – are considered to be part of compensatory or antagonistic responses to cellular damage. These responses initially mitigate the damage, but eventually, if chronic or exacerbated, they become deleterious themselves. Finally, the integrative hallmarks – stem cell exhaustion and altered intercellular communication – directly impair homeostasis when the process that leads to accumulation of damage becomes irreversible (López-Otín *et al.*, 2013).

Full understanding of aging is far from being attained due to the complexity of the mechanisms involved in this process, but defining hallmarks of aging is an important input to build a framework for future studies on the molecular mechanisms of aging and to design interventions to improve human healthspan (López-Otín *et al.*, 2013).

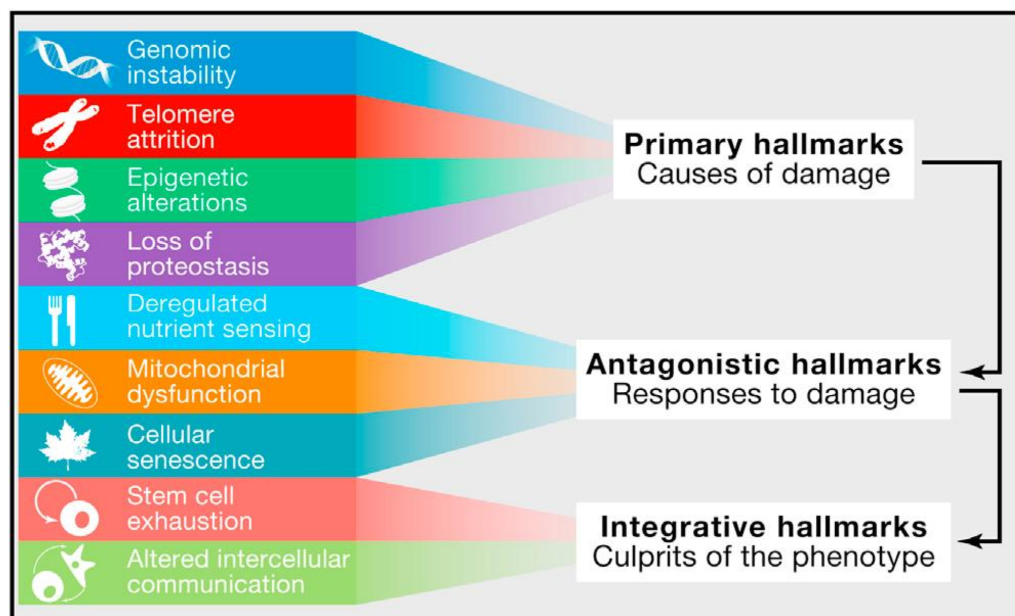

Fig. 2. The hallmarks of aging and their functional interconnections. The proposed nine hallmarks of aging are grouped into three categories: primary, antagonistic, and integrative hallmarks. Reproduced from (López-Otín *et al.*, 2013).

1.2.2.1. Genomic instability

Genomic instability is recognized as one of the main drivers of the aging process (Moskalev *et al.*, 2013). Indeed, genomic DNA is constantly being challenged by endogenous and exogenous threats that undermine its integrity and functionality. Endogenous sources of DNA damage include hydrolysis, oxidation, alkylation, and mismatch of DNA bases; and exogenous sources of DNA damage include ionizing radiation, ultraviolet radiation, and various chemicals agents (Hakem, 2008; Aguilera and García-Muse, 2013). Given the potentially devastating effects of genomic instability, cells have developed versatile complex mechanisms to detect, signal and repair the damaged DNA in a coordinated manner and, thus, maintain genomic integrity (Hakem, 2008; Jackson and Bartek, 2009; Lord and Ashworth, 2012). However, excessive DNA damage or insufficient DNA repair leads to accumulation of DNA damage that plays a crucial role in triggering the aging process (Moskalev *et al.*, 2013; Belancio *et al.*, 2014). DNA damage accumulation has also been implicated in premature aging (progeroid) syndromes, including Hutchinson–Gilford progeria syndrome (Burtner and Kennedy, 2010). Moreover, experimental reinforcement or debilitation of DNA repair mechanisms delays or accelerates aging, respectively (Baker *et al.*, 2013).

Mitochondrial DNA (mtDNA) mutations are also heavily implicated in age-associated diseases and aging (Park and Larsson, 2011; Pinto and Moraes, 2015). In fact, the mtDNA mutation rate is believed to be ten times higher than that of nuclear DNA and multiple factors have been proposed to explain this phenomenon, including the oxidative microenvironment of the mitochondria, the lack of protective histones in the mtDNA, and the limited efficiency of the mtDNA repair mechanisms compared to those of nDNA (López-Otín *et al.*, 2013; Pinto and Moraes, 2015). Evidence supporting that mtDNA damage is important for aging, resulted from studies that showed that mice expressing a deficient mitochondrial DNA polymerase γ accumulate mtDNA mutations and display features of accelerated aging (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Vermulst *et al.*, 2008). However, further studies are necessary to determine whether genetic manipulations that decrease mtDNA mutations are able to extend lifespan.

Genomic instability can also be caused by defects in the nuclear lamina, which is a filamentous structure that participates in genome maintenance by providing a scaffold for tethering chromatin and protein complexes that regulate genomic stability (Gonzalez-

Suarez *et al.*, 2009; Gonzalo, 2014). Alterations of the nuclear lamina and production of an aberrant prelamin A isoform have been detected during normal human aging (Scaffidi and Misteli, 2006; Ragnauth *et al.*, 2010). In addition, mutations in genes encoding protein components of the nuclear lamina or factors affecting their maturation and dynamics cause accelerated aging syndromes such as the Hutchinson-Gilford and the Néstor-Guillermo progeria syndromes (De Sandre-Giovannoli *et al.*, 2003; Eriksson *et al.*, 2003; Cabanillas *et al.*, 2011).

1.2.2.2. Telomere attrition

Telomeres are protective nucleoprotein structures located at the ends of linear chromosomes that have been implicated in the aging process. Telomeres shorten with each cell division because replicative DNA polymerases are unable to fully replicate the ends of linear chromosomes and telomerase, a specialized DNA polymerase capable of replicate telomeres, is not expressed in normal mammalian somatic cells. Eventually, this gradual telomere shortening (attrition) during consecutive rounds of replication leads to critically short telomeres, that induce replicative senescence, the irreversible loss of division potential of somatic cells (Blackburn *et al.*, 2006, 2015; Hewitt *et al.*, 2012).

Telomerase deficiency in humans is associated with premature development of diseases that involve the loss of the regenerative capacity of different tissues, such as pulmonary fibrosis, dyskeratosis congenita and aplastic anemia (Armanios and Blackburn, 2012).

Several studies using genetically modified animal models have demonstrated a correlation between telomere shortening, cellular senescence, and aging. For instance, mice models with shortened or lengthened telomeres exhibit decreased or increased lifespans, respectively (Tomás-Loba *et al.*, 2008; Armanios *et al.*, 2009).

1.2.2.3. Epigenetic alterations

Features of the epigenetic architecture, including DNA methylation, histone modification, and chromatin structure, change during aging (Ford, 2016). Indeed, increased histone H4K16 acetylation, H4K20 or H3K4 trimethylation, and decreased H3K9 methylation or H3K27 trimethylation, constitute age-associated epigenetic marks (Fraga and Esteller, 2007; Han and Brunet, 2012).

The relationship between DNA methylation and aging is highly complex. Early studies described an age-associated global hypomethylation, but subsequent analyses revealed that several loci actually become hypermethylated with age. Mapping of changes in DNA methylation to specific sites has revealed both increased and reduced age-associated local DNA methylation (Maegawa *et al.*, 2010). Cells from patients and mice with progeroid syndromes exhibit DNA methylation patterns similar to those found in normal aging (Osorio *et al.*, 2010). Although aging is strongly correlated with changes in DNA methylation, there is no direct experimental demonstration so far that lifespan can be extended by altering patterns of DNA methylation (López-Otín *et al.*, 2013).

Among the histone modifications that are known to affect the aging process, the most prominent ones are methylation and acetylation of lysine residues (Pal and Tyler, 2016). Indeed, deletion of components of histone methylation complexes (for H3K4 and for H3K27) extends longevity in nematodes and flies, respectively (Greer *et al.*, 2010; Siebold *et al.*, 2010). Sirtuins, a family of NAD^+-dependent protein deacetylases and ADP ribosyltransferases, have been tested for their ability to extend lifespan and healthspan. SIRT6 regulates genomic stability NF-kB signaling, and glucose homeostasis through histone H3 lysine 9 (H3K9) deacetylation (Kawahara *et al.*, 2009; Kanfi *et al.*, 2010; Zhong *et al.*, 2010). Mutant mice deficient or overexpressing *Sirt6*, display accelerated aging or longer lifespan, respectively (Mostoslavsky *et al.*, 2006; Kanfi *et al.*, 2012).

These epigenetic alterations in DNA methylation and histones determine changes in chromatin architecture, such as global heterochromatin loss and redistribution, which constitute characteristic features of aging (Tsurumi and Li, 2012). The relevance of these chromatin alterations in aging is supported by the finding that flies with loss-of-function mutations in heterochromatin protein 1α (HP1α) exhibit a dramatic shortening of lifespan, whereas overexpression of this protein extends longevity (Larson *et al.*, 2012).

Furthermore, gain- and loss-of-function studies have confirmed that transcriptional alterations of miRNAs modulate longevity in flies and worms (Liu *et al.*, 2012; Shen *et al.*, 2012; Smith-Vikos and Slack, 2012).

Epigenetic alterations are reversible hence offering opportunities for the design of novel therapeutic approaches to delay aging and age-related diseases (Pal and Tyler, 2016).

1.2.2.4. Loss of proteostasis

Emerging evidence shows that aging and diverse age-related pathologies are associated with impaired protein homeostasis (proteostasis). Proteostasis involves mechanisms of protein stabilization by molecular chaperones and mechanisms of protein degradation by the lysosome and the proteasome. These mechanisms function in a coordinated manner to restore the structure or to remove and degrade misfolded proteins, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins. Several studies have demonstrated that proteostasis is altered with aging and that chronic expression of aberrant proteins contributes to the development of various age-related pathologies, such as Alzheimer's, Parkinson's, and Huntington's disease (Powers *et al.*, 2009; Koga *et al.*, 2011; Höhn *et al.*, 2017).

Regarding chaperone-mediated protein folding and stability, flies, worms, and mice overexpressing chaperones exhibit an increased longevity (Walker and Lithgow, 2003; Morrow *et al.*, 2004; Swindell *et al.*, 2009), whereas mice deficient in a ubiquitin ligase/cochaperone (carboxyl terminus of Hsp70-interacting protein) exhibit decreased longevity (Min *et al.*, 2008).

Regarding proteolytic systems, induction of autophagy (autophagy-lysosomal system) promotes longevity in yeast, flies, worms, and mice (Eisenberg *et al.*, 2009; Soda *et al.*, 2009); and enhancement of proteasome activity (ubiquitin-proteasome system) extends lifespan in yeast and nematodes (Kruegel *et al.*, 2011; Liu *et al.*, 2011).

1.2.2.5. Deregulated nutrient sensing

Lifespan is regulated by highly conserved nutrient sensing pathways which are controlled by AMP-activated protein kinase (AMPK), insulin/insulin-like growth factor 1 (IGF1), mechanistic target of rapamycin (mTOR), and sirtuins (Haigis and Yankner, 2010; Kenyon, 2010). Several studies have shown that increasing or restricting dietary intake affects the aging process and the onset of several age-related diseases. High nutrient intake shortens lifespan and accelerates age-associated disorders, while moderate nutrient intake extends lifespan and delays or attenuates age-related diseases (Haigis and Sinclair, 2010; Haigis and Yankner, 2010). Moreover, dietary restriction increases lifespan or healthspan in all investigated model organisms, supporting that deregulated nutrient sensing is a relevant characteristic of aging (Fontana *et al.*, 2010).

Insulin and IGF-1 signaling (IIS) pathway was the first nutrient sensing pathway discovered to affect the aging process (Mathew *et al.*, 2017). However, the role of this glucose sensing pathway in longevity remains controversial (van Heemst, 2010). On the one hand, the activity level of this pathway experiences a decline during normal aging, as well as mouse models of premature aging (Schumacher *et al.*, 2008) but, on the other hand, genetic manipulations that decrease the activity of the IIS pathway consistently extend the lifespan of worms, flies, and mice (Fontana *et al.*, 2010; Ortega-Molina *et al.*, 2012; Foukas *et al.*, 2013). These apparently contradictory observations can be reconciled under the hypothesis that the downregulation of the IIS pathway reflects a defensive response that aims reduce the cell growth and metabolism and, thus reduce cellular damage (Garinis *et al.*, 2008). According to this hypothesis, organisms with a constitutively decreased IIS can live longer because they have lower rates of cell growth and metabolism and, therefore, lower rates of cellular damage; and aged organisms decrease IIS pathway in an attempt to extend their lifespan. However, defensive responses against aging may eventually become deleterious and aggravate aging and, as a result, extremely low levels of IIS are incompatible with life (Renner and Carnero, 2009).

Other nutrient sensing systems have been the subject of intense investigation, particularly the mechanistic target of rapamycin (mTOR) that senses amino acid concentrations. Genetic or pharmacological inhibition of mTOR complex 1 (mTORC1) extends lifespan in several model organisms (Harrison *et al.*, 2009; Lamming *et al.*, 2012; Johnson *et al.*, 2013).

IIS and mTOR signal nutrient abundance and anabolism, whereas AMPK and sirtuins signal nutrient scarcity and catabolism. Accordingly, the upregulation of AMPK and sirtuins favors healthy aging (Alers *et al.*, 2012).

1.2.2.6. Mitochondrial dysfunction

Mitochondrial dysfunction has long been associated with aging. The deterioration of the mitochondrial respiratory chain function that is observed during aging leads to decreased ATP generation and increased electron leakage, which increases ROS production and oxidative damage (Green *et al.*, 2011). However, the exact influence of ROS in mammalian aging is debatable. The mitochondrial free radical theory of aging (MFRTA) postulates that the progressive mitochondrial dysfunction that occurs with aging results in increased production of ROS, which in turn induces further mitochondrial

deterioration and cellular damage (Harman, 1965). Multiple data support a role for ROS in aging, but recent developments have forced a profound reassessment of MFRTA (Hekimi *et al*., 2011). Recent results that appear incompatible with the MFRTA have demonstrated that increased ROS production prolongs lifespan in model organisms (Van Raamsdonk and Hekimi, 2009; Mesquita *et al.*, 2010; Schmeisser *et al.*, 2011, 2013) and that antioxidants fail to extend lifespan in model organisms (Doonan *et al.*, 2008; Pérez *et al.*, 2009). These and other data have prompted a reconsideration of the role of ROS in aging (Ristow and Schmeisser, 2011).

Currently, there is increasing evidence indicating that ROS do not only cause oxidative stress, but rather may function as signaling molecules that promote healthspan and ultimately extend lifespan (Sena and Chandel, 2012). High levels of ROS are generally accepted to cause cellular damage and to promote aging, whereas low levels of ROS may rather improve defense mechanisms by inducing an adaptive response. This concept has been denominated mitochondrial hormesis or mitohormesis (Ristow and Schmeisser, 2014).

1.2.2.7. Cellular senescence

Cellular senescence refers to the irreversible growth arrest that occurs when cells are exposed to senescence-causing inducers, including DNA damage, oncogenic mutations, reactive metabolites, high mitogen and nutrient signals; and proteotoxic stress. These inducers activate the $p16^{INK4a}$/Rb, p53/p21, and other pathways that initiate a senescence response (Tchkonia *et al.*, 2013; Loaiza and Demaria, 2016). The senescent phenotype is not limited to an arrest of cell proliferation. In fact, cells undergoing senescence exhibit profound phenotypic alterations, including flattened cellular morphology, increased senescence-associated β-galactosidase (SA-β-gal) activity, and in many instances the appearance of heterochromatin domains known as senescence-associated heterochromatic foci (SAHF). Moreover, senescent cells can secrete multiple pro-inflammatory cytokines, chemokines, and extracellular matrix proteases, which together constitute the senescence-associated secretory phenotype (SASP) (Coppé *et al.*, 2010; Kuilman *et al.*, 2010; Campisi, 2013).

Senescent cells accumulate in multiple tissues and organs over time and have been hypothesized to play a role in aging and aging-related diseases. It is not clear if this accumulation of senescent cells reflect an increase in the rate of generation of senescent

cells and/or a decrease in their rate of clearance (Wang *et al.*, 2009; López-Otín *et al.*, 2013). Nevertheless, recent studies demonstrated that elimination of senescent cells delays aging-associated disorders in progeroid mice and, more importantly, extends medium lifespan and delays aging-associated disorders in non-progeroid mice. This evidence unequivocally establishes that cellular senescence is causally implicated in age-related dysfunction, which suggests that therapeutic interventions to eliminate senescent cells or block their effects may be a novel approach to delay age-related diseases and extend healthspan (Baker *et al.*, 2011, 2016; Zhu *et al.*, 2015) .

1.2.2.8. Stem cell exhaustion

Stem cell exhaustion contributes the functional decline associated with aging and age-related diseases by reducing the regenerative potential of tissues. Stem cell exhaustion may be driven by an imbalance of stem cell quiescence and proliferation (Oh *et al.*, 2014). Indeed, deficient proliferation of stem and progenitor cells is detrimental for the organism, but an excessive proliferation can also be deleterious by accelerating the exhaustion of stem cells pools. Therefore, the ability of stem cells to balance quiescence with proliferative activity is crucial to sustain the stem cell pool and to maintain their regenerative potential, as demonstrated by studies in *Drosophila* intestinal stem cells (Rera *et al.*, 2011), and in hematopoietic stem cells and neural stem cells of p21-null mice (Cheng *et al.*, 2000; Kippin *et al.*, 2005).

Recent studies using both physiological (e.g. heterochronic transplantation and parabiosis) and pharmacological (e.g. rapamycin treatment) approaches suggest that aged stem cell functionality can be rejuvenated, restoring more youthful regenerative potential to aged tissues (Rando and Wyss-Coray, 2014). In particular, transplantation (in which cells derived from a donor of one age are transplanted into a recipient of a different age) of muscle-derived stem cells from young mice to progeroid mice extends lifespan and healthspan (Lavasani *et al.*, 2012). Additionally, parabiosis experiments (in which two mice of different ages are adjoined to create a shared circulatory system, thus exposing cells in one animal to the systemic environment of the other) have demonstrated that the decline in neural and muscle stem cell function in old mice can be reversed by systemic factors from young mice (Conboy *et al.*, 2005; Conboy and Rando, 2012; Goodell and Rando, 2015). Several studies implicate mTOR signaling in stem cell exhaustion and, accordingly, mTOR inhibitors such as rapamycin may improve stem cell function

(Castilho *et al.*, 2009; Chen *et al.*, 2009; Yilmaz *et al.*, 2012). Recent advances suggest that stem cell rejuvenation may reverse the aging phenotype at the organismal level (Rando and Chang, 2012).

1.2.2.9. Altered intercellular communication

Aging involves changes not only at the individual cell level but also at the level of intercellular communications, be it endocrine, neuroendocrine, or neuronal (López-Otín *et al.*, 2013). An important aging-associated change in intercellular communications is inflammaging, which refers to the low-grade, chronic, systemic inflammation that accompanies aging and multiple age-related diseases (Franceschi *et al.*, 2000; Salminen *et al.*, 2012; Franceschi and Campisi, 2014).

In addition to inflammaging, the function of the adaptive immune system declines with age. This immunosenescence may aggravate the aging phenotype at the systemic level due to the failure of the immune system to remove infectious agents, infected cells, and premalignant cells (Gruver *et al.*, 2007; Deeks, 2011; Poland *et al.*, 2014). Furthermore, one of the functions of the immune system is to identify and eliminate senescent cells and hyperploid cells that accumulate in aging tissues and premalignant lesions (Senovilla *et al.*, 2012).

Another example of altered intercellular communication associated with aging is ‘‘contagious aging’’ or bystander effects, in which an aged cell, tissue or organ, leads to aging-specific deterioration of another cell, tissue or organ. For example, continuous exposure to senescent cells induce senescence in intact bystander cells (Nelson *et al.*, 2012).

Fortunately, it is possible to restore the defective intercellular communication underlying the aging process through genetic, nutritional, or pharmacological interventions that may improve the cell-cell communication properties that are lost with aging (Freije and López-Otín, 2012; Rando and Chang, 2012).

2. Aging intervention

As acknowledged in the previous section, aging is characterized by molecular, cellular, and organismal changes that culminate in the inability of an organism to maintain physiological integrity, which leads to impaired function and increased vulnerability to death (López-Otín *et al.*, 2013). Accordingly, the main objective of aging research is to develop interventions that can delay the onset of multiple age-related diseases and prolong healthspan (Longo *et al.*, 2015; Kumar and Lombard, 2016). Over the past decades, remarkable progress has occurred in the science of aging in model organisms. Several studies have demonstrated that cellular signaling pathways modulate healthspan in diverse species across great evolutionary distance and established that aging-related pathways constitute a target for intervention (Fontana *et al.*, 2010; Fontana and Partridge, 2015). Lifespan, and also healthspan, has been reliably modulated by genetic, pharmacologic, and behavioral interventions in multiple model systems (Longo *et al.*, 2015; Barzilai *et al.*, 2016).

In the past decades, hundreds of genes that modulate lifespan have been identified in model organisms. There is interesting evidences that single gene mutations in nutrient sensing pathways, such as IIS or mTOR signaling pathways, can extend lifespan and healthspan in invertebrates and, in some cases, those mutations can extend prolong by almost 10-fold. However, genes that can regulate aging in model organisms cannot be directly applied to humans through genetic manipulations for numerous legal, ethical and technical reasons (de Magalhães *et al.*, 2009, 2012). Several investigators have been suggesting that the technical limitations of gene therapy can be overcome by the CRISPR (clustered regularly interspaced short palindromic repeats) technology. Indeed, the CRISPR/Cas9 (CRISPR associated protein 9) is a revolutionary gene editing technology that enables precise and efficient genomic modifications in human cells and in a wide variety of organisms, including bacteria, yeast, fruit fly, worm, zebrafish, frog, mouse, and rat (Hsu *et al.*, 2014; Guan *et al.*, 2016). Although CRISPR/Cas9 technology has the potential to permanently cure diseases through disrupting disease-causing genes, correcting disease-causing mutations or inserting new protective genes, there are some important issues in this technology (e.g. off-target mutations) that need to be addressed before it can become a practical approach to treating disease (Zhang *et al.*, 2014; Naldini, 2015).

Several pharmacological compounds that target aging-related pathways and processes have demonstrated great promise in the laboratory setting in enhancing the lifespan and healthspan of multiple species, raising the possibility that effective pharmacologic anti-aging therapy in people may be possible. However, screening for novel molecules with anti-aging effects in mammals in an unbiased fashion represents an enormous challenge (de Cabo *et al.*, 2014; Longo *et al.*, 2015; Kumar and Lombard, 2016). Therefore, the NIH developed the NIA-sponsored Interventions Testing Program (ITP) that is a multi-institutional study investigating treatments with the potential to extend lifespan and delay disease and dysfunction in genetically heterogeneous (outbred) mice (https://www.nia.nih.gov/research/dab/interventions-testing-program-itp). Given the promising results in ITP, metformin was chosen to be the subject of a first-of-its-kind clinical trial. Metformin is not necessarily more promising than other drugs that have shown signs of extending life and reducing age-related diseases, but it has been widely and safely used for many years. The goal of this landmark clinical trial is to test the ability of metformin to delay age-associated disease in humans. Indeed, the U.S. Food and Drug Administration (FDA) recently approved the Targeting Aging with Metformin (TAME) for the evaluation of metformin as an anti-aging drug. The TAME project will involve approximately 3000 participants with ages between 70 and 80 years who already have one, two or all of the three conditions: cancer, heart disease or cognitive impairment, or are at risk of developing them. The trial will take place at roughly 15 centers around the United States during 5 to 7 years and will cost approximately $50 million. The success of TAME may determine a paradigm shift, moving from treating individual medical conditions to targeting aging per se, which is expected to facilitate the development of even better pharmacologic approaches that will ultimately reduce the burden of age-related diseases (Barzilai *et al.*, 2016; Kumar and Lombard, 2016).

Most of the health problems of older age are the result of diseases that can be prevented or delayed by adopting healthy behaviors (World Health Organization, 2015). Indeed, caloric restriction (CR) and exercise have been shown to delay these age-related diseases. CR, a reduction in the intake of calories without malnutrition, is the most effective and reproducible intervention known to extend healthspan and/or lifespan in various model organisms, including yeast, worms, flies, rodents, and primates (Lee and Longo, 2016; Mattison *et al.*, 2017). In humans, CR interventions have been shown to lower some risk factors for age-related diseases (Ravussin *et al.*, 2015). However, CR with adequate nutrition is not an option for most people, because it is difficult to practice

and sustain. Moreover, CR with inadequate nutrition can result in some adverse health effects such as osteoporosis, functional disability, infertility, and amenorrhea (Dirks and Leeuwenburgh, 2006; Fontana and Partridge, 2015). Given the disadvantages of CR, more studies have begun investigating the health benefits of alternative dietary interventions that avoid unrealistic levels of self-deprivation (e.g. fasting and fasting mimicking diets) and pharmacological interventions that mimic beneficial effects of DR (e.g. caloric restriction mimetics) (Mercken *et al.*, 2012; Fontana and Partridge, 2015). In lower eukaryotes, fasting regimens extend longevity in part by reprogramming metabolic and stress resistance pathways. The two major forms of fasting are intermittent fasting, which usually refers to a water only or very low calorie period that lasts less than 24 h and is followed by a normal feeding period of one to two days, or periodic fasting, which lasts 2 or more days and is separated by the next cycle by at least one week. In rodents, intermittent or periodic fasting protects against diabetes, cancers, heart disease and neurodegeneration, and help reduce obesity, hypertension, asthma and rheumatoid arthritis in humans (Longo and Mattson, 2014). Fasting mimicking diets have been shown to decrease risk factors/biomarkers of aging, diabetes, cardiovascular disease and cancer without major adverse effects, providing support for their use to promote healthspan (Brandhorst *et al.*, 2015). Thus, fasting has the potential to delay aging and help prevent and treat diseases, but additional studies are needed before fasting-associated interventions can be integrated in standard medical care (Longo and Mattson, 2014; Longo and Panda, 2016). Currently, there is also great interest in CR mimetics, i.e. compounds that provide the beneficial effects of CR without the need for diet limitations. Many research groups are attempting to develop compounds that activate the same metabolic- and stress-response pathways induced by CR, without restriction of food intake, which could have an enormous potential impact for aging intervention. Several CR mimetics have been studied, including resveratrol, rapamycin, and metformin (Mercken *et al.*, 2012; Willcox and Willcox, 2014). Similarly to CR, regular exercise is associated with an improved quality of life (Mercken *et al.*, 2012).

2.1 Aging intervention through hormesis

Several researchers are making legitimate attempts to develop various means of intervention to delay the onset of multiple age-related diseases and prolong healthspan, as discussed above. Another promising approach is that of applying hormesis in aging

research and interventions, which is based on the principle of stimulation of maintenance and repair pathways by repeated exposure to mild stress (Rattan, 2008b, 2010).

Aging is characterized by the progressive accumulation of molecular damage in nucleic acids, proteins, lipids, and carbohydrates, whose main cause is the inefficiency and failure of cellular maintenance and repair mechanisms systems (MARS). The failure of MARS leads to failure of homeodynamics that is the ability of all living systems to respond to internal and external stress, and to counteract by neutralization and/or by adaptation any disturbances threatening their survival. This, in turn, leads to altered cellular functioning, reduced stress tolerance, increased disease incidence and ultimately, death. Therefore, mild stress-induced stimulation of MARS has been increasingly recognized as an important approach for aging intervention (Rattan, 2004, 2005, 2008a). The process in which exposure to a low level stress elicits adaptive beneficial responses that protect against subsequent exposure to severe stress is a phenomenon known as hormesis (Calabrese *et al.*, 2007; Mattson, 2008).

2.1.1. Hormesis: a biphasic dose response

Hormesis is a dose response phenomenon characterized by a low dose stimulation and a high dose inhibition. Graphically it can be represented by a U- or inverted U-shaped dose response, depending on the endpoint measured. If the endpoint is a biological dysfunction such as disease incidence, the dose response would be described as U-shaped, whereas if the endpoint is a normal biological function such as growth or longevity, the dose response would be an inverted U-shape (Fig. 3) (Calabrese, 2004).

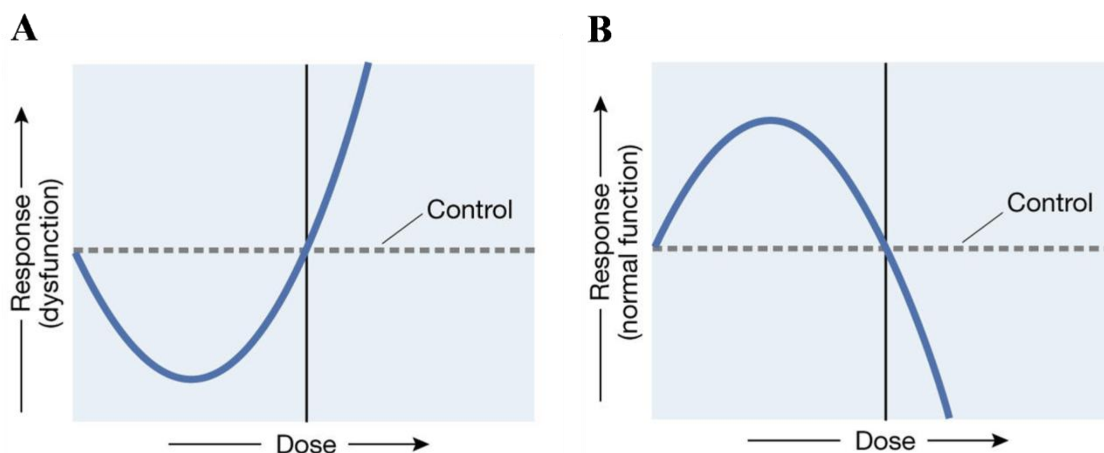

Fig. 3. Dose-response relationships described by (A) the U-shaped hormetic model and (B) the inverted U-shaped hormetic model. Adapted from (Calabrese, 2004).

The occurrence of hormesis was first demonstrated by Schulz (1888) who reported biphasic dose responses in yeast following exposure to a broad range of chemical disinfectant agents. The work of Schulz inspired numerous investigators in diverse fields to assess whether such low dose effects may be a general feature of biological systems. Indeed, similar types of biphasic dose responses were reported by numerous researchers assessing chemicals and radiation, with investigators adopting different names such as the Arndt-Schulz Law, Hueppe's Rule, and other terms to describe these similar dose response phenomena. The term hormesis, from the Greek meaning "to excite", was introduced in the scientific literature by Southam and Ehrlich (1943) who reported that low doses of red cedar extracts enhanced the proliferation of various fungal species but higher doses strongly inhibited proliferation (Calabrese *et al.*, 2010; Cornelius *et al.*, 2013).

Despite considerable research documenting the occurrence of hormetic dose responses during these early years, the inclusion of this concept into drug safety assessment and pharmacological investigations was difficult, mainly because of the following reasons: (1) failure to carry out a more rigorous evaluation in the low dose zone; (2) failure to understand its clinical significance; (3) failure to appreciate the quantitative features of the hormetic dose response; (4) failure to understand the limitations of its agricultural and industrial applications; (5) predominant interest in responses at relatively high doses during most of the 20th century; and (6) inappropriate tendency to associate hormesis with the medical practice of homeopathy. In the last decades, however, there has been a growing interest in hormetic-like biphasic dose responses across the broad spectrum of biomedical sciences. That results from a variety of factors, including the capacity to measure progressively lower doses of chemicals, the adoption of cell culture methods that allow more efficient testing of numerous doses, the need to reconsider the validity of using linear no-threshold model for low dose cancer risk assessment, and the astute observations of numerous independent investigators to generalize their hormetic finding across biological systems (Calabrese *et al.*, 2010; Cornelius *et al.*, 2013).

These research initiatives from highly diverse biomedical areas resulted in the recognition that hormetic dose responses are common and highly generalizable, being independent of biological model, endpoints measured and chemical class and/or physical agent studied. Of further significance were observations that these broad ranging dose response relationships share the same general quantitative features. Particularly, the low

dose stimulation which becomes manifested immediately below the pharmacological and toxicological thresholds is modest in magnitude being at most only about 30–60% greater than the control group response. The width of the hormetic stimulation is usually about 10–20 fold starting immediately from the zero equivalent dose (i.e. estimated threshold) (Fig. 4). The hormetic dose response may result from either a direct stimulation or via an overcompensation stimulatory response following disruption of homeodynamics. Regardless of the mode of action by which the stimulation occurs the quantitative features of hormetic dose responses are similar. These observations are based on abundant data derived from the published literature ranging from plants to humans (Calabrese *et al.*, 2010; Cornelius *et al.*, 2013).

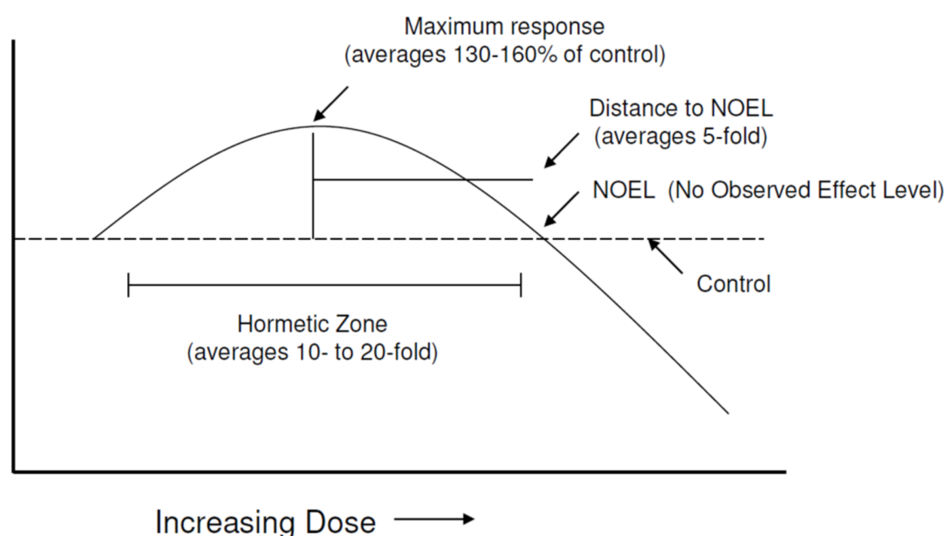

Fig.4. Dose-response curve illustrating the quantitative features of hormesis. Reproduced from (Cornelius *et al.*, 2013).

2.1.2. Hormesis: an adaptive stress response

The main conceptual features of hormesis are the disruption of homeodynamics, the modest overcompensation, the reestablishment of homeodynamics, and the adaptive nature of the process (Rattan, 2010). More specifically, the disruption of homeodynamics caused by a physical, chemical, or biological stressor, leads to a stress response to counteract the disruption. The main molecular stress responses, their potential stressors, and various sensors and effectors are listed in Table 1. The molecular and physiological processes initiated by the stress response are not strictly limited to match the level of disruption, and almost always lead to modest overcompensation. A successful stress response not only results in the reestablishment of homeodynamics, but also strengthens

the homeodynamic space (Rattan, 2010, 2013, 2017). The term homeodynamic space refers to the survival ability of a system. The main characteristics of the homeodynamic space are: (1) effective stress response; (2) damage control and management; and (3) constant remodeling and adaptation. A large number of molecular, cellular and physiological pathways and their interconnected networks, including MARS mentioned above, determine the nature and extent of the homeodynamic space (Rattan, 2017).

Table 1
Major molecular pathways of stress response in human cells. Adapted from (Rattan, 2017).

Stress response	Common stressors	Sensors and effectors
Heat shock response (HSR)	Heat, exercise, heavy metals, natural and synthetic small molecules, antibiotics	Heat shock transcription factors (HSF), heat shock proteins (HSP), proteasome
Unfolded protein response	Unfolded and misfolded proteins, cytokines	Chaperones, chaperonins, HSP, proteasome
Autophagic response	Nutritional limitation, hypoxia, damaged organelles	Autophagosomes, lysosomes
Oxidative stress response	Oxidants, free radicals, reactive oxygen species	Transcription factors (Nrf2, FOXO), heme oxygenase 1, antioxidant enzymes (SOD, catalase)
DNA damage response	Radiation, reactive oxygen species	DNA damage sensors (ATM, ATR), p53, DNA repair proteins
Inflammatory response	Pathogens, allergens, damaged macromolecules	NF-κB transcription factors, cytokines, nitric oxide synthase
Sirtuin-mediated response	Energy depletion, metabolic imbalance	Sirtuins

Considering previous concepts, Rattan (2017) define aging as the progressive shrinkage of the homeodynamic space that leads to an increased zone of vulnerability and to increased probabilities of the onset of aging-related diseases. Hormesis in aging is characterized by the life-supporting beneficial effects resulting from the cellular responses to single or multiple rounds of mild stress (Rattan, 2008a). The homeodynamic ability of a biological system is strengthened in a hormetic zone (H) during mild stress, whereas chronic and severe stress results in the progressive weakening of homeodynamics and an increased zone of disruption (D) leading to functional impairments, diseases and eventual death (Fig.5) (Demirovic and Rattan, 2013). It is noteworthy that although the hormetic zone is usually small, with respect to the dose and the effect, its biological consequences are cumulative, amplified and physiologically significant (Rattan, 2012).

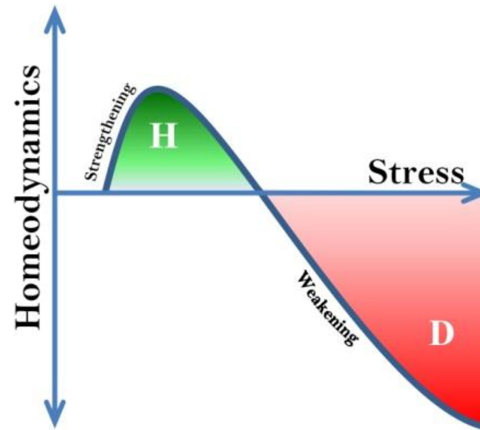

Fig. 5. The homeodynamic ability of a biological system is strengthened in a hormetic zone (H) at low levels of stress by stimulating maintenance and repair processes, whereas chronic and severe stress results in the progressive weakening of homeodynamics and an increased zone of disruption (D), by exhausting the energy sources. Reproduced from (Demirovic and Rattan, 2013).

2.2. Hormetins

Hormetins are conditions that induce hormesis i.e. that bring about health beneficial effects by initially causing molecular damage, which then leads to the activation of one or more stress response pathways and thereby strengthens the homeodynamics. These may be further categorized as: (1) physical hormetins, such as exercise, thermal shock, and irradiation; (2) psychological hormetins such as mental challenge and focused attention or meditation; and (3) nutritional hormetins, such as micronutrients, spices, and other interventions including caloric restriction and fasting (Rattan, 2013, 2015, 2017).

A very important observation in studies of hormesis is that a single hormetin can strengthen the overall homeodynamics of cells by initiating a cascade of processes resulting in a biological amplification of the beneficial effects. Moderate and repeated exercise as a hormetin is the best example of stress-induced hormesis. Exercise initially increases the production of free radicals, acids, and aldehydes, which leads to the activation of a series of stress response pathways, and eventual health beneficial effects are achieved (Radak *et al.*, 2008; Rattan, 2013, 2017).

Various hormetins have been reported to modulate aging and longevity in cells and model organisms, including exercise, heat shock, irradiation, heavy metals, pro-oxidants, acetaldehyde, alcohols, hypergravity, mechanical stretching, electromagnetic field, mental challenge, and food restriction (Rattan, 2013, 2017).

2.2.1. Nutritional hormetins

Nutritional hormetins, especially those of plant origin, have generated much scientific interest for their health beneficial effects. This is because of the realization that not all chemicals found in plants are beneficial for animals in a simple and straightforward manner. Instead, these often cause molecular damage by virtue of their electrochemical properties and have a typical hormetic biphasic dose response (Rattan, 2013). Although the exact nature of the initial molecular damage caused by such compounds may not be easily identified, an activation of one or more stress responses, as listed in the Table 1, is a good indicator of the primary action of the compound.

For example, the oxidative stress response by the activation of Nrf2 transcription factor follows the electrophilic modification/damage of its inhibitor protein Keap1, which then leads to the accumulation, nuclear translocation, heterodimerization, and binding of Nrf2 to ARE, resulting in the downstream expression of a large number of cytoprotective genes (Surh *et al.*, 2008). Nrf2-mediated oxidative stress response has been shown to be induced by several phytochemicals (e.g. carnosol, sulforaphane, and curcumin) and plant extracts (e.g. coffee, broccoli, turmeric, rosemary, thyme, clove, and oregano) (Balstad *et al.*, 2011). It has been suggested that screening for other inducers of Nrf2-mediated oxidative stress response in natural compounds isolated from nutritional sources, in synthetic compounds with nutritional utility, and in complex and multiple food extracts will discover novel hormetins useful for healthy aging and longevity (Rattan, 2013).

2.3. Nrf2-mediated stress response pathway

Nrf2 (Nuclear factor erythroid 2-related factor 2) is a member of the cap'n'collar transcription factor family (CNC) family of basic leucine zipper (bZip) transcription factors (Itoh et al., 2004; Kobayashi and Yamamoto, 2006; Surh *et al.*, 2008). Nrf2 consists of 589 amino acids and contains six evolutionarily highly conserved domains known as Nrf2-ECH homologies (Neh), designated as Neh1-6 (Fig. 6A). Each Neh domain has its own function. The N-terminal Neh2 domain includes two highly conserved peptide sequences: the lower-affinity DLG motif and the high-affinity ETGE, which are required for the interaction with Keap1 (Kelch-like ECH-associated protein 1), and a hydrophilic region of lysine residues (7 K), which are indispensable for the Keap1-dependent polyubiquitination and degradation of Nrf2. Neh4, Neh5, and the C-terminal Neh3 domains are important for the transactivation activity of Nrf2. Neh6 domain is a

serine-rich region that controls Nrf2 stability in a Keap1-independent manner. Neh1 contains a CNC-type bZip motif, a basic region – leucine zipper (L-Zip) structure, where the basic region is responsible for DNA recognition and the L-Zip mediates dimerization with other transcription factors (Jaramillo and Zhang, 2013; Kansanen *et al.*, 2013). Recently, a seventh Neh domain – Neh7 – was described, however its function has not yet been completely clarified (Wang *et al.*, 2013; Canning *et al.*, 2015).

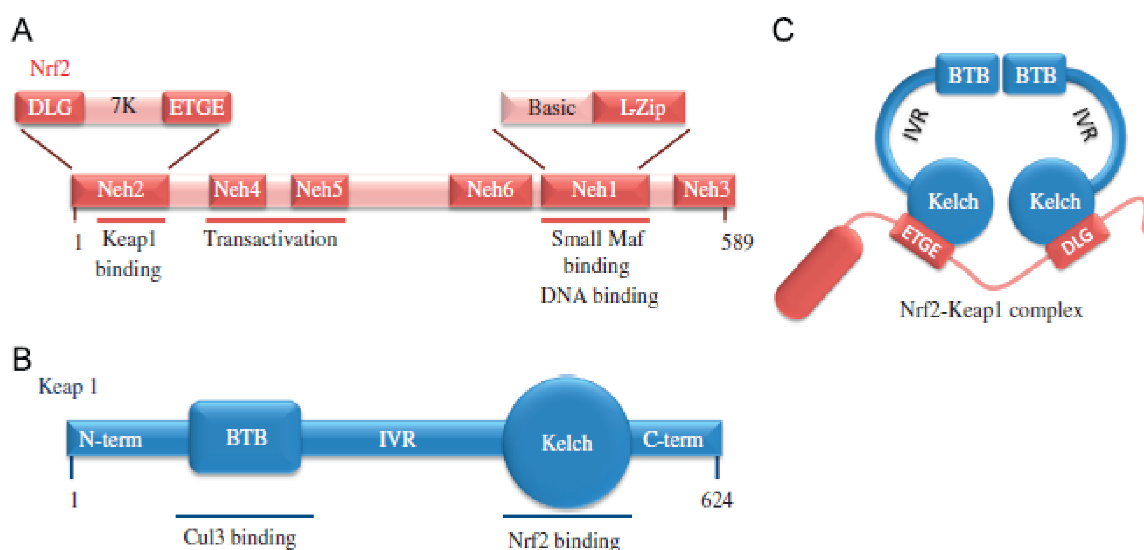

Fig. 6. Schematic structures of Nrf2 and Keap1. (A) Nrf2 consists of 589 amino acids and has six domains: Neh1–Neh6. (B) Keap1 consists of 624 amino acids and has five domains: N-terminal, BTB, IVR, Kelch and C-terminal. (C) Nrf2 interacts with two molecules of Keap1 through its Neh2 domain. Both ETGE and DLG bind to similar sites on the bottom surface of the Keap1 Kelch domain. Adapted from (Kansanen *et al.*, 2013).

Nrf2 regulates both constitutive (or basal) and inducible expression of hundreds of target genes, which suggests that its activity is controlled by a tightly regulated pathway (Nguyen *et al.*, 2005).

Under basal or unstressed conditions, Nrf2 is sequestered in the cytosol by Keap1, a repressor protein that promotes the ubiquitination and proteasomal degradation of Nrf2. Keap1 consists of 624 amino acids and has five domains (Fig. 6B). The two protein-interacting domains, the BTB (Broad complex, Tramtrack and Bric-a-brac) domain and the Kelch domain, are separated by the intervening region (IVR). The BTB domain together with the N-terminal portion of the IVR mediates homodimerization of Keap1 and binding with Cullin3 (Cul3). The Kelch domain and the C-terminal region mediate the interaction with the Neh2 domain of Nrf2 (Fig. 6C). Keap1 associates with Cul3 and Rbx1 (RING box protein 1) to form a functional E3 ubiquitin ligase complex that

promotes Nrf2 ubiquitination and subsequent recognition and degradation by the 26S proteasome, resulting in basal expression of Nrf2 target genes (Fig. 7A and B).

Under oxidative or electrophilic stress conditions, inducers can modify critical cysteine residues (e.g. Cys151 Cys273 and Cys288) of Keap1, which results in conformational changes in Keap1, leading to stabilization and nuclear translocation of Nrf2 and subsequent target gene expression. The exact mechanism through which Keap1 modifications contribute to Nrf2 stabilization is not known but two models, not mutually exclusive, have been proposed: the hinge and latch model and the Keap1-Cul3 dissociation model. In the hinge and latch model, conformational changes in Keap1 disrupt the interaction between the DLG domain of Nrf2 and the Kelch domain of Keap1, thus inhibiting Nrf2 ubiquitination (Fig. 7D), whereas in the Keap1-Cul3 dissociation model, conformational changes in Keap1 disrupt binding of Keap1 and Cul3, leading to the escape of Nrf2 from the ubiquitination system (Fig. 7E). In both of the models, Keap1 becomes saturated with Nrf2 that is no longer targeted for degradation and newly synthesized Nrf2 accumulates in the cytosol and, consequently, Nrf2 translocates to the nucleus (Fig. 7F) (Kansanen *et al.*, 2013; Ma, 2013). Recent studies have revealed that Nrf2 can also be regulated by Keap1-independent mechanisms, including the phosphorylation of Nrf2 by several signal transduction pathways such as PI3K/Akt and JNK, the involvement of epigenetic factors such as microRNAs, and the interaction of Nrf2 with other proteins may also play a role in Nrf2 activation (Bryan *et al.*, 2013).

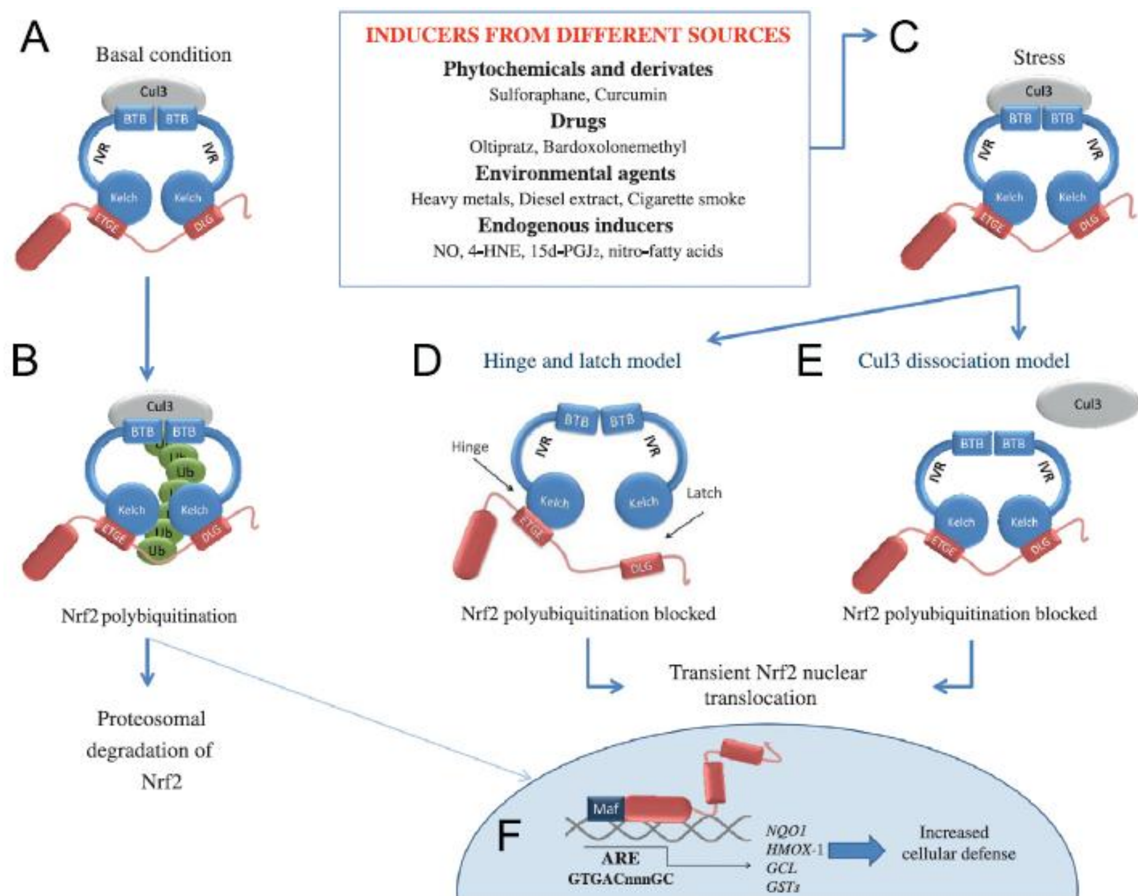

Fig. 7. Schematic representation of the Nrf2/ARE signaling pathway. (A and B) Under basal conditions, Keap1 homodimer sequesters Nrf2 in the cytosol and promotes its polyubiquitination by the Cul3-based E3 ubiquitin ligase complex, which results in proteasomal degradation of Nrf2. (C) Under stress conditions, inducers modify specific cysteine residues of Keap1, leading to inhibition of Nrf2 ubiquitination through dissociation of the inhibitory complex. (D) According to the hinge and latch model, conformational changes in Keap1 disrupt the interaction between the DLG domain of Nrf2 and the Kelch domain of Keap1, thus inhibiting Nrf2 ubiquitination. (E) According to the Keap1-Cul3 dissociation model, conformational changes in Keap1 disrupt binding of Keap1 and Cul3, leading to the escape of Nrf2 from the ubiquitination system. (F) Nrf2 translocates to the nucleus, forms a heterodimer with small Maf proteins and then binds to the ARE, which activates the transcription of genes encoding a wide variety of cytoprotective proteins, including NQO1, HMOX1, GCL, and GSTs. Reproduced from (Kansanen *et al.*, 2013).

After translocation into the nucleus, Nrf2 forms a heterodimer with small Maf proteins and binds to ARE (antioxidant response element). The ARE is a *cis*-acting transcription regulatory element with the consensus sequence 5′-TMAnn<u>RTGAY</u>nnn<u>GC</u>R-3′ (where M = A or C; R = A or G; Y = C or T; n = A, C, G, or T, and the "core" consensus is underlined) (Wasserman and Fahl, 1997; Wang *et al.*, 2016). Once bound to ARE, the Nrf2/small Maf heterodimer recruits the transcriptional machinery to activate transcription of genes encoding stress-responsive and cytoprotective enzymes and related proteins (Surh *et al.*, 2008).

Nrf2 target genes are involved in: (1) glutathione (GSH) production and regeneration, which is regulated by glutamate–cysteine ligase catalytic and modifier subunits (GCLC and GCLM, respectively), cystine/glutamate transporter (XCT) and glutathione reductase (GSR); (2) GSH utilization, which is regulated by the glutathione S-transferases (GSTs) and glutathione peroxidase 2 (GPX2); (3) thioredoxin (TXN) production, regeneration and utilization, which is regulated by TXN1, thioredoxin reductase 1 (TXNRD1), peroxiredoxin 1 (PRDX1), and sulfiredoxin-1; and (4) NADPH production, which is controlled by glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate dehydrogenase (PHGDH), malic enzyme 1 (ME1) and isocitrate dehydrogenase 1 (IDH1). Additionally, Nrf2 regulates the transcription of numerous other stress response proteins, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HMOX1), ferritin heavy chain (FTH), and ferritin light chain (FTL). By inducing the expression of these genes, Nrf2 is able to improve endogenous antioxidant defenses, thereby enhancing the capacity of cells to detoxify potentially harmful substances. Therefore, the Nrf2/ARE signaling pathway is generally considered a major cellular defense pathway (Gorrini *et al.*, 2013; Jaramillo and Zhang, 2013).

Further evidence supporting the protective role of Nrf2 comes from studies with Nrf2 deficient mice, which displayed lower basal expression of cytoprotective enzymes and negligible inducible expression of cytoprotective enzymes, and also, increased susceptibility to benzo[*a*]pyrene-induced neoplasia than wild-type mice (Ramos-Gomez *et al.*, 2001).

3. Motivation

People around the world are living longer, but longer lives do not necessarily mean healthier lives. Indeed, age-related chronic diseases such as arthritis, type 2 diabetes, cancer, osteoporosis, cardiovascular and neurodegenerative disorders are more prevalent than ever. For many years, research has focused predominantly on the pathogenesis and treatment of individual diseases, particularly those with large impacts upon morbidity and mortality. This disease-specific focus has been unquestionably successful at helping people live longer today than ever before, but largely unsuccessful at postponing, ameliorating, or preventing the accumulation of morbidities during aging. Consequently, people are living longer but often suffer from multiple diseases or disabilities related to aging, and this has important social and economic implications. Many families struggle to care for elderly relatives who survive with reduced quality of life for years or even decades, while nations devote an increasing proportion of finite resources toward medical care for aging populations. This emphasizes the urgent need to identify interventions that are holistic and move away from the single disease model. Accordingly, the main objective of aging research is to design interventions that target common mechanisms of aging, in order to delay the onset of more than one age-related disease at the same time and to improve human healthspan (Kaeberlein *et al.*, 2015; Figueira *et al.*, 2016).

Epidemiological studies have demonstrated significant associations of regular consumption of fruits, vegetables and other plant foods with improved health outcomes, including reduced risk for cardiovascular disease, stroke, diabetes, some cancers, asthma, rheumatoid arthritis, and neurodegenerative disorders. These improved health outcomes have been ascribed to phytochemicals, the bioactive non-nutrient plant compounds in fruits, vegetables and other plant foods. Several phytochemicals have been demonstrated to have clear antioxidant properties *in vitro*, and many of their biological actions have been attributed to their free radical scavenging activity. However, direct antioxidant activity is unlikely to explain all cellular effects because micromolar concentrations of phytochemicals are required to effectively scavenge free radicals. Since phytochemicals are poorly absorbed and rapidly metabolized *in vivo*, such high concentrations have not been shown to be achieved by the consumption of fruits, vegetables, or other plant foods. Therefore, the "antioxidant hypothesis" for explaining the health benefits of phytochemicals is being questioned. Moreover, emerging evidence suggests that high

doses of antioxidants may not be beneficial for health and might even be harmful. Indeed, clinical trials of vitamins A, C and E, and beta carotene have failed to show a positive outcome in patients with a range of disorders (Lee *et al.*, 2014).

Phytochemicals may also exert their effects by modulating cell signaling pathways and gene expression. Recent evidence suggest that at least some of the phytochemicals in fruits, vegetables, and other plant foods may prevent or mitigate various chronic diseases by activating adaptive stress response signaling pathways in cells. This "hormesis hypothesis" postulates that cells recognize some phytochemicals as potentially dangerous, and thus respond adaptively by engaging one or more stress signaling pathways that enhance the resistance of cells, organs, and the organism to a range of stressors that can cause or exacerbate disease(s) (Lee *et al.*, 2014). Hormesis has been defined as "a process in which exposure to a low dose of a chemical agent or environmental factor that is damaging at higher doses induces an adaptive beneficial effect on the cell or organism" (Mattson, 2008). Applying the concept of hormesis in testing the effects of compounds and extracts, by analyzing stress response pathways, can help to screen and select potentially useful compounds with specific targets (Rattan, 2012). One of the most important adaptive stress response pathways in animals is the one mediated by Nrf2, which plays a crucial role in the coordinated induction of genes encoding cytoprotective proteins (Son *et al.*, 2008). Since Nrf2 transcriptional activity declines during aging and in disease, it has been suggested that interventions that activate Nrf2 may impact the aging process and, consequently, contribute to delay the onset of multiple age-related diseases and prolong healthspan (Lewis *et al.*, 2010).

As previously mentioned, several phytochemicals and plant extracts have been shown to induce the Nrf2-mediated stress responses (Balstad *et al.*, 2011). Among them, curcumin has been extensively studied and found to have several biological activities, which are dependent on its ability to modulate numerous molecular targets, including some in the context of the hallmarks of aging. Therefore, it became essential to review the literature about the mechanisms of action of curcumin in order to clarify if this phytochemical can be useful for aging intervention. This task has led to the publication presented in Chapter 2.

Many other phytochemicals have been also shown to induce Nrf2-mediated stress responses, including the phenolic diterpenes carnosic acid and carnosol (Takahashi *et al.*, 2009). It has been suggested that screening compounds and extracts for their ability to induce one or more stress response pathways in human cells in culture can be a good

starting point to discover novel potential hormetins useful for healthy aging. This screening gains from being done in normal diploid cells because immortal cell lines usually have one or more genetic and metabolic deviations, which are rarely comparable with normal cells (Rattan, 2012, 2015). Therefore, the potential beneficial effects of these phenolic diterpenes in improving healthy aging was studied in chapter 3 in an integrated way in normal diploid skin fibroblasts, and in particular the involvement of activation of redox stress.

Plant extracts also have the potential to induce the Nrf2-mediated stress response. Indeed, several plant extracts have been found to show strong direct and indirect antioxidant activity and to protect against oxidant-induced damage. Accordingly, many of them demonstrated to confer significant protection against several chronic diseases. The origin of many therapeutic substances is due to secondary metabolism in the plant, which is activated after exposure to biotic and abiotic stress. This supports the perception that although secondary metabolites play a role in plant defense, they may also be beneficial for improving human health. Therefore, the biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material. In recent years, various strategies have been developed for the synthesis of secondary compounds, such as strain improvement, optimization of medium and culture environments, elicitation and many others (Naik and Al-Khayri, 2016). In chapter 4, a methanolic extract from *Hypericum perforatum* suspension cultures elicited by co-cultivation with *Agrobacterium tumefaciens* (biotic stress), which significantly increases xanthone production (Franklin *et al.*, 2009), was used to test its ability to induce cytoprotective responses in HepG2 human cells.

Collectively, this work intends to give a significant contribution for the perception that the interventions that target common mechanisms of aging may be in a near future an important way to improve human healthspan.

4. Objectives

The main objective of this doctoral thesis was to clarify if the hormetic induction of cellular stress responses by phytochemicals, in particular the Nrf2/ARE signaling pathway, can provide anti-aging effects and, therefore explain the health beneficial effects of consumption of fruits and vegetables. To accomplish this goal, the following specific goals were defined:

- To test the ability of selected natural compounds and plant extracts to induce stress responses. The induction of stress responses will then be correlated with possible hormetic effect of a given compound/extract by testing pre-conditioned cells to a further toxic/oxidant insult.
- To study the involvement of the Nrf2/ARE signaling in the induction of cellular antioxidant defenses
- To investigate the anti-aging effects of the selected compounds/extracts in a stress-induced premature senescence model.
- To explore the anti-aging effects of the selected compounds/extracts during the replicative senescence of normal human fibroblasts.

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CHAPTER 2

Mechanisms of action of curcumin on aging: nutritional and pharmacological applications

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Publication

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Mechanisms of action of curcumin on aging: nutritional and pharmacological applications

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Abstract

Curcumin is an active polyphenol from the rhizome of *Curcuma longa* (turmeric) that has been used in traditional medicine for centuries and widely investigated in the last decades. The numerous activities reported for curcumin are related with its peculiar structure and modulation of multiple molecular and biochemical targets. Many of them impact in the recently proposed hallmarks of aging. Curcumin is viewed nowadays as a potential phytochemical useful for aging interventions as shown by its actions on healthspan and longevity in different model organisms. This polyphenol is also increasingly associated with health promoting effects by its potential in the prevention and treatment of aging-related diseases such as cancer, diabetes, cardiovascular, and neurodegenerative diseases. To solve the low bioavailability of curcumin, new formulations are being developed to envisage its use in functional foods and for pharmacological applications to promote healthy aging. This will be the step forward for this golden nutraceutical.

Keywords: Curcumin; Aging; Age-related diseases; Molecular mechanisms; Senescence; Longevity; Nutritional interventions; Curcumin applications.

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1. Introduction

The polyphenol curcumin (diferuloylmethane) is an active compound found in the rhizome of *Curcuma longa*, which is used since ancient times as spice, coloring ingredient and component in traditional medicine. Native from tropical Asia, *C. longa* is used in these countries, from India to China, to treat several ailments including several inflammatory symptoms, respiratory conditions, sinusitis, and abdominal pain. Extensive research over the last decades has increased remarkably our knowledge about curcumin. Indeed, more than 7000 articles are listed in the US National Institutes of Health PubMed database (consulted in March 2015) [1], being most of them published in the last 10 years (Fig. 1A). The data gathered in these studies confirmed many of the attributed biological effects to *C. longa,* and to curcumin in particular, such as their anti-septic, anti-inflammatory and wound healing ability. The exponential growth of biomedical research with curcumin was also observed in the area of aging (Fig. 1B). In fact, in recent years, the effects of curcumin on the aging process and on several age-related diseases, including cancer, diabetes, cardiovascular, and neurodegenerative diseases, have been investigated with the aim for their treatment and/or prevention. In this chapter we will review the current knowledge on the effects of curcumin in the biology of aging, mainly the effects in senescence, the lifespan of experimental organisms, and the potential therapeutic benefits in age-related diseases. The impact of curcumin in several molecular targets related with hallmarks of aging will also be reviewed. The research in progress intended to increase the bioavailability of curcumin and its impact in the current and future nutritional and pharmacological applications for improving healthspan and longevity will be extensively discussed.

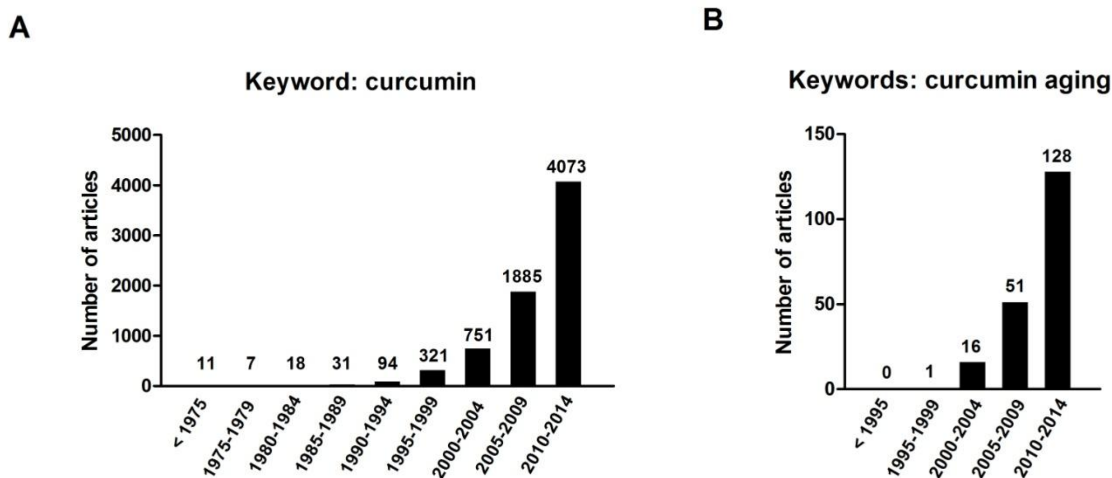

Fig. 1. Number of publications found in the US National Institutes of Health PubMed database [1] using the keyword "curcumin" (A) or the keywords "curcumin, aging" (B).

2. Curcumin and its traditional uses

Curcumin is the main curcuminoid found in the rhizome of the perennial plant *Curcuma longa* Linn, a member of the ginger family (Zingiberaceae). *Curcuma longa* is indigenous to South and Southeast Asia but is nowadays widely cultivated in tropical areas of Asia and Central America. Curcuminoids, including curcumin, demethoxycurcumin and bisdemethoxycurcumin, have also been isolated from *Curcuma mangga*, *Curcuma zedoaria*, *Costus speciosus*, *Curcuma xanthorrhiza*, *Curcuma aromatica*, *Curcuma phaeocaulis*, *Etlingera elatior*, and *Zingiber cassumunar* [2]. Together they are responsible for the yellow color of turmeric (the powder that results from the ground dried rhizome of *C. longa*), which is commonly used as a spice and in phytomedicine [2,3]. Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) corresponds to about 2–5% of turmeric and was first isolated in 1815 by Vogel and Pelletier, with its chemical structure determined by Milobedzka and colleagues in 1910 (Fig. 2) [4]. Curcumin is a yellow-orange hydrophobic compound insoluble in water and ether but soluble in dimethylsulfoxide, acetone, ethanol, and oils. It generically consists of two ferulic acid residues joined by a methylene bridge, and exhibit two main tautomeric forms (Fig. 2): the keto-enol form (also known as enol form) and the diketo form (also known as keto form). The enol form of curcumin is the predominant in most solvents and has important implications in the activity of this polyphenol, such as metal quelation and interaction with proteins [5,6]. Recently it was shown that in water

containing mixtures, the keto tautomeric form of curcumin increases and dominates with a high percentage of water [6].

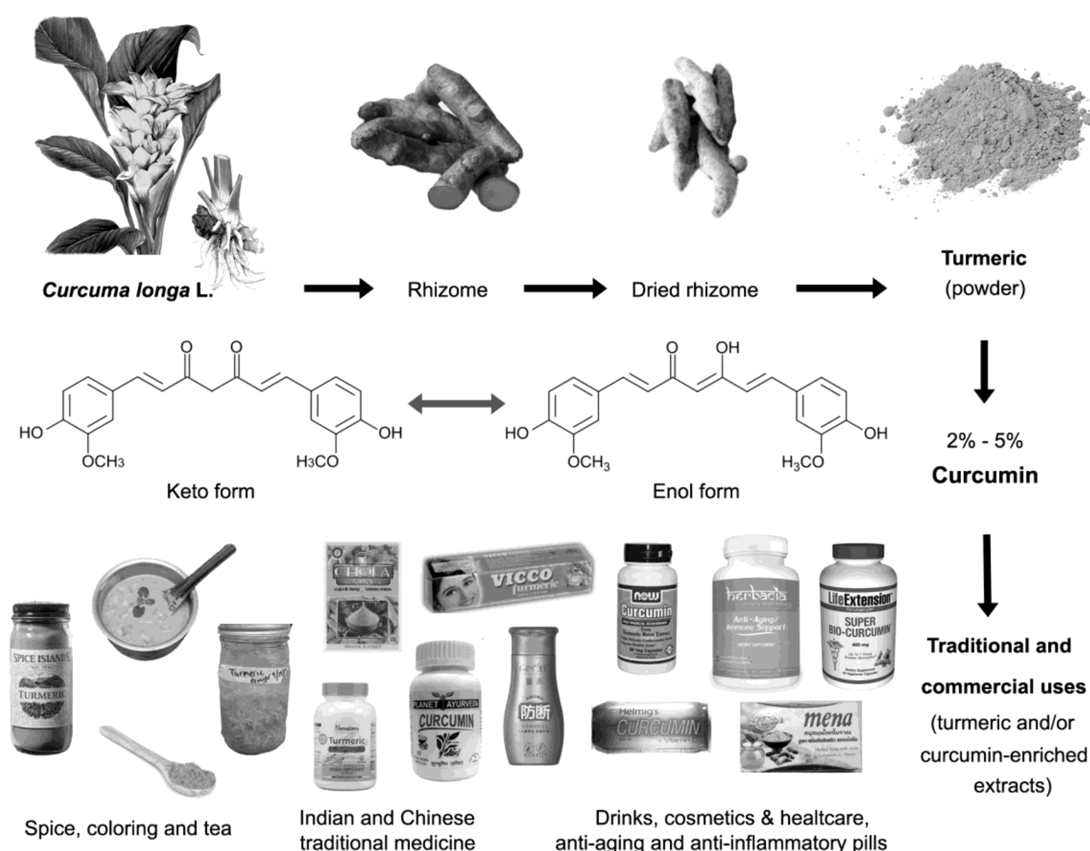

Fig. 2. From *Curcuma longa* to turmeric to curcumin: its traditional and commercial uses.

Turmeric has been used for thousand years as a dietary spice, food preservative, and as a coloring agent of food and other materials, such as cosmetics, textile fibers, and paper [2,3,7]. Turmeric is frequently used in Asian cooking, particularly in India, Pakistan and Thailand, to improve the palatability and presentation of food preparations. It is one of the ingredients of the curry powder, which gives its distinctive yellowish color and flavor [2,3,8]. In fact, curcumin is an approved natural food coloring (E100) additive that is used at low concentrations in butter, canned fish, cheese, mustard, pastries, and other foods [9]. Turmeric is also used in tea preparations, particularly by the Japanese Okinawa population [8]. Turmeric is also included for centuries in preparations of traditional Indian and Chinese medicine to treat various conditions such as allergy, anorexia, asthma, biliary disorders, bronchial hyperactivity, cough, diabetic wounds, liver disorders, rheumatism, rhinitis, sinusitis, sprains, and swelling [10]. In the old Hindu texts of traditional medicine of Ayurveda (meaning knowledge of long life), turmeric is

recommended for its aromatic, stimulant, and carminative properties [11]. In traditional Chinese medicine, it is used to treat diseases associated with abdominal pain [2], and as a major constituent of Jiawei-Xiaoyao-san medicinal formula, *C. longa* has been used to manage dyspepsia, stress, and depression/mood-related ailments [12]. In addition, in the Indian subcontinent, turmeric mixed with slaked lime has been used topically as a household remedy for the treatment of wounds, inflammation, burns, and skin diseases [11,13].

The importance of this medicinal plant as spice and in folk medicine, not only in Asian countries but also in the western world, prompted the growing research with turmeric extracts and their main constituents over the past 30 years (see Fig. 1). Many of the biological activities attributed to turmeric were confirmed by experimental scientific studies, such as its antimicrobial, antioxidant and anti-inflammatory properties [2,8,14,15]. Curcumin has been identified as one of the main active compounds responsible for turmeric's effects. For example, the promotion of wound healing by turmeric was confirmed for curcumin from preclinical to intervention studies in humans [2,16–18]. The pleiotropic and multitargeting capability of curcumin combined with its apparently safe profile identified it as a potential therapeutic phytochemical against cancer, lung and liver diseases, aging, neurological diseases, metabolic diseases, and cardiovascular diseases [7,12]. The public interest in the wide range of effects of curcumin paved the way to its commercial use worldwide in several products (Fig. 2) including anti-aging and immunomodulatory pills, functional foods, soaps, and cosmetics [12,15].

3. Biochemical and molecular targets of curcumin: an overview

Extensive research over the last decades revealed that curcumin has antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative and pro-apoptotic effects [2]. How a single compound can exhibit all these effects is a subject of intense investigation [19]. Accumulating evidences suggest that the pleiotropic effects of curcumin are dependent on its ability to interact and regulate multiple biological molecular targets. Mechanistic investigations attribute the diverse biological activities to the peculiar chemical structure of curcumin, in particular the two aromatic o-methoxy phenolic groups, the α, β-unsaturated β-diketo moiety and the seven carbon linker [5]. These chemical structural features allow curcumin to possess antioxidant activity and to

interact directly with different biomolecules through non-covalent and covalent binding [5,20]. Apart from the direct binding of curcumin to key components of cellular signaling pathways, various molecular targets are also indirectly modulated resulting in either increased or decreased expression/activity [20]. The main biochemical and molecular targets of curcumin include transcription factors, growth factors and receptors, inflammatory cytokines, enzymes and protein kinases, adhesion molecules, cell cycle and apoptosis-related proteins, among others (Table 1). Effects of curcumin on these targets were recently reviewed by different authors, for example, see Gupta et al. [20], Zhou et al. [21], Shishodia [7], Prasad et al. [4], and Shanmugam et al. [22].

Table 1. Different known molecular targets of curcumin

Molecular targets of curcumin
Transcription factors
AHR, AP-1, ATF3, β-catenin, C/EBP, CHOP, CTCF, EGR1, EpRE, HIF1, HSF1, NF-κB, NOTCH1, Nrf2,PPAR-γ, STAT1, 3, 4, 5, WT1
Growth factors and receptors
AR, CTGF, CXCR1, 2, 4, EGF, EGFR, ESR, FasR, FGF, HGF, HRH2, INSR, ITPR, LDLR, NGF, PDGF, TF, TGF-β1, VEGF
Inflammatory cytokines
IFN-γ, IL-1, 2, 5, 6, 8, 12, 18, MIP-1, 2, MCP-1, TNF-α
Enzymes and protein kinases
5-LOX, ATPase, CDPK, COX-2, FAK, GCL, GST, HO-1, INOS, IRAK, JAK, MAPK, MMP, MTOR, NQO1, ODC, PHK, PKA, PKB/AKT, PKC, SIRT1, SRC, SYK
Adhesion molecules
ELAM-1, ICAM-1, VCAM-1
Cell cycle and Apoptosis-related proteins
Cyclin D1, p53, BCL2, BCL-XL, IAP, BAX, BAK1, CASP3, 7, 8, 9, DR4, 5

Adapted from Zhou et al. [21], Shishodia [7], and Prasad et al. [4].

3.1. Aging-related molecular targets

At the cellular level, aging is characterized by the progressive accumulation of molecular damage, leading to impaired function and increased vulnerability to cell death [23–25] (See also chapter "Molecular and Cellular Basis of Aging" for details). The main causes of aging are attributed to the deterioration and failure of genetic pathways and biochemical processes conserved in evolution involved in the cellular maintenance and

repair mechanisms [23]. The loss of physiological integrity during aging is the primary risk factor for major human pathologies, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases [25]. Recently, Lopez-Otin et al. proposed nine cellular and molecular hallmarks of aging, which are genomic instability, telomere attrition, epigenetic alterations, proteostasis imbalance, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [25]. These hallmarks that characterize the aging process aim to help design further studies on the molecular mechanisms of aging and on the development of interventions that will improve human healthspan [25]. In the following subsections we review the effects of curcumin on biochemical and molecular targets that impact on some of the hallmarks of aging (Fig. 3).

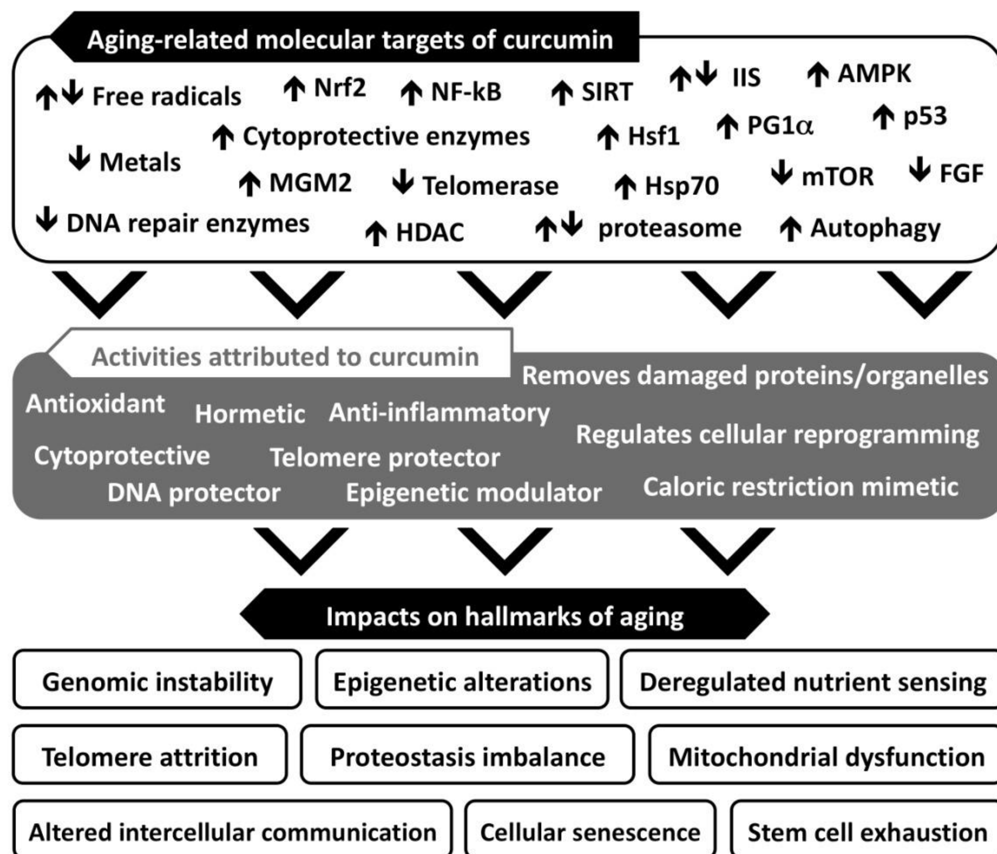

Fig.3. Molecular targets of curcumin that impact on the hallmarks of aging, with the associated attributed activities. Arrows in the first box indicate increase/activation (↑) or decrease/inhibition (↓) of the target by curcumin.

Genomic instability

The genome is constantly being challenged by endogenous and exogenous threats that result in DNA damage and genomic instability [26]. A highly conserved and complex DNA repair machinery are usually able to maintain the integrity of the genome [25]. However, excessive DNA damage or insufficient DNA repair favors the aging process. The accumulation of genetic damage and alterations during aging inevitably also associate aging and cancer [27].

In several experimental settings, curcumin showed a higher antioxidant activity that can be useful to protect DNA and other biomolecules from oxidative damage. The direct antioxidant activity of curcumin is dependent on its ability to work as free radical scavenger and to chelate transition metal ions. The first activity is mainly dependent on the electron donation of the phenolic OH groups, whereas the latter is dependent on the β-diketo group [5]. Curcumin also possesses indirect antioxidant activities by up-regulating several antioxidant and cytoprotective enzymes [28–30]. This effect is known to be mediated by Nrf2, a basic leucine zipper (bZIP) transcription factor, that upon activation accumulates in the nucleus and, in heterodimeric combination with small Maf transcription factors, binds to ARE and recruits the basal transcriptional machinery to activate the transcription of genes encoding stress-responsive and cytoprotective enzymes and related proteins [31,32]. Under normal conditions, the Kelch-like ECH-associated protein (Keap1) forms a complex with cullin3 (Cul3) and represses Nrf2 by presenting it for ubiquitination and proteasomal degradation. Upon stimulation with curcumin, the cysteine residues of Keap1 are modified resulting in conformational changes that eliminate the capacity of Keap1 to repress Nrf2 [31,32]. Curcumin contains electrophilic α, β-unsaturated carbonyl groups (β-diketo moiety) that can react selectively with nucleophiles such as thiols, leading to formation of Michael adducts [29]. This Michael addition ability of curcumin allows its covalent binding to nucleophilic cysteine sulfhydryls and the selenocysteine moiety, affecting the activity of several proteins/enzymes [5,20], including the inhibition of Keap1. Curcumin was also shown to interact with glutathione (GSH) forming glutathionylated products [33]. Moreover, the induction of antioxidant defenses by curcumin was associated with a transient decrease of GSH levels that impact on cellular redox state [30,34]. This transient mild impairment of thiol-disulfide redox state by curcumin may also influence indirectly redox signaling through activation of Nrf2, resulting in a compensatory antioxidant response in cells, including the increase in GSH synthesis to restore cellular redox state [30]. Both the

direct and indirect antioxidant effects of curcumin are thus regarded as useful in the slow down of aging and prevention of age-related diseases that have been associated with the deleterious effects of free radicals and oxidative stress, such as cancer, neurodegenerative disorders, and cardiovascular diseases [35].

The O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein that protects the cellular genome and critical oncogenic genes from the mutagenic action of endogenous and exogenous alkylating agents, was also shown to be induced by curcumin, probably by the ability of this polyphenol to increase the availability of cysteine [36]. Higher content of cysteine drive the synthesis of cysteine-rich and cysteine-sufficient proteins including MGMT [36].

Curcumin at high concentrations, however, is also known to induce reactive oxygen species (ROS), DNA damage and cytotoxicity, which are linked to one of its anticancer mechanisms [37]. Curcumin is also known to inhibit several DNA repair enzymes and mechanisms [37–39]. However, when curcumin induces mild stress and DNA damage to normal human skin fibroblasts, cells recover and repair DNA in a few hours [30]. It is possible that in this case hormesis is involved [40]. According to this concept of intervention, mild toxic treatments may generate a beneficial compensatory response by increasing the maintenance and repair mechanisms of the cells [41,42]. Thus, although higher concentrations of curcumin are deleterious, which may be useful for cancer treatment, lower doses may slow down aging and be chemopreventive by inducing a hormetic response. Interestingly, in a human intervention study, the ability of curcumin to prevent DNA damage and to enhance the repair potential was shown in a human population in West Bengal (India) chronically exposed to arsenic [43].

Telomere attrition

Telomeres are specialized nucleoprotein structures on the extremities of eukaryotic chromosomes that protect them and are particularly implicated in the aging process [44]. Gradual telomere shortening (attrition) in normal somatic cells during consecutive rounds of replication leads to critically short telomeres that induce replicative senescence, the irreversible loss of division potential of somatic cells [45,46]. In addition, it was shown that telomeres are a preferential target of genotoxic stress and ROS-induced DNA damage, which has important consequences for the aging process [44]. The antioxidant actions of curcumin may therefore have important implications in healthy aging by preventing oxidative damage to telomeres.

Telomerase, a reverse transcriptase that maintains telomere length, is practically absent in normal somatic cells and highly activated in most tumor cells enabling their replicative immortality [47,48]. In recent years, telomerase has been proposed as a potential target for cancer therapy and thus extensive investigations have been carried out in the search of compounds capable of inhibiting telomerase [49]. Several studies have demonstrated that curcumin inhibits telomerase activity in a dose and time-dependent manner by suppressing the translocation of human telomerase reverse transcriptase (hTERT) from the cytosol to the nucleus [49] and by decreasing hTERT expression [50,51].

Both telomeric and non-telomeric DNA damage has been shown to induce and stabilize senescence contributing to the aging phenotype [52]. Recently, it was shown that systemic chronic inflammation in mice accelerates aging via ROS-mediated exacerbation of telomere dysfunction and cell senescence [53]. The preferential accumulation of telomere-dysfunctional senescent cells in this mouse model of chronic inflammation was blocked by anti-inflammatory or antioxidant treatments [53]. Therefore, the known anti-inflammatory action of curcumin (see Ref. [21] for review) may help to prevent telomere attrition contributing to the potential promotion of healthspan by this phytochemical. Many age-related diseases as well as normal and pathological aging have been for some time associated with chronic low-grade inflammation [54,55]. In fact, when the expression of an NF-κB inhibitor was activated in the aged skin of transgenic mice this tissue was rejuvenated and that was accompanied by the restoration of the transcriptional signature associated to young age [56]. In addition, genetic and pharmacological inhibition of NF-kB signaling was shown to prevent age-associated parameters in different mouse models of accelerated aging [57,58]. Interestingly, Zhang and colleagues showed that mice with blocked NF-kB activation live longer than untreated mice [59]. Therefore, the global imbalance between the lifelong inflammatory processes and the anti-inflammatory networks is proposed to be a major driving force for frailty and common age-related pathologies [54]. Thus, curcumin's action as an anti-inflammatory agent and an efficient inhibitor of NF-kB suggests its potential contribution to the promotion of health aging [60]. In particular, the inhibition of the NF-κB transcriptional activity by curcumin suppresses expression of various cell survival and proliferative genes and consequently leads to cell cycle arrest, inhibition of proliferation, and induction of apoptosis, all of which may have positive implications in the combat against cancer [21].

The anti-inflammatory action of curcumin may also influence another hallmark of aging – altered intercellular communication. Such alterations during aging have been frequently associated with pro-inflammatory signals that result, for example, from the secretome of senescent cells, dysfunctional immune system, tissue damage and enhanced NF-kB activaction [25,52]. The anti-inflammatory activity of curcumin has been associated to its ability to inhibit various pro-inflammatory cytokines, including interferon-γ (IFN-γ), interleukin (IL)-1, 2, 5, 6, 8, 12 and 18, macrophage inflammatory protein (MIP)-1 and 2, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor alpha (TNF-α) (reviewed in Refs [21] and [7]).

Epigenetic alterations

Epigenetic alterations, including changes in DNA methylation, histone modifications and chromatin remodeling, are associated with aging and several age-related diseases [61]. Epigenetic modulation constitutes an important mechanism by which dietary components can selectively activate or inactivate gene expression and exert their biological activities [62,63]. Recent evidences have shown that curcumin possesses different epigenetic effects including the modulation of histone deacetylases (HDAC 1, 3, 8 and SIRT1, 7), histone acetyltransferases (p300/CBP), DNA methyltransferase (DNMT1, 2), and several miRNAs [63–66]. Therefore, the exploitation of curcumin as a regulator of the epigenome holds great promise for promotion of healthy aging and prevention of diseases. Deserving special attention are the effects of curcumin on sirtuins - NAD^+-dependent HDACs. Sirtuins and their functions in aging have been the subject of intense research because increased expression of sirtuins has been shown to have beneficial effects on aging or even increase the lifespan in different model organisms [25,67]. In view of the increased longevity afforded by caloric restriction in several laboratory organisms, in particular through activation of sirtuins and other epigenetic effects, mounting evidence is being gathered to show that many phytochemicals might have health benefits by mimicking caloric restriction [66]. This can also be the case for curcumin.

Proteostasis imbalance

Recent studies show that aging and diverse age-related pathologies, such as diabetes, Alzheimer's and Parkinson's disease, are associated with impaired protein homeostasis – proteostasis [68,69]. Proteostasis involves mechanisms of protein

stabilization by molecular chaperones, including the heat shock family of proteins (Hsp), and mechanisms of protein degradation by the lysosome and the proteasome [68,70]. These mechanisms function in a coordinated manner to restore the structure or to remove and degrade misfolded proteins, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins [25]. Several studies demonstrated that proteostasis is altered with aging [68] and that its perturbation is detrimental to aging whereas genetic manipulations that improve proteostasis have been shown to delay aging in mammals [25].

Curcumin has been shown to modulate several effectors of proteostasis that may have profound impact in improving healthy aging. In particular, curcumin was able to stimulate the heat stress-induced expression of Hsp70 [71]. Curcumin alone was also reported to induce the heat shock response, in particular causing the nuclear translocation of the heat shock transcription factor 1 and increasing the expression of Hsp70 at transcriptional and translational levels [72–75]. Several studies indicate that curcumin inhibits the proteasome, usually at higher concentrations and linked with induction of cell death in cancer cells [74,76,77]. On the other hand, others have shown the opposite effect with lower curcumin concentrations [78]. This is consistent with the principle of hormesis and may have positive implications for longevity. Contrarily to reports identifying the anticancer effects of curcumin through proteasome inhibition, other studies demonstrated its anticancer potential by inducing the proteasome to promote the degradation of some oncogenic and angiogenic proteins [74,79]. Curcumin has also been shown to efficiently induce autophagy in numerous studies [74,80,81], and also because of that, curcumin is has been classified as a caloric restriction mimetic [82]. The induction of autophagy by curcumin can, however, be a double-edged sword in cancer treatment. Whereas chronic induction may lead to autophagic cancer cell death, moderate induction may promote autophagic survival in nutrient-limiting and low-oxygen conditions characteristic of internal regions of tumours [83,84].

Deregulated nutrient sensing

Lifespan is regulated by highly conserved nutrient sensing pathways that are controlled by insulin/insulin-like growth factor 1 (IGF1) signaling (IIS; that participates in glucose sensing), mammalian target of rapamycin (mTOR; related with aminoacid sensing), and AMP-activated protein kinase (AMPK) and sirtuins that sense low-energy states (by detecting high levels of AMP and NAD^+, respectively) [25,85,86]. Several

studies have shown that increasing or restricting dietary intake affects the aging process and the onset of several age-related diseases. High nutrient intake shortens lifespan and accelerates age-associated disorders, while moderate nutrient intake extends lifespan and delays (or attenuates) age-related diseases [86,87]. Moreover, dietary restriction increases lifespan in all investigated model organisms, supporting the idea that deregulated nutrient sensing is a relevant characteristic of aging [88].

The effects of curcumin on IIS have been controversial, since some studies reported that curcumin activates this pathway when stimulated by mitogenic factors [30,89–91], whereas others report the opposite [92–94]. Some of these later studies link Akt inhibition with decreased mTOR signaling and eventually induction of autophagy by curcumin [92,93]. Other authors observed that mTOR inhibition by curcumin was independent of Akt [89,95]. The increased sensitivity to insulin conferred by curcumin has been linked with its beneficial effects on diabetes [90,96]. AMPK activation is also being described as a mechanism for the health beneficial effects of curcumin [90,97,98]. In fact, this particular effect of curcumin was associated with neuroprotection and improvement of aging-related cerebrovascular dysfunction in mice [99,100]. Overall, the regulation of nutrient sensing pathways by curcumin mimics a state of limited nutrient availability that is known to extend longevity, and therefore the connection between this polyphenol and the potential promotion of human healthspan is not surprising.

Mitochondrial dysfunction

Mitochondrial dysfunction has a profound impact on the aging process and contributes to multiple aging-associated pathologies [25]. The progressive mitochondrial dysfunction that occurs with aging results in decreased ATP generation and increased ROS production, which in turn causes further mitochondrial and global cellular damage [101]. Some studies demonstrated that mitochondrial dysfunction could in fact accelerate aging in mammals [25]. Curcumin, through the described antioxidant effects, and induction of Nrf2, autophagy and sirtuins, might in fact control mitochondria function conferring protection against aging and age-associated diseases. For example, activation of SIRT1 by curcumin [102,103] may impact on mitochondrial biogenesis through the involvement of the peroxisome proliferator-activated receptor-γ (PPAR- γ) coactivator-1α (PGC-1α) and the removal of damaged mitochondria by autophagy [25]. In fact, curcumin was shown to induce the transcriptional coactivator PGC-1α [104–106] and to induce autophagy (see above).

Cellular senescence

Cellular senescence refers to the state of irreversible growth arrest that occurs due to DNA damage, mitogenic and oncogentic stress, and/or epigenomic perturbations that derepress the INK4/ARF locus [107,108]. Although senescence has an important underlying tumor suppressive role, it is also involved in aging and related pathologies by contributing to the age-related loss of tissue renewal and function [52]. Senescence can, however, in the long run, have adverse effects promoting cancer growth by virtue of the senescence-associated secretory phenotype (SASP), characterized by the released of ROS and an array of pro-inflammatory molecules [107]. Senescence is usually associated with the activation of the $p16^{INK4a}$/pRb and p53/p21 pathways that drive the irreversible growth arrest [107,108]. Due to its antioxidant, anti-inflammatory and DNA repair promoting activity, curcumin may help to restrain the increase in senescent cells during aging and thereby help control the impact of the senescence phenotype in the surrounding tissue micro-environment.

Curcumin has also been shown to induce p53 [109–111]. This transcriptional factor plays a central role in maintaining genome stability and integrates and responds to a multitude of stresses to exert its function in tumor suppression, such as by inducing cell cycle arrest, apoptosis and/or senescence [112]. p53 is also an important player in the regulation of aging and longevity: it may accelerate aging in case of severe and chronic activation of p53, but its low grade and regulated activation may extend lifespan [25,113]. Therefore, curcumin may confer hormetic health benefits by moderately inducing p53 in a transient and regulated manner, thus preventing cancer and promoting healthy aging. At higher doses and in continuous administration, curcumin is reported to be cytostatic and to induce senescence in normal cells [114] (and our own unpublished data). The senescence induction by curcumin at higher concentrations may be useful in cancer treatment [110,111,115].

Stem cell exhaustion

Although the beneficial compensatory response of activation of p53 and INK4a/ARF aims to avoid the propagation of damaged and potentially oncogenic cells and its consequences on aging and cancer, under persistent activation of these senescence pathways the regenerative capacity of progenitor cells can be exhausted or saturated [25]. Under these extreme conditions, p53 and INK4a/ARF responses can become deleterious

and accelerate aging which, in conjugation with the perturbations of the immunological system during aging, eventually results in the accumulation of senescent cells and consequent loss of the tissue's normal functions [25,107]. Like rapamycin that enhances the generation of mouse induced pluripotent stem cells and restoration of self-renewal in hematopoietic stem cells of aged mice by mTORC1 inhibition [116,117], curcumin was also shown to have some capacity to regulate cellular reprogramming [117], which may counteract stem cell exhaustion that is associated with aging.

Recently, loss of quiescence in the aged muscle stem cell niche of mice was also shown to be due to increased of fibroblast growth factor 2 (FGF2) signaling [118]. This eventually results in stem cell depletion and diminished regenerative capacity [25,118]. Therefore, the recognized inhibition of FGF signaling by curcumin that is associated with angiogenesis and tumorigenesis [7,25,119,120], may also be a promising approach to reduce stem cell exhaustion during aging.

4. Curcumin and aging

Curcumin has been viewed as a promising phytochemical in future interventions for improving human healthspan and longevity. Although there are no epidemiologic studies with curcumin intake being the sole variable, several investigations with animal models have associated it with the prevention and treatment of age-related diseases and the potential to delay the aging process [121].

4.1. Curcumin in aging and longevity

Over the past years, experiments in model organisms demonstrated that dietary curcumin can prolong lifespan by inducing cellular stress response pathways that are controlled by highly conserved signaling mechanisms, including AMPK, IIS, mTOR, and sirtuin pathways [85,86]. Until recently, different studies demonstrated that curcumin and its metabolite, tetrahydrocurcumin (THC), increase mean lifespan of three model organisms useful to identify compounds that can prolong the lifespan of humans – *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus* [122–128]. These effects on increased lifespan were, however, not observed in yeast [129].

The roundworm *C. elegans* and the fruit fly *D. melanogaster* are popular models for studying aging and longevity because of their short lifespan, rapid generation time and well-defined genetics [130]. In 2011, curcumin was shown to prolong in about 45% the

mean lifespan of the nematode *C. elegans* through a mechanism that involves the regulation of protein homeostasis [131]. In the same year, Liao and colleagues reported an increase in mean and maximal lifespan of *C. elegans* when the synchronized L1 larvae were allowed to develop to adulthood in the presence of curcumin [123]. This lifespan extension was associated with decreased levels of ROS and age-related lipofuscin content, and increased resistance to heat stress. Several mutant strains in selected stress- and lifespan-relevant genes did not show prolonged lifespan suggesting their gene products were required for curcumin-mediated increased longevity in *C. elegans*, unraveling the involvement of diverse modes of action and signaling pathways [123].

Several studies have also investigated the effects of curcumin on the lifespan and aging of *D. melanogaster*. Suckow and Suckow reported an increase of 12 days (18% increase) in the mean lifespan of wild type fruit flies maintained on media containing curcumin 1 mg/ml [122]. The authors suggested that the effect of curcumin was mediated by induction of superoxide dismutase (SOD) activity. Lee et al. reported the gender and genotype specific lifespan extension in *D. melanogaster*: by 19% in females of Canton-S strain exposed to 100 mM curcumin, but not in males; by 16% in males of Ives strain given 250 mM curcumin, but not in females [124]. The increase in lifespan of Canton-S flies by curcumin was associated with increased expression of several aging and stress-associated genes [124]. Other authors also confirmed the ability of curcumin to increase the lifespan in fruit flies [125,128,132]. Shen and colleagues confirmed that curcumin increases SOD and that was associated with decreased lipid peroxidation [125]. Two independent studies reported no negative effects on flies' fecundity by curcumin [128,132]. Turmeric was also shown to increase mean and maximal lifespan of *D. melanogaster* [133]. The THC metabolite of curcumin was also shown to extend mean lifespan in both male and female flies and to inhibit the oxidative stress response by regulating the evolutionarily conserved signaling pathways foxo and Sir2 [127]. Although curcumin did not cause lifespan extension in the mammalian mouse model [134], THC supplementation did in male C57BL/6 mice as reported by Kitani and colleagues [126].

Curcumin has also been studied in normal human cells, a model used to study the molecular basis of cellular aging by serial cell subcultivation, which undergo progressive aging and culminate in irreversible growth arrest (replicative senescence) [135]. Our group demonstrated that curcumin induces stress responses in normal human skin fibroblasts through induction of redox stress and associated with activation of Nrf2 signaling [30]. This effect resulted in the induction of several antioxidant and

cytoprotective enzymes as well as in the increase of GSH content, effects that were substantially impaired in late passage senescent cells. This hormetic response by curcumin conferred significant protection against a further oxidant challenge [30]. The ability of curcumin to induce Nrf2 might have important implications in slowing down the aging process. Several studies demonstrated that the expression of Nrf2 and its target genes decline during aging and disease [136–139]. In fact, the decline of Nrf2 transcriptional activity causes the age-related loss of glutathione synthesis, which adversely affects cellular thiol redox balance, leaving cells highly susceptible to different stresses [136]. In addition, the orthologue of mammalian Nrf2 has been associated with oxidative stress tolerance and aging modulation in *Drosophila* [139–141]. Therefore, modulation of Nrf2 signaling pathway by curcumin may positively regulate healthspan by hormesis. In fact, stimulation of maintenance and repair mechanisms by repeated exposure to mild stressors has been recognized as a promising strategy to prevent the age-related accumulation of molecular damage [23,24]. Many lines of evidences, including our own studies and those described in the previous section on curcumin's molecular targets, indicate that it acts as a hormetin. From the point of view of aging prevention and healthspan, curcumin has positive effects at lower concentrations but is detrimental at higher concentrations. This hormetic response elicited by curcumin was also observed in most studies with model organisms cited earlier. The higher tested doses did not cause lifespan extension, and only mild and appropriate doses of curcumin were effective as compared with untreated controls (see for example Refs [123] and [128]).

Considering the beneficial and hormetic effects of curcumin in human normal cells, we recently investigated whether curcumin would protect human skin fibroblasts from undergoing replicative senescence *in vitro*. For that, middle passage cells were grown either continuously (except in the day for cell attachment after subcultivation) or intermittently (two times a week for 3 h with 5 μM of curcumin) in the presence of curcumin. No positive effects were observed and, in fact, curcumin was even detrimental inducing the stoppage of cell division and the appearance of morphological and physiological features typical of replicative senescence. In the continuous treatment with curcumin, among the concentrations tested, only the lower one (1 μM) did not affect the growth curve (the cumulative population doublings). Therefore, contrary to the positive effects with carnosol that we recently reported [142], curcumin at the tested conditions was not capable of ameliorating the physiological state of cells during replicative senescence. The fact that curcumin decreases GSH levels in the initial hours of incubation

[30] as part of its hormetic response may explain the unfavorable impact in the growth of human fibroblasts, an hypothesis raised by Satoh and collaborators [143]. The ability of curcumin to induce senescence was also recently shown in primary human cells (vascular smooth muscle and endothelial cells derived from aorta) [114]. We also suggested previously the possible induction of premature senescence in normal cells by high concentrations of curcumin due to chronic induction of oxidative stress and Akt signaling [30]. It may happen that the beneficial effects of curcumin in healthspan are not attained by itself but when consumed mixed with different bioactive phytochemicals that will antagonize the drawbacks of curcumin. These possibilities should be explored in the design of future studies from preclinical to animal models and to human intervention trials.

4.2. Curcumin in aging and disease

By virtue of its multitarget ability demonstrated in numerous studies, curcumin has been advocated as effective against a wide range of diseases including cancer, cardiovascular, inflammatory, liver, lung, metabolic, neurological, renal, and other diseases [2,4], which are summarized in Table 2. The molecular and biochemical targets of curcumin on aging hallmarks have been linked with the protection against several age-related diseases using animal models, specifically on cancer (reviewed in Refs [22] and [144]), diabetes (reviewed in Refs [145], [146], and [147]), cardiovascular disorders (reviewed in Refs [145] and [148]), and neurodegenerative diseases (reviewed in Refs [9], [12], and [149]).

Table 2. Diseases potentially targeted by curcumin according to the literature review done by Aggarwal et al. [2] and Prasad et al. [4].

Diseases and symptoms targeted by curcumin
Cancer
Bone, Brain, Breast, Gastrointestinal cancers (esophagus, stomach, liver, pancreas, intestine, rectum), Genitourinary cancers (bladder, kidney, prostate), Gynecologic cancers (cervix, ovary, uterus), Hematologic cancers (leukemia, lymphoma, myeloma), Lung, Oral, Skin
Cardiovascular diseases
Atherosclerosis, Cardiomyopathy, Myocardial infarction, Stroke
Inflammatory diseases
Allergy, Colitis, Crohn's disease, Eczema, Gallstone, Inflammatory bowel disease, Multiple sclerosis, Pancreatitis, Psoriasis, Rheumatoid arthritis, Sinusitis, Systemic sclerosis, Ulcer
Kidney diseases
Chronic kidney disease, Diabetic nephropathy, Renal failure, ischemia and reperfusion
Liver diseases
Alcoholic liver disease, Cirrhosis, Fibrosis
Lung diseases
Asthma, Bronchitis, Chronic obstructive pulmonary disease, Cystic fibrosis,
Metabolic diseases
Diabetes, Hyperlipidemia, Hypoglycemia, Hypothyroidism, Obesity
Neurological diseases
Alzheimer’s disease, Depression, Epilepsy, Parkinson’s disease
Other diseases and symptoms
Cataract, Fatigue, Fever, Hemorrhage, Osteoporosis, Septic shock, Wound healing

Curcumin and cancer is a major area of research, and data largely agree that this polyphenol inhibits carcinogenesis at multiple levels [22]. In a systematic review of published results, Ghorbani et al. concluded that curcumin has also antihyperglycemic and insulin sensitizer effects [96]. In addition, curcumin regulates the expression of AMPK, PPARγ, and NF-κB in livers of diabetic db/db mice, suggesting its beneficial effect for treatment of diabetes complications [98]. Recently, pretreatment with curcumin was shown to confer cardioprotection by attenuating mitochondrial oxidative damage in ischemic rat hearts through activation of SIRT1 signaling [102].

More recently, a strong effort has been channeled to exploiting these effects of curcumin for tackling neurodegenerative diseases. Using different transgenic *Drosophila* as models of Alzheimer’s disease, curcumin led to up to 75% extended lifespan

associated with improvement of amyloid fibril conversion, locomotor activity, and reduced neurotoxicity [150]. Wang et al. also showed the ability of curcumin to protect both morphological and behavioral defects in these models [151]. In *Drosophila* expressing human wild type α-synuclein in neurons as a model of Parkinson's disease, curcumin significantly delayed the loss of activity pattern, reduced oxidative stress and apoptosis, and increased the lifespan of transgenic flies [152]. Dietary curcumin supplementation also regulated molecules involved in energy homeostasis, such as AMPK, in rat brain tissue after induced trauma, which may be important for brain functional recovery [153]. Using rats and mice, Pu et al. demonstrated that curcumin improves aging-related cerebrovascular dysfunction via the AMPK/mitochondrial uncoupling protein 2 (UCP2) pathway [99]. Recently, curcuminoids intake by female rats also demonstrated several improvements on brain age-related mitochondrial dysfunction parameters [154]. Curcumin given intragastrically attenuated cognitive deficits of senescence-accelerated mouse prone 8 (SAMP8 mice) [155]. Overall, these reports validate curcumin as an interesting drug candidate for the prevention of age-associated neurodegenerative disorders.

5. Nutritional and pharmacological applications of curcumin in aging

Although there is intense research regarding different effects associated to curcumin, epidemiological and clinical studies showing health beneficial effects from its consumption or use as a phytopharmaceutic are scarce. The pharmacological application of curcumin is constrained by its poor solubility and low bioavailability. New delivery systems are being developed to overcome this limitation and may be a significant step forward towards its applicability in the treatment and prevention of several age-related diseases.

5.1. Epidemiological data and clinical trials

Therapeutic and preventive effects against several age-related diseases have been attributed to curcumin and, therefore, it has acquired great importance as a possible anti-aging therapeutic. Although many preclinical studies support these health promoting effects, there are no consistent epidemiological data and clinical trials that unequivocally link curcumin with healthy aging. Although Indians and Chinese have used turmeric since ancient times, systematic collection of epidemiological data is influenced by other factors

that affect the drawing of reliable conclusions. These include the presence of other ingredients commonly found in the diet of these ethnic groups, such as other spices and food additives, high percentages of vegetarianism, and use of alternative medicines [156].

Relevant prospective epidemiological research studies that link curcumin (turmeric) consumption and aging are being performed in one of world's longest-lived populations – the Okinawans [157]. This Japanese population from the Ryukyu Islands has the world's longest life and health expectancy with the lowest mortality rates from a multitude of chronic diseases of aging [157]. The traditional Okinawan diet is characterized by low fat intake, particularly saturated, and high carbohydrate intake together with a very abundant ingestion of calorie-poor and antioxidant-rich orange-yellow root vegetables (turmeric), sweet potatoes, and green leafy vegetables [158,159]. This nutritional data support the view that mild caloric restriction (10-15%) and high consumption of foods with antioxidant and caloric restriction-mimetic properties play a role in the extended healthspan and lifespan of the Okinawans [159]. More research is nevertheless needed to clearly understand the function of curcumin in this equation. Present research data support that curcumin has in fact some activities that mimic the biological effects of caloric restriction [66,82,132].

Epidemiological data have been collected supporting a positive relationship between turmeric consumption and cognitive function in the elderly. It has been reported that the prevalence of Alzheimer's disease in India among people of ages between 70 and 79 years is 4.4 fold lower than that of the United States [160]. In addition, a meta-analysis showed that dementia incidence in East Asian countries (that have the highest consumption of curry) is lower than in Europe, where a higher incidence of Alzheimer's disease tends to exist [161]. In another study with the multiethnic population of Singapore, the association between curry consumption and cognitive function in elderly was investigated in more than 1000 older adults over 60 years of age. The study found a significant beneficial effect on cognitive functioning associated with low-to-moderate levels of curry consumption in elderly Asian subjects [162]. The authors stated however that these findings should be interpreted with caution since they come from a cross-sectional data analysis and do not establish a clear and direct causal effect of curry consumption and improvement of cognitive function [162].

The overall lower cancer rates in Indian population, especially colorectal, prostate, pancreatic, and lung cancers, as compared with western countries, have also been used as an argument for the possible correlation between curry consumption and decreased cancer

risk [156,163]. However, to better explore the relationship between curcumin consumption and healthspan well designed epidemiological studies are needed, in particular longitudinal follow up cohorts of elderly persons and that specifically investigate parameters and biomarkers relevant for aging and age-related diseases. Though, several confounding factors such as lifestyle, genetics, and other dietary components, will make it difficult to have a clear picture.

Supported by the promising activities and multitargets identified for curcumin in *in vitro* and animal studies, several clinical trials and human intervention studies have already been performed or are underway. In the website www.clinicaltrials.gov are documented more than 50 phase I and/or phase II clinical trials with turmeric and more than 100 using curcumin. To date, human intervention studies with curcumin have focused mainly in the treatment of an existing health problem or disease, such as cancer and neurodegeneration, demonstrating in some cases its potential as a promising drug. Considering the cardioprotection and lipid lowering ability of curcumin established in pre-clinical studies, a meta-analysis was recently performed with data from randomized controlled trials that measured parameters related with blood lipids [164]. No effects could be associated with curcumin supplementation on serum total cholesterol, LDL-C, triglycerides and HDL-C levels.

The clinical use of curcumin is greatly limited by its poor oral bioavailability (see details below). In fact, Sahebkar left some suggestions for a more robust assessment of the lipid-modulating properties of curcumin, such as the use of bioavailability-improved formulations of curcumin with longer supplementation duration in randomized controlled trials conducted in dyslipidemic subjects [164]. Recently, DiSilvestro et al. reported the use of lipidated curcumin in a low dose supplementation study on healthy middle aged people [165]. In this form curcumin is expected to have better absorption performance and the authors found a variety of potentially health promoting effects. Among them, curcumin decreased triglyceride levels but did not affect cholesterol parameters; decreased plasma beta amyloid protein concentrations, which may impact on Alzheimer's disease development; and, increased salivary radical scavenging capacity (direct antioxidant activity) and plasma catalase enzyme activity (indirect antioxidant activity) [164]. With better designed and longer intervention studies as well as the development of different approaches to increase curcumin bioavailability we may attain very soon promising results demonstrating the beneficial effects of this polyphenol for the treatment/prevention of age-related diseases and the promotion of healthy aging.

5.2. Curcumin bioavailability and pharmacokinetics

From the studies conducted in humans, curcumin has a good safety profile with no toxicities reported in phase I clinical trials at dosages as high as 8 g/day [166,167]. Together with the safety profile documented for curcumin present in the diet and in phytomedicinal extracts, its use at high dosages that far exceed the ones in ancient tradition seems also to be well tolerated by humans. For that it may contribute the reduced water solubility of curcumin, its limited intestinal absorption and rapid metabolism and excretion, which results in low bioavailability [168]. After oral ingestion of 8 g of curcumin the peak serum concentration was observed after 1 to 2 hours with an average value of 1.8 ± 1.9 µM [166]. At dosages below 3.6 g/day no detectable amount of curcumin and metabolites were observed [167]. Consistent with the findings in animal models, curcumin was also shown to be efficiently metabolized, particularly in the intestine, resulting in, for example, glucuronide and sulfate conjugates [13]. The metabolite tetrahydrocurcumin has been reported to retain some of the activities of the parent compound, and may therefore contribute to the pharmacological effects of curcumin taken orally [169]. Although some pilot studies with humans suggest poor systemic availability of curcumin when administered orally, probably not enough to exert pharmacologic activity in tissues such as liver and brain, such effects may be possible in gastrointestinal tissues and oral mucosa [13,167]. Patients with colorectal cancer receiving 3.6 g of curcumin daily in capsules resulted in detectable levels of curcumin (together with its sulfate and glucuronide metabolites) in normal and malignant colorectal tissue at concentrations compatible to exert pharmacologic activity [170]. In view of its lipophilicity, topical application of curcumin might also be effective. Topical treatment of burned and photo-damaged skin with curcumin in a gel base was shown to possess healing and repair effects [18].

Maximal dietary intake has been estimated at 1.5 g of turmeric per person per day in certain South East Asian communities [171], and therefore the consumption of curcumin is much lower. Thus, based on the prospective human studies reported above, exposure to curcumin or its active metabolites (apart from gastrointestinal tract) from normal diet consumption is expected to be insufficient to reach any biological activity. However, these phase I and phase II clinical trials do not consider the possible involvement of other factors present in the diet that may boost curcumin bioavailability.

Compounds and other polyphenols present in the food matrix may interact positively for enhancement of bioavailability of phenolic compounds. Upon epithelial uptake, certain flavonoids may reduce and/or inhibit phase II metabolic enzymes and influence efflux transporters such as p-glycoprotein [172]. Dietary lipids may also increase polyphenol bioaccessibility, specially for hydrophobic molecules such as curcumin and other polyphenol aglycones [172]. These notions are already being explored to increase curcumin bioavailability and might be used in the future in functional foods for prevention and pharmacological strategies in aging interventions. Rather than a high dose of curcumin, a low dose supplement of lipidated curcumin showed diverse health promoting effects in healthy middle-aged people [164]. Zou et al. are also developing an excipient corn oil-in-water emulsion as a food matrix to increase oral bioavailability of curcumin [173]. Although excipient foods have no bioactivity themselves, they may promote that of co-ingested bioactives [174]. These excipients may increase the release of lipophilic curcumin from food matrices, improve its solubility in gastrointestinal fluids, and enhance epithelium cell permeability. On the other hand, the piperine compound from black pepper has been shown to synergize with curcumin to increase significantly its bioavailability. Piperine, which inhibits the intestinal and hepatic metabolic enzymes involved in the glucuronidation, was successfully used to increase serum concentration of curcumin in rats and humans [175]. This principle was already tested in clinical trials in patients with multiple myeloma and against mild cognitive impairment in the United States (see www.clinicaltrials.gov; search for curcumin), but without significant or available results. The use of adjuvants to enhance curcumin bioavailability by increasing absorption and decreasing metabolic clearance are also being explored in new drug delivery systems, such as nanoparticles and self-microemulsions [176,177].

5.3. New delivery systems

Different approaches have been developed to overcome the poor oral bioavailability of curcumin due to various physicochemical and physiological processes. Apart from the use of adjuvants to decrease curcumin metabolization (see above), synthetic analogues and conjugates are being developed to be metabolically stable [12,168,178,179]. Nevertheless, much of the effort has been focused in the development of new and improved drug delivery systems for curcumin by means of nanotechnology. Several types of biocompatible and biodegradable nanoparticles are being developed to

suitably encapsulate and deliver curcumin at higher concentrations to target organs or tissues in order to improve curcumin's therapeutic efficacy. These include liposomes, polymeric nanoparticles, nanoemulsions, phospholipid formulations, cyclodextrins, and nanogels (for review see Refs [15] and [180]).

Nanoparticles can increase solubility and stability of polyphenols in biological fluids, enhance their absorption in several epithelia, prevent them from premature metabolization and excretion from the body, and allow specific targeting, ultimately resulting in improved bioactivity with minor side effects [181]. Most of the nanodelivery studies are related with the effects of curcumin in the treatment of several ailments, mainly cancer [182–184], brain damage [185–188], and inflammation [189–191]. Some of these new bioavailable curcumin formulations are being already tested in phase I clinical trials [192].

These nanotechnological innovative approaches might also be useful in nutraceutical applications of curcumin to prevent age-related diseases and to improve healthspan. However, as a very recent field, nanoparticles still presents many difficulties and limitations to their use. Major challenges are the potential toxicity of nanoparticles, their efficient absorption through the different biological barriers until the target tissue is reached, and cost [181]. Another possible limitation of the use of these drug delivery systems is the potential side effects of highly effective curcumin delivery to target organs. The improvement of curcumin bioavailability by these new formulations might induce toxic effects that have not been addressed yet in the interventions done with free curcumin [9]. For example, some adverse events experienced by volunteers that ingested very high doses of curcumin, such as nausea, diarrhea and rash [193], might be exponentially exacerbated. In addition, toxic effects and strong prooxidant effects observed for high concentrations of curcumin in *in vitro* studies may also happen *in vivo* with these efficient delivery vehicles [9]. Instead, other alternative concepts of intervention, such as exploiting the hormetic effects of curcumin and possible involvement of the gut microbiome are worthy of future investigation regarding the promotion of healthy aging, and should be considered in the design of novel clinical trials.

5.4. Applications

As mentioned before, due to the millennial use of turmeric as food condiment and in phytomedicine, and its attributed beneficial effects against several ailments, curcumin and curcumin-containing plant extracts have been already applied in several commercial products sold worldwide, but especially in South East Asian countries and USA [15]. Although the efficacy of some of these products has never been scientifically proven, the pleiotropic biological activities of curcumin have been supported in the last years by hundreds of *in vitro* and *in vivo* preclinical studies as well as some human intervention studies. Although its chemical proprieties (lipophilicity and intense yellow color) and low bioavailability are hampering the development of curcumin for clinical applications, novel formulations and drug delivery systems may lead the way to a promising future for the use of this compound. Its multitargeting faculty enables curcumin to modulate the activity of several proteins and signaling pathways, which together with its apparent safe profile and low cost, make curcumin an ideal candidate for nutritional interventions to improve healthspan and longevity. Data collected in animal models and in humans are also encouraging for the use of curcumin in the treatment of cancer, stroke, Alzheimer's disease, and Parkinson's disease [12].

An interesting application of curcumin is its use against skin aging and photoaging. Its cosmetic use for skin care and aging and wound healing is already a reality [15]. These applications are supported by several studies, from animals to humans. Curcumin has shown *in vitro* the ability to hormetically stimulate wound healing of human fibroblasts [16], and to be effective in the treatment of burn wounds in rats and humans [17,18]. Evidences from the literature indicate the ability of curcumin to enhance collagen deposition, tissue remodeling and wound contraction as important processes for proper wound healing [194]. Application of curcumin in new formulations has been identified as essential for optimizing its therapeutic effect in wound healing [194]. Turmeric extracts and curcumin also significantly inhibited the ultraviolet-induced photodamage in mice and humans [18,195–198]. Anti-inflammatory and antioxidant actions of curcumin are proposed to mediate not only the burn wound healing capacity of this polyphenol but also its anti-photoaging effects [18,194,199]. Therefore, all these data support the use of curcumin in cosmeceuticals, which combined with the development of new formulations may help to maximize its beneficial skin anti-aging effects.

The hormetic effects of curcumin discussed above can also have tremendous relevance on the preventive potential of this polyphenol against aging and age-related diseases. The hormetic modulation of aging by nutritional factors by inducing mild stress and increasing antioxidant defenses and other cellular maintenance and repair pathways has been increasingly recognized as potentially applicable to aging intervention strategies [200]. However, in order to fully explore the mild stress-induced hormesis attained by curcumin, the problem of ensuring that tissues and organs are exposed to the correct dose to attain positive effects needs to be overcome. Further research in new formulations and nanotechnologies to improve bioavailability of curcumin and to ensure the correct dosage to obtain beneficial effects from mild stress and avoid toxicity are needed to envisage the use of curcumin as a hormetin.

Fig. 4 schematically show the possible future applications of curcumin for stimulation of a healthy aging, which may depend on forthcoming research in relevant formulations, nanoparticles, food matrices, and routes of applications. The different approaches, whether with the aim to treat age-related diseases, preventing them or intervening in the basic process of aging, will need to be tested through adequate experimental studies with animal models and with human subjects. This will be essential to validate the ancient and novel attributed nutritional and pharmacological actions of curcumin, ultimately seeking the achievement of healthy aging and healthspan extension.

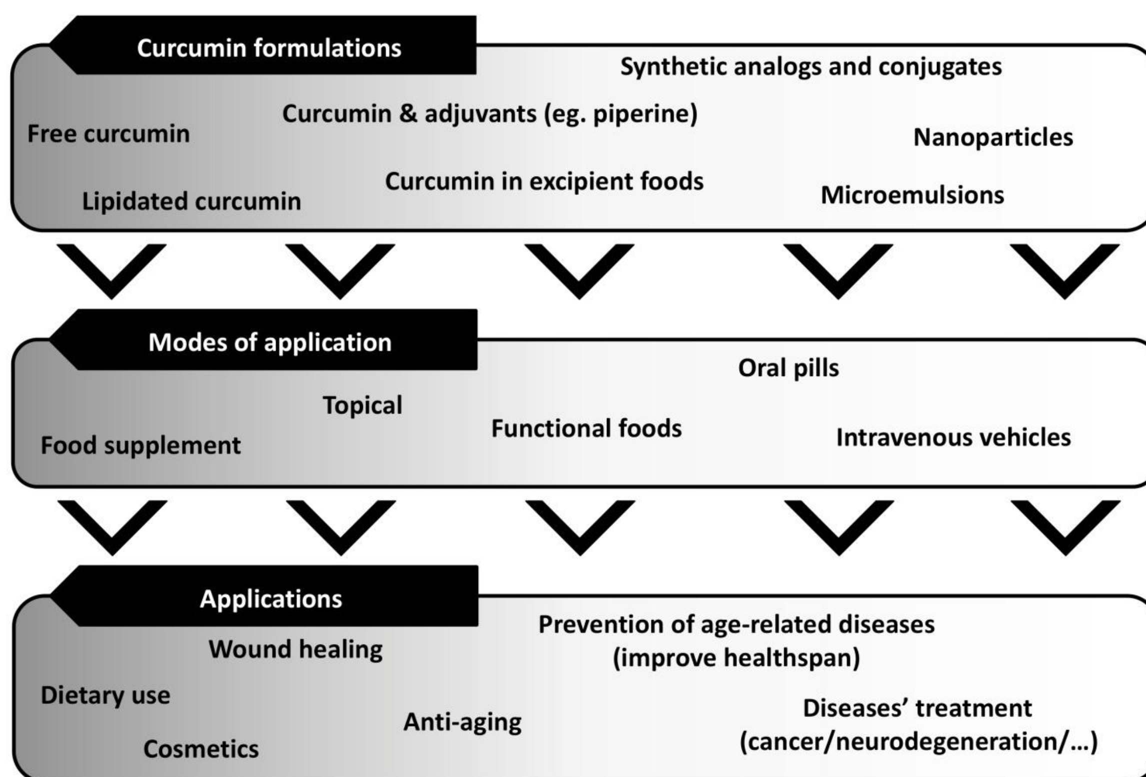

Fig.4. Actual and possible future applications of curcumin for stimulation of healthy aging: formulations involved and modes of application.

6. Concluding remarks

The ancient use of turmeric for treatment of several ailments and the discovery that the polyphenolic curcumin is one of its most active compounds have guided in the last decades thousands of studies regarding this natural compound. Several biological activities, putative cellular targets and potential therapeutic effects were identified in numerous pre-clinical studies confirming many folk uses of turmeric and attributed new effects to curcumin. The potential beneficial effects of curcumin on aging arise from the modulation of several molecular and biochemical targets relevant in the context of the recent proposed aging hallmarks, as well as by its lifespan extension capabilities in different model organisms. There is a lack, however, of epidemiological and clinical evidences with human subjects of the health promoting effects and increased longevity provided by curcumin. In view of its low bioavailability that is hampering its clinical application, different curcumin formulations, including drug nanodelivery systems, are being developed to target specific tissues at therapeutic concentrations.

Considering the hormetic effects of curcumin and potential to induce senescence at high doses, appropriate tissue exposure needs to be established with proper

experimental design in order to foresee the use of curcumin in nutritional interventions for improving healthspan and longevity. The potential interactions with other phytochemicals, the effect of food matrices and the use of new curcumin formulations should be explored in the context of functional foods. Considering the aging of global population and increased incidence of age-related diseases and morbidity, both in developed and developing countries, new and cost-effective strategies need to be employed. The multitargeting ability of curcumin may constitute a challenging opportunity as a golden nutraceutical for a healthy aging.

Key facts

• The ancestral use of turmeric in traditional medicine and the extensive research over the last decades suggest that curcumin can be exploited for future nutritional and pharmacological interventions.

• The peculiar chemical structure of curcumin is behind its diverse biological activities and ability to interact and regulate multiple molecular targets.

• The effects of curcumin in aging and longevity are related with its multitargeting capacity.

• Intense research is underway to envisage the future application of curcumin in the improvement of human healthspan and longevity.

Summary

• Turmeric has been used in traditional medicine for thousands of years to treat several medical conditions.

• Curcumin is the main active constituent of turmeric and it has powerful antioxidant and anti-inflammatory properties.

• Numerous biochemical and molecular targets relevant to the hallmarks of aging are regulated by curcumin.

• Curcumin extends the lifespan of model organisms, including worms and flies.

• Many lines of evidence demonstrate the potential of curcumin for the prevention and/or treatment of age-related diseases, such as cancer and neurodegenerative diseases.

• Human intervention studies and clinical trials are being conducted to demonstrate that curcumin increases healthspan.

• The nutritional and pharmacological applications of curcumin are restricted by its poor solubility and low bioavailability.
• Different formulations are being developed to increase curcumin bioavailability, including new delivery systems like nanoparticles.
• Applications of curcumin are already a reality in wound healing and cosmetics against skin aging.
• The caloric restriction mimetic and hormetic effects of curcumin may be explored for future aging nutraceutical applications.
• Considering the global population aging, curcumin may constitute an interesting low cost and effective natural compound for interventions to achieve healthy aging.

Dictionary of terms

• Curry powder: Curry powder is a blend of up to 30 (average 10) different spices and herbs; turmeric is one of the basic components, which gives curry its characteristic golden color.
• Functional food: A natural or processed food that contains biologically active components that provide health benefits beyond that of basic nutrients it contains.
• Apoptosis: A type of programmed cell death in which an active sequence of events regulated by different groups of executioner and regulatory molecules leads to the self-destruction of the cell without releasing intracellular constituents to intercellular space.
• Glutathione: A tripeptide of glycine, cysteine, and glutamic acid, existing in reduced (GSH) and oxidized (GSSG); glutathione disulfide) forms, which is an important intracellular antioxidant and cofactor of different enzymes that has numerous roles in protecting cells from oxidants and in maintaining cellular thiol-disulfide redox state.
• Hormesis: In the biology/medicine field, hormesis is a process in which exposure to a low dose or moderate stressor (that is damaging at higher concentrations) elicits adaptive beneficial responses, which may result in health promotion effects.
• Epigenome: Potentially heritable chemical modifications to the DNA and associated structures (eg, histone proteins) that can result in changes of genetic expression independent of the DNA sequence of a gene. Unlike the genome, the epigenome can be modified by environmental factors including dietary constituents.
• Proteasome: Is a multicomponent enzymatic system incorporating different regulators and a catalytic core with different proteases responsible for the degradation of a large

portion of soluble intracellular proteins. The proteasome is an important component of the intracellular system for the turnover of proteins.

• Autophagy: A catabolic process of cellular selfdigestion in which proteins and organelles are engulfed by double-membrane autophagosomes and degraded in lysosomes by proteases, leading to recycling of macromolecular constituents.

• Mammalian target of rapamycin (mTOR): Evolutionarily conserved serine/threonine protein kinase that regulates protein synthesis and degradation processes important for cell growth, proliferation, motility and survival. mTOR is a key autophagic regulator, whose inhibition activates autophagy.

• Tetrahydrocurcumin (THC): A metabolite of curcumin that retains many effects of curcumin, such as the antioxidant and anti-inflammatory activities. Curcumin is metabolized to THC after oral ingestion by reductases found in the intestinal epithelium, and the structures vary only by the lack of the double bonds in the seven carbon linker of curcumin.

• Nanoparticle: A small particle generally between 1 and 100 nanometers in size. In biomedical research, several types of nanoparticles are being developed to carry and deliver compounds to particular biological targets.

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CHAPTER 3

Redox-dependent induction of antioxidant defenses by phenolic diterpenes confers stress tolerance in normal human skin fibroblasts: Insights on replicative senescence

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Redox-dependent induction of antioxidant defenses by phenolic diterpenes confers stress tolerance in normal human skin fibroblasts: Insights on replicative senescence

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Abstract

Mild stress-induced hormesis represents a promising strategy for targeting the age-related accumulation of molecular damage and, therefore, for preventing diseases and achieving healthy aging. Fruits, vegetables, and spices contain a wide variety of hormetic phytochemicals, which may explain the beneficial health effects associated with the consumption of these dietary components. In the present study, the induction of cellular antioxidant defenses by the phenolic diterpenes carnosic acid (CA) and carnosol (CS) were studied in normal human skin fibroblasts, and insights into the aging process at the cellular level investigated. We observed that CA and CS induced several cytoprotective enzymes and antioxidant defenses in human fibroblasts, whose induction was dependent on the cellular redox state for CS and associated with Nrf2 signaling for both compounds. The stress response elicited by preincubation with CS conferred a cytoprotective action against a following oxidant challenge with *tert*-butyl hydroperoxide, confirming its hormetic effect. Preincubation of normal fibroblasts with CS also protected against hydrogen peroxide-induced premature senescence. Furthermore, cultivation of middle passage normal human skin fibroblasts in the presence of CS ameliorated the physiological state of cells during replicative senescence. Our results support the view that mild stress-induced antioxidant defenses by CS can confer stress tolerance in normal cells and may have important implications in the promotion of healthy aging.

Keywords: Phenolic diterpenes; Nrf2; Normal human skin fibroblasts; Cytoprotection; Aging.

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1. Introduction

Population aging was one of the most distinctive demographic events marking the twentieth century and will certainly remain an important tendency throughout this century [1,2]. The worldwide prolongation of the mean life expectancy has resulted in a rapid increase in the size of the elderly population, both in absolute numbers and as a proportion and, consequently, increased the incidence of age-related diseases [1]. As such, population aging presents new and serious medical, social, and financial challenges for modern societies [1,3].

Aging is characterized by the progressive accumulation of molecular damage, whose main cause is the inefficiency and failure of cellular maintenance and repair mechanisms. Therefore, mild stress-induced stimulation of these mechanisms has been recognized as a promising strategy to prevent age-related diseases and achieve healthy aging [4–6]. The process in which exposure to a low level of stress elicits adaptive beneficial responses that protect against subsequent exposure to severe stress is a phenomenon known as hormesis [7,8]. The paradigm for hormesis is physical exercise, which stresses the muscle cells but induces adaptation when practiced moderately. Indeed, moderate exercise is beneficial to health by increasing the physiological function of different organs. On the other hand, physical inactivity and arduous exercise decrease physiological function and increase the risk of diseases [9].

Fruits, vegetables, and spices contain a wide variety of hormetic phytochemicals, such as resveratrol, sulforaphane, and curcumin, that stimulate cell stress-induced maintenance and repair mechanisms and which may explain the health benefits associated with the consumption of these dietary components [10–16]. Many of these hormetic phytochemicals are inducers of the nuclear factor erythroid 2-related factor (Nrf2), a basic leucine zipper transcription factor that plays a key role in orchestrating the induction of several cytoprotective genes containing at least one antioxidant response element (ARE) within their promoters [17,18]. Under normal conditions, the Kelch-like ECH-associated protein (Keap1) forms a complex with cullin 3 (Cul3) and represses Nrf2 by presenting it for ubiquitination and proteasomal degradation. On stimulation, the highly reactive cysteine residues of Keap1 are modified, resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2. Under these conditions, the transcription factor accumulates in the nucleus and, in heterodimeric combination with small Maf transcription factors, binds to ARE and recruits the basal transcriptional machinery to

activate transcription of genes encoding stress-responsive and cytoprotective enzymes and related proteins [18–20].

Several studies on different species demonstrated that expression of Nrf2 and its target genes declines during aging and disease [21–24]. Indeed, the decline of Nrf2 transcriptional activity causes the age-related loss of glutathione synthesis, which adversely affects cellular thiol redox balance, leaving cells highly susceptible to different stresses [21]. In some animal models, such as *Drosophila* and *Caenorhabditis elegans*, Nrf2 orthologues have been associated with oxidative stress tolerance and aging modulation [24–27]. Therefore, the Nrf2 signaling pathway has been pointed out as a regulator of health span and its induction by hormetic phytochemicals may explain their health-promoting effects [14,26].

In the present study, carnosic acid (CA) and carnosol (CS), two phenolic diterpenes found in *Rosmarinus officinalis* (rosemary) that have been reported to activate the Nrf2/ARE signaling pathway [28– 30], were used. Their ability to induce antioxidant defenses and cytoprotective enzymes, as well as to confer stress tolerance, was investigated in normal diploid human skin fibroblasts to better link the effects to the aging process at the cellular level. For that, both compounds were also studied using a model of stress-induced premature senescence and during replicative senescence.

2. Materials and methods

Chemicals and antibodies

Carnosic acid and carnosol were purchased from LGC standards (Teddington, UK). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, kinase inhibitors LY294002, PD98059, and SP600125, *tert*-butyl hydroperoxide (*tert*-BOOH), hydrogen peroxide (H_2O_2), anti-β-actin antibody (dilution 1:2000), and all other not specified reagents were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany), complete protease inhibitor cocktail from Roche (Penzberg, Germany), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) from Apollo Scientific Ltd (Stockport, UK), 2',7'-dichlorodihydrofluorescein diacetate from Molecular Probes (Eugene, OR, USA), and SYBR Gold nucleic acid gel stain was purchased from Invitrogen (Paisley, UK).

An SV Total RNA isolation system was purchased from Promega (Madison, WI, USA), iScript cDNA synthesis kit and SsoFast EvaGreen Supermix from Bio-Rad

Laboratories (Hercules, CA, USA), primers from STAB VIDA (Caparica, Portugal), and TransAM Nrf2 kit from Active Motif (Carlsbad, CA, USA).

Antibodies against NQO1 (dilution 1:1000), ferritin (dilution 1:250), and histone H1 (dilution 1:250) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GCLM antibody (dilution 1:500) developed by the Clinical Proteomics Technologies for Cancer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology (Iowa City, IA, USA). Anti-HO-1 (dilution 1:500) and anti-GST-Pi (dilution 1:1000) antibodies were from Enzo Life Sciences (Farmingdale, NY, USA), anti-caspase-3 (dilution 1:5000) from EMD Millipore Corporation (Billerica, MA, USA), and anti-Nrf2 (dilution 1:5000) from Novus Biologicals (Littleton, CO, USA). Finally, horse anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and experimental conditions

Normal diploid adult human skin fibroblasts (ASF-2 cells) isolated from a breast biopsy specimen of a consenting young healthy Danish woman (aged 28 years) were used [31]. Cells were maintained in DMEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, and 10 mM Hepes, at 37 °C in a humidified incubator containing 5% CO_2. Culture medium was renewed every 2 days until cells reached 90–95% confluence and then they were subcultivated. At each subcultivation, harvested cells were counted in a hemocytometer and the number of population doublings was calculated using the following equation: log10 [(number of cells harvested) / (number of cells seeded)] / log10 (2). The calculated population doublings were then added to the previous population doublings, to yield the cumulative population doublings (CPDs) [32]. All experiments were performed in low passage fibroblasts (cumulative population doublings between 15 and 25), except the replicative senescence experiments that were performed in middle passage fibroblasts (cumulative population doublings between 30 and 35), at a density of 50,000 cells/ml. CA and CS were dissolved in dimethyl sulfoxide (DMSO) at such concentrations that the final solvent concentration did not exceed 0.5% (v/v) when added to the cell culture medium. A similar concentration of DMSO (up to 0.5% v/ v) was added to controls.

Glutathione content

Glutathione (GSH) content was determined by the DTNB-GSSG reductase recycling assay as previously described [33], with some modifications [34]. Briefly, after protein precipitation with 5-sulfosalicylic acid, samples were centrifuged and the resultant supernatants used for measurement of glutathione following the TNB formation at 415 nm and compared with a standard curve. The results were expressed as nanomole GSH per milligram of protein.

Total protein extraction

Total protein extracts were performed as previously described [12]. Briefly, cells were rinsed with PBS and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% v/ v NP-40) containing 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF, and 1 × complete protease inhibitor cocktail. Protein concentration was quantified using the DC protein assay (Bio-Rad Laboratories) and BSA used as protein standard.

Cytosolic and nuclear protein extraction

Cytosolic and nuclear protein extracts were prepared as previously described [12]. Briefly, cells were incubated with ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5 mM DTT, 1 mM PMSF, and 1 × complete protease inhibitor cocktail and then NP-40 was added to a final concentration 0.7% (v/v). The homogenate was centrifuged and the cytosolic supernatant harvested. The nuclear pellet was resuspended in ice-cold nuclear buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) containing 0.5 mM DTT, 1 mM PMSF, and 1 × complete protease inhibitor cocktail, and after centrifugation, the supernatant containing the nuclear proteins was harvested. Protein concentration was quantified as described above.

Western blotting

For Western blot analysis, equal amounts of protein were separated in a SDS polyacrylamide gel and then transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were blocked in TPBS (PBS with 0.05% Tween 20) containing 5% (w/v) nonfat dry milk, washed in TPBS, and then incubated with primary antibody of interest. After washing, membranes were incubated with the appropriate secondary antibody conjugated with IgG horseradish

peroxidase. Blots were visualized using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories). Band area intensity was analyzed using Quantity One software (Bio-Rad Laboratories).

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted from cells with the SV Total RNA isolation system (Promega) and cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories), according to the manufacturer's instructions.

Quantitative real-time PCRs were prepared according to the SsoFast EvaGreen Supermix manufacturer's instructions (Bio-Rad Laboratories). Briefly, each reaction contained 10 μl of SsoFast EvaGreen Supermix, 1 μl of forward (Fw) and reverse (Re) primers (10 pmol), 1 μl of template cDNA (50 ng), and the appropriate volume of nuclease-free water to complete 20 μl. Primer sequences used to detect human transcripts are shown in Table 1. Standard curves for each primer set were generated using a dilution series of a mixture of cDNA samples. No template controls were included in all assays. PCRs were run in duplicate in the CFX96 real-time PCR detection system (Bio-Rad Laboratories) and the conditions used were: denaturation program (95 °C for 3 min), amplification and quantification program repeated 40 times (95 °C for 10 s, 60 °C for 30 s with a single fluorescence measurement), and melting curve program (65 to 95 °C with increments of 0.5 °C/s and continuous fluorescence measurements).

Relative gene expression was calculated by a mathematical method based on the PCR efficiencies (calculated from standard curves) and the Ct deviation of an unknown sample versus a control, and expressed in comparison to a reference gene [35]. β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes, with data calculated in relation to the β-actin gene and verified with the GAPDH gene. The averages of the duplicates of each independent experiment were used for statistical analysis.

Table 1
Primer sequences used for quantitative real-time PCR.

Gene	Primer sequences (5′-3′)	References
GCLC	Fw: GTCTTCAGGTGACATTCCAAGC Re: TGTTCTTCAGGGGCTCCAGTC	[21]
NQO1	Fw: CTGGTTTGAGCGAGTGTTCA Re: TTCCATCCTTCCAGGATTTG	[65]
FTH1	Fw: CTTTGACCGCGATGATGTGGCTTT Re: TTTGTCAGTGGCCAGTTTGTGCAG	[66]
TXNRD1	Fw: ATCAGGAGGGCAGACTTCAA Re: CCCACATTCACACATGTTCC	[65]
β-actin	Fw: GAGCGGGAAATCGTGCGTGAC Re: GCCTAGAAGCATTTGCGGTGGAC	
GAPDH	Fw: AGGTCGGTGTGAACGGATTTG Re: TGTAGACCATGTAGTTGAGGTCA	

Nrf2 activation

Nrf2 activation was assessed using the ELISA-based TransAM Nrf2 kit (Active Motif), according to the manufacturer's guidelines. Briefly, nuclear extracts (5 μg) were added to wells coated with an oligonucleotide containing the ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3'), to which the active form of Nrf2 specifically binds. A primary antibody against Nrf2 was then used to detect bound Nrf2. A secondary antibody conjugated to horseradish peroxidase (HRP) allowed a colorimetric readout at 450 nm of Nrf2 activation. Nuclear extract from COS-7 cells transfected with Nrf2 was included as positive control.

Cell viability and growth

Cell viability was evaluated by the lactate dehydrogenase (LDH) release assay or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as previously described by Lima et al. [36] and by Xavier et al. [37] with some modifications, respectively.

In the LDH assay, the activity of LDH was measured at 30 °C by quantification of NADH consumption by continuous spectrophotometry (at 340 nm) on a microplate reader (SpectraMax 340 pc, Molecular Devices, Sunnyvale, CA, USA) using pyruvate as substrate. Cell viability was calculated using the following equation: cell viability (%) = (100 – extracellular LDH) / (100 – total LDH)) × 100.

In the MTT assay, cells were incubated with MTT to a final concentration of 0.5 mg/ml for 2 h. Then, the medium was removed, the formazan crystals formed by the cell's capacity to reduce MTT were dissolved with a 50:50 (v/v) DMSO:ethanol solution, and the absorbance was measured at 570 nm (using 690 nm as reference wavelength).

Cell growth was determined 4 days after compound incubation by the MTT assay. During this time period cells significantly grew and this increase in cell number results in an increase in the amount of formazan formed and an increase in absorbance.

In replicative senescence experiments, cell growth was analyzed by calculating the CPDs at each subcultivation [38]. Cells were considered senescent (irreversibly growth arrested) when cell density did not increase within 3 weeks.

3.2.10. Cytochemical staining for senescence-associated β-galactosidase (SA-β-gal)

Cytochemical staining for SA-β-Gal at pH 6.0 was performed as previously described [39]. Briefly, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 5 min, washed three times in PBS, and then incubated with freshly prepared SA-β-Gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$, 40 mM citric acid/sodium phosphate buffer at pH 6.0) for 24 h at 37 °C. Phase-contrast images of representative fields were captured using an inverted microscope equipped with a DP72 digital camera (Olympus IX70, Olympus, Hamburg, Germany) and the percentage of SA-β-Gal-positive cells was calculated by the following formula: 100 × (number of blue cells/total number of cells in the same field).

Analysis of cell size and lipofuscin content by flow cytometry

Senescent-related parameters such as the cell size and lipofuscin content were analyzed by flow cytometry as previously described [40]. For that, ASF-2 cells were trypsinized, collected in complete medium at 4 °C and immediately used for analysis in a flow cytometer (EPICS XL, Beckman Coulter, Brea, CA, USA). Cell size was monitored by forward scatter (FSC) and yellow-green autofluorescence, a marker of cellular lipofuscin content, was measured in FL1 channel.

Statistical analysis

Data were expressed as mean ± SEM of at least three independent experiments. Statistical significances were assessed by the Student's *t* test, one-way ANOVA followed by Dunnett´s multiple comparison test, or two-way ANOVA followed by the Bonferroni

posttest, as appropriate, using GraphPad Prism 5.0 software (Graph-Pad Software, San Diego, CA, USA). Differences between groups were considered to be significant when $P \leq 0.05$.

3. Results

3.1. Carnosic acid and carnosol induce antioxidant defenses

To test whether phenolic diterpenes induce antioxidant defenses in normal diploid human skin fibroblasts (ASF-2 cells) without toxic effects, cells were incubated with 2.5–40 μM CA or CS for 48 h and cell viability evaluated by the MTT assay. CA and CS did not significantly inhibit cell growth at concentrations up to 40 and 20 μM, respectively (see Suppl. Fig. 1), which were therefore the highest concentrations applied in subsequent experiments.

GSH is a well-studied cellular antioxidant and has numerous roles in protecting cells from oxidants and in maintaining cellular thiol-disulfide redox status. Previous studies have reported that CA and CS induce GSH levels, in different cell types [30,41,42]. Here, using normal human ASF-2 cells, CA and CS significantly induced GSH levels, in a concentration-dependent manner. CA and CS at 20 μM significantly induced GSH levels after 24 h of incubation by around 105 and 59%, respectively (Fig. 1A). In time, GSH levels increased after 16 h of incubation with phenolic diterpenes and persisted for over 24 h. After 16 h of incubation, CA (20 μM) and CS (20 μM) significantly induced GSH levels by around 77 and 57%, respectively (Fig. 1B).

In addition to GSH levels, the ability of CA and CS to modulate the levels of cytoprotective enzymes in ASF-2 cells was studied by Western blot. Both compounds induced heme oxygenase-1 (HO-1) and glutamate cysteine ligase modulatory subunit (GCLM) in a concentration- and time-dependent manner (Fig. 1C and D and Suppl. Fig. 2). Although the highest expression of HO-1 induced by these phenolic diterpenes was observed after 8–16 h of incubation, for GCLM this occurred after around 16–24 h of incubation (Fig. 1D and Suppl. Fig. 2C and D).

Treatment of ASF-2 cells with CA (10 and 20 μM) and CS (5 and 10 μM) for 16 h also induced protein levels of NADP(H):quinone oxidoreductase-1 (NQO1), glutathione S-transferase P1 (GST-P1), and ferritin heavy chain (FTH1) (Fig. 2A and Suppl. Fig. 3). The mRNA levels of some cytoprotective enzymes were also evaluated by real-time PCR. As shown in Fig. 2B, CA (40 μM) and CS (20 μM) for 4 h increased the mRNA levels of

glutamate cysteine ligase catalytic subunit (GCLC), NQO1, FTH1, and thioredoxin reductase 1 (TXNRD1).

To test whether the induction of GSH and cytoprotective proteins levels by these phenolic diterpenes was due to induction of reactive oxygen species (ROS), the ASF-2 cells were incubated with CA (40 μM) and CS (20 μM) for 30 min and the ROS levels were measured using the DCF probe. Neither compound altered significantly ROS levels, contrary to the oxidant *tert*-BOOH (see Suppl. Table 1.). In addition, using the alkaline version of the comet assay, CA and CS did not induce DNA damage (see Suppl. Table 1), which indicates that stress responses elicited by the phenolic diterpenes were not due to the induction of ROS.

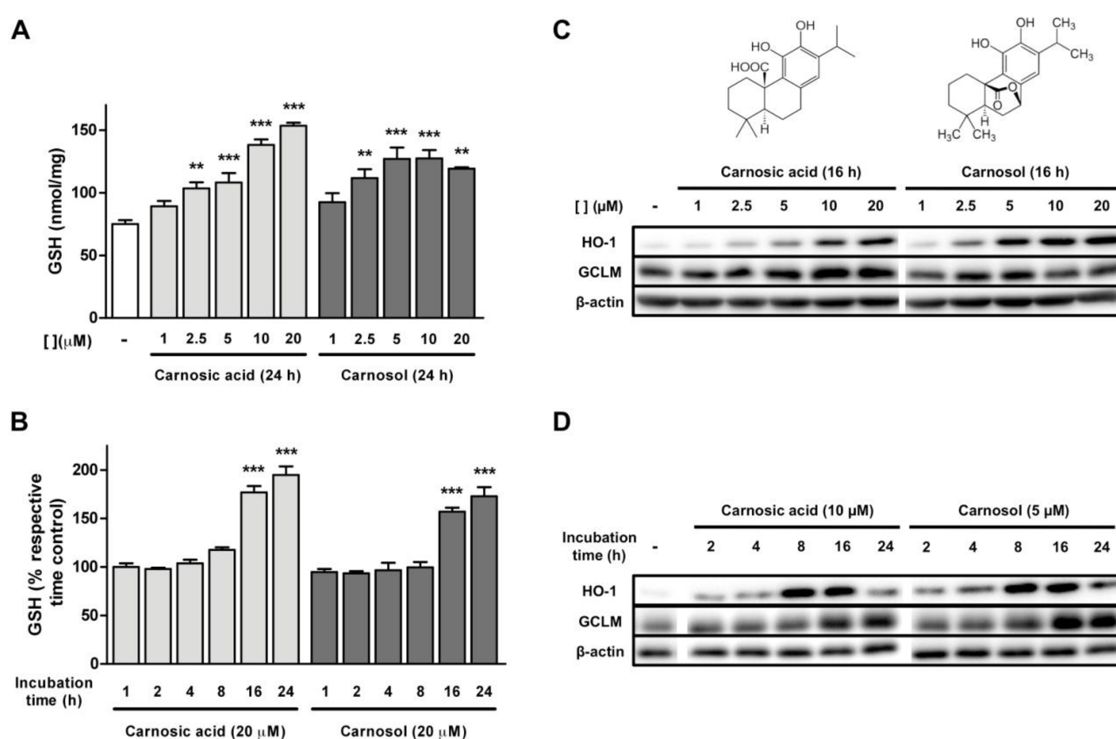

Fig. 1. Effects of carnosic acid (CA) and carnosol (CS) on the levels of GSH and cytoprotective proteins in ASF-2 cells. Cells were incubated with increasing concentrations of CA and CS for 24 h (A), or with CA (20 μM) and CS (20 μM) for different incubation times (B), and GSH levels were measured. Values are mean ± SEM of at least three independent experiments. ** $P \leq 0.01$; *** $P \leq 0.001$ when compared with control (A) or with the respective 1 h incubation (B) by one-way ANOVA. The expression of different cytoprotective enzymes (HO-1, heme oxygenase-1; GCLM, glutamate cysteine ligase modulatory subunit) was also analyzed by Western blot after incubation of cells with increasing concentrations of CA and CS for 16 h (C), or with CA (10 μM) and CS (5 μM) for different incubation times (D). Blots are representative of three independent experiments and β-actin was used as loading control. The band intensities were quantified by densitometric analysis and the expression levels relative to that of β-actin are presented in Suppl. Fig. 2. The chemical structures of carnosic acid and carnosol are represented in (C).

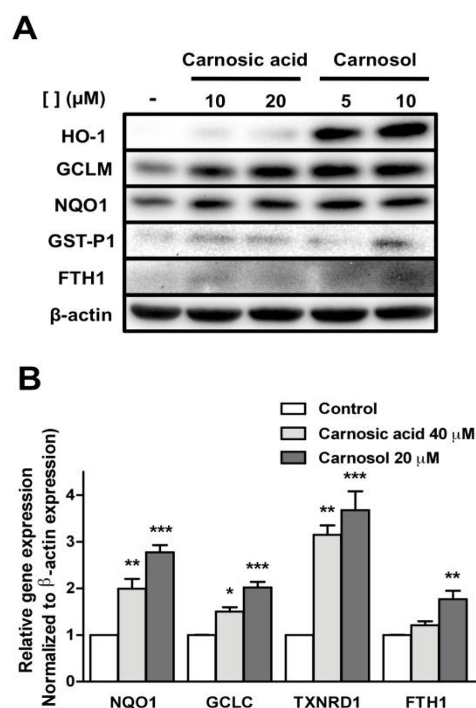

Fig. 2. Effects of carnosic acid (CA) and carnosol (CS) on the levels of cytoprotective proteins in ASF-2 cells. (A) Cells were incubated with CA (10 and 20 μM) and CS (5 and 10 μM) for 16 h, and the protein levels of several cytoprotective enzymes (HO-1, heme oxygenase-1; GCLM, glutamate cysteine ligase modulatory subunit; NQO1, NADP(H):quinone oxidoreductase-1; GST-P1, glutathione S-transferase P1; FTH1, ferritin heavy chain) were measured by Western blot. Blots are representative of three independent experiments and β-actin was used as loading control. The band intensities of HO-1, GCLM, NQO1, and β-actin were quantified by densitometric analysis and the expression levels relative to that of β-actin are presented in Suppl. Fig. 3. (B) Cells were incubated with CA (40 μM) and CS (20 μM) for 4 h, and the mRNA levels of cytoprotective proteins (GCLC, glutamate cysteine ligase catalytic subunit; NQO1, NADP(H):quinone oxidoreductase-1; FTH1, ferritin heavy chain; TXNRD1, thioredoxin reductase 1) were measured by real-time PCR. Results were normalized to the β-actin relative expression. Values are mean±SEM of three independent experiments. Statistical significances were evaluated for each gene. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ when compared with respective control by one-way ANOVA.

3.2. The effects of carnosic acid and carnosol are dependent on redox stress and associated with Nrf2 signaling

The induction of antioxidant and phase 2 enzymes by phytochemicals is usually coordinated by activation of the Nrf2/ARE pathway, a stress response regulated by the cells' redox state [18]. In order to investigate whether redox stress was involved in the stress response elicited by the phenolic diterpenes, ASF-2 cells were incubated with CA (20 μM) and CS (10 μM) for 8 h with or without the antioxidant NAC (10 mM), after which the levels of HO-1 were measured. As shown in Fig. 3A (see also Suppl. Fig. 4), NAC inhibited the induction of HO-1 by CA and CS, indicating that a disturbance of cellular redox state contributes to the effects associated with CA and CS. To determine whether Nrf2 signaling was associated with the induction of cytoprotective enzymes by

the phenolic diterpenes, ASF-2 cells were incubated with CA (40 μM) and CS (20 μM) for 4 h, and the nuclear extracts were obtained. As shown in Fig. 3B, both compounds increased the levels of Nrf2 in the nuclear fraction. Finally, the transcriptional activity of Nrf2 was measured with an assay using an immobilized oligonucleotide containing the ARE consensus binding site. Treatment of ASF-2 cells with CA and CS significantly increased the transcriptional activity of Nrf2 (Fig. 3C), corroborating therefore that Nrf2 signaling is associated with the induction of cytoprotective enzymes by the phenolic diterpenes in normal human cells.

Activation of upstream kinases, such as phosphatidylinositol 3-kinase (PI3K)/Akt, has been considered to facilitate nuclear translocation and transcriptional activation of Nrf2 [18]. Therefore, ASF-2 cells were incubated with CA (20 μM) or CS (10 μM) for 8 h with or without inhibitors of different signaling pathways and the levels of HO-1 were measured by Western blot. LY (LY294002, 25 μM), inhibitor of PI3K; PD (PD98059, 25 μM), inhibitor of MAPKK (in MAPK/ERK pathway); and SP (SP600125, 10 μM), inhibitor of JNK were tested. LY remarkably decreased the expression of HO-1 induced by CA or CS, indicating that the PI3K/Akt pathway may be involved in the induction of HO-1 by the phenolic diterpenes (see Suppl. Fig. 5).

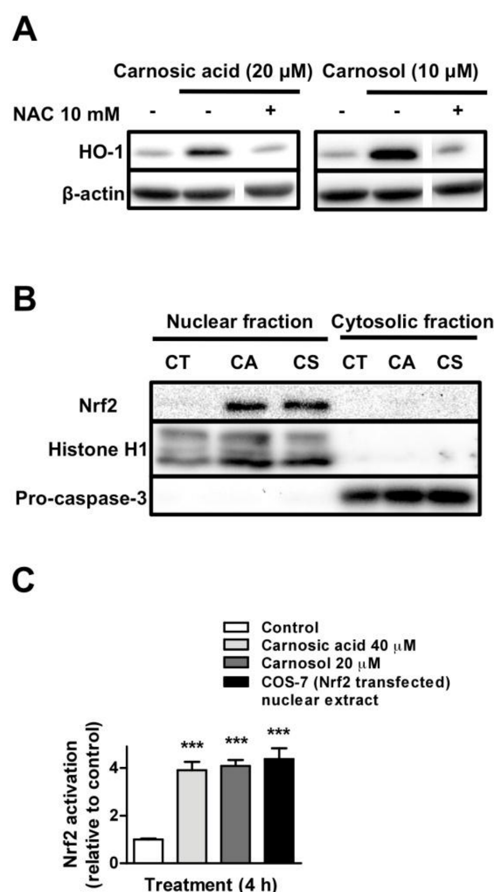

Fig. 3. Effects of carnosic acid (CA) and carnosol (CS) on redox and Nrf2/ARE signaling. (A) Cells were incubated with CA (20 μM) and CS (10 μM) for 8 h with or without the antioxidant NAC (10 mM), and the levels of heme oxygenase-1 (HO-1) were measured by Western blot. Blots are representative of three independent experiments and β-actin was used as loading control. The band intensities were quantified by densitometric analysis and the expression levels relative to that of β-actin are presented in Suppl. Fig. 4. (B) Cells were incubated with CA (40 μM) and CS (20 μM) for 4 h, and the levels of Nrf2 were measured by Western blot. Blots are representative of four independent experiments. Histone H1 was used as loading control for nuclear fraction and pro-caspase-3 as loading control for cytosolic fraction. (C) Cells were incubated with CA (40 μM) and CS (20 μM) for 4 h, and Nrf2 activation was assessed using the TransAM Nrf2 kit. Nuclear extract from COS-7 cells transfected with Nrf2 was included as positive control. Values are mean ± SEM of three independent experiments. *** $P \leq 0.001$ when compared with control by one-way ANOVA.

3.3. Carnosol increases stress tolerance in human fibroblasts

Considering the stress response elicited by the tested phenolic diterpenes in ASF-2 cells, we next tested whether this effect would protect cells against a following oxidant challenge. For that, cells were incubated with CA and CS for 4 h, followed by a recovery period of 6 h with fresh medium, and then exposed to the oxidant *tert*-BOOH (200 μM) for 3 h. This pulse incubation (4 h) with CA (40 μM) or CS (20 μM) followed by a recovery period (6 h) was enough to increase cells' antioxidant defenses, as shown by the

increase of GSH levels by 30 and 25%, respectively, under the CA and CS treatment conditions (Fig. 4A). However, only the pre-incubation treatment with CS conferred significant protection against *tert*-BOOH-induced cell death (Fig. 4B), demonstrating its hormetic effect. Moreover, NAC (10 mM) significantly prevented the protection conferred by CS, but not by CA, against *tert*-BOOH-induced cell death, which suggests that the induction of the stress response by CS may be due to the induction of cysteine-related redox stress (Fig. 4C and Suppl. Scheme 1).

In addition, a functional assay with fibroblasts was also performed after treatment with either CA or CS. When ASF-2 cells were preincubated with both compounds for 4 h, followed by a recovery period of 6 h with fresh medium, they significantly improved the *in vitro* wound healing capacity of the human fibroblasts (see Suppl. Fig. 6). Under coincubation conditions, using higher concentrations, the effect was inhibitory (data not shown), and using lower concentrations, the increase in wound healing ability was smaller than under the preincubation conditions (see Suppl. Fig. 6).

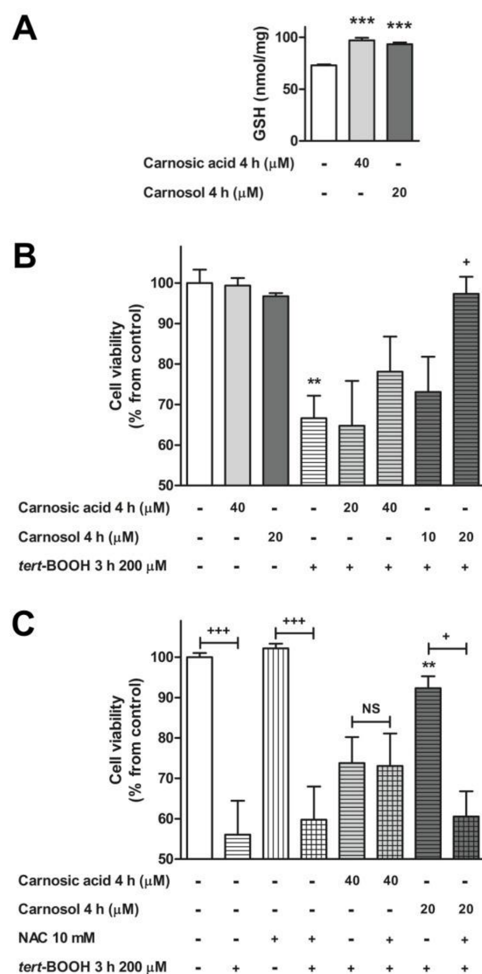

Fig. 4. Hormetic effects of carnosic acid (CA) and carnosol (CS) in ASF-2 cells. (A) Cells were incubated with CA (40 μM) and CS (20 μM) for 4 h, followed by a recovery period of 6 h with fresh medium, and GSH levels were measured. Values are mean ± SEM of four independent experiments. *** $P \leq 0.001$ when compared with control by one-way ANOVA. (B) Cells were incubated with CA (20 and 40 μM) and CS (10 and 20 μM) for 4 h, followed by a recovery period of 6 h with fresh medium. Then, cells were incubated with *tert*-BOOH (200 μM) for 3 h and cell viability was measured by the LDH leakage method. Values are mean ± SEM of four independent experiments. ** $P \leq 0.01$ when compared with control by the Student's *t*-test.;$^{+}$ $P \leq 0.05$ when compared with *tert*-BOOH alone by one-way ANOVA. (C) Cells were incubated with CA (40 μM) and CS (20 μM) with or without NAC for 4 h, followed by a recovery period of 6 h with fresh medium. Then, cells were incubated with *tert*-BOOH (200 μM) for 3 h and cell viability was measured by the LDH leakage method. Values are mean ± SEM of four independent experiments. Statistical significances were calculated using three independent two-way ANOVA (see details in Suppl. Scheme 1). ** $P \leq 0.01$ when compared with *tert*-BOOH alone. NS, not significant ($P > 0.05$); $^{+}P \leq 0.05$; $^{+++}$ $P \leq 0.001$ when compared with each other.

3.4. Carnosic acid and carnosol protect human fibroblasts against stress-induced premature senescence

In view of the induction of antioxidant defenses and cytoprotective enzymes provided by the phenolic diterpenes, we next tested whether it would protect the normal

fibroblasts against stress-induced premature senescence. For that, ASF-2 cells were incubated with CA or CS for 4 h, followed by a recovery period of 6 h with fresh medium, and then exposed to the oxidant H_2O_2 (200 μM) for 1 h to induce premature senescence [39,43]. H_2O_2 treatment led to the appearance of a senescent phenotype in the human fibroblasts within 4 days, as judged by the following criteria: inhibition of cell growth (Fig. 5A), appearance of SA-β-gal-positive cells (Fig. 5B and C), and enlargement and flattening of cells (Fig. 5C). As shown in Fig. 5A, the preincubation with CA or CS conferred a significant protection against H_2O_2-induced cell growth inhibition (Fig. 5A) and the appearance of SA-β-gal-positive cells (Fig. 5B and C). The effect was higher for cells treated with CS, even at lower concentrations than CA.

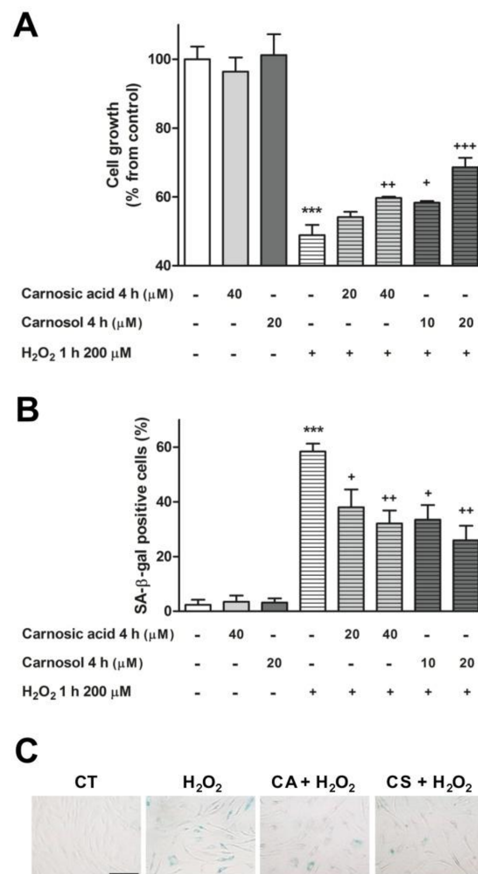

Fig. 5. Antiaging effects of carnosic acid (CA) and carnosol (CS) on H_2O_2-induced premature senescence of ASF-2 cells. Cells were incubated with CA (20 and 40 μM) and CS (10 and 20 μM) for 4 h, followed by a recovery period of 6 h with fresh medium. Then, cells were incubated with H_2O_2 (200 μM) for 1 h and 4 days later growth inhibition was determined by the MTT assay (A) and senescence-associated β-galactosidase staining was performed (B). (A, B) Values are mean ± SEM of at least three independent experiments. *** $P \leq 0.001$ when compared with control by the Student's *t* test. $^{+}$ $P \leq 0.05$; $^{++}$ $P \leq 0.01$; $^{+++}$ $P \leq 0.001$ when compared with H_2O_2 alone by one-way ANOVA. (C) Representative images of SA-β-gal staining of cells incubated with vehicle control, H_2O_2, CA + H_2O_2, and CS + H_2O_2. Bar indicates 200 μm.

3.5. Carnosol prevents age-associated changes of human fibroblasts due to replicative senescence

Normal human fibroblasts cultured in vitro have a limited proliferation potential and undergo replicative senescence as a result of serial passage. Therefore, middle passage ASF-2 cells were used to explore whether CA and CS would protect cells against replicative senescence. For that, cells were grown continuously (except in the day for cell attachment after subcultivation) in the presence of CA or CS. The concentrations used for each compound were much lower than those used in the previous experiments to ensure that the continuous presence of CA and CS in the cell culture medium would not inhibit cell growth. Based on preliminary experiments, the concentration chosen was 1.5 μM for CS and 2 μM for CA. The normal human skin fibroblasts were grown for about 160 days in the presence of the phenolic diterpenes after passage 28, but the growth curve with the CPDs was not significantly affected by either CS (Fig. 6A) or CA (data not shown), when compared with control condition. However, CS significantly improved the fibroblasts morphology by reducing considerably the senescent-related enlargement and flattening of cells, when compared with control (Fig. 6B). Throughout the experiment, cells were analyzed by flow cytometry for cell size and green autofluorescence to quantify cells with a senescent phenotype [40], which was shown to increase significantly with the number of cell passages (Fig. 6C). In each time point, a region that includes 25% of the control cell population with higher cell size and green autofluorescence was created and considered to have a senescent phenotype (SEN), and the change induced by CS was compared (Fig. 6D). We observed that SEN-like cells decreased from 25 to 19.2% after 70 days (T1) of treatment with CS, and from 25 to 14.4% after 160 days (T2), when compared with control cells (Fig. 6D). In addition, the percentage of SA-β-gal-positive cells was quantified along the time, and shown to increase from 1% (T0, day 0) to 73% (T2, day 160) (Fig. 6E and F). CS remarkably decreased the number of SA-β-gal-positive cells when compared with the respective control, although at the end of the experiment (T2) that inhibition was not significant (Fig. 6E and F).

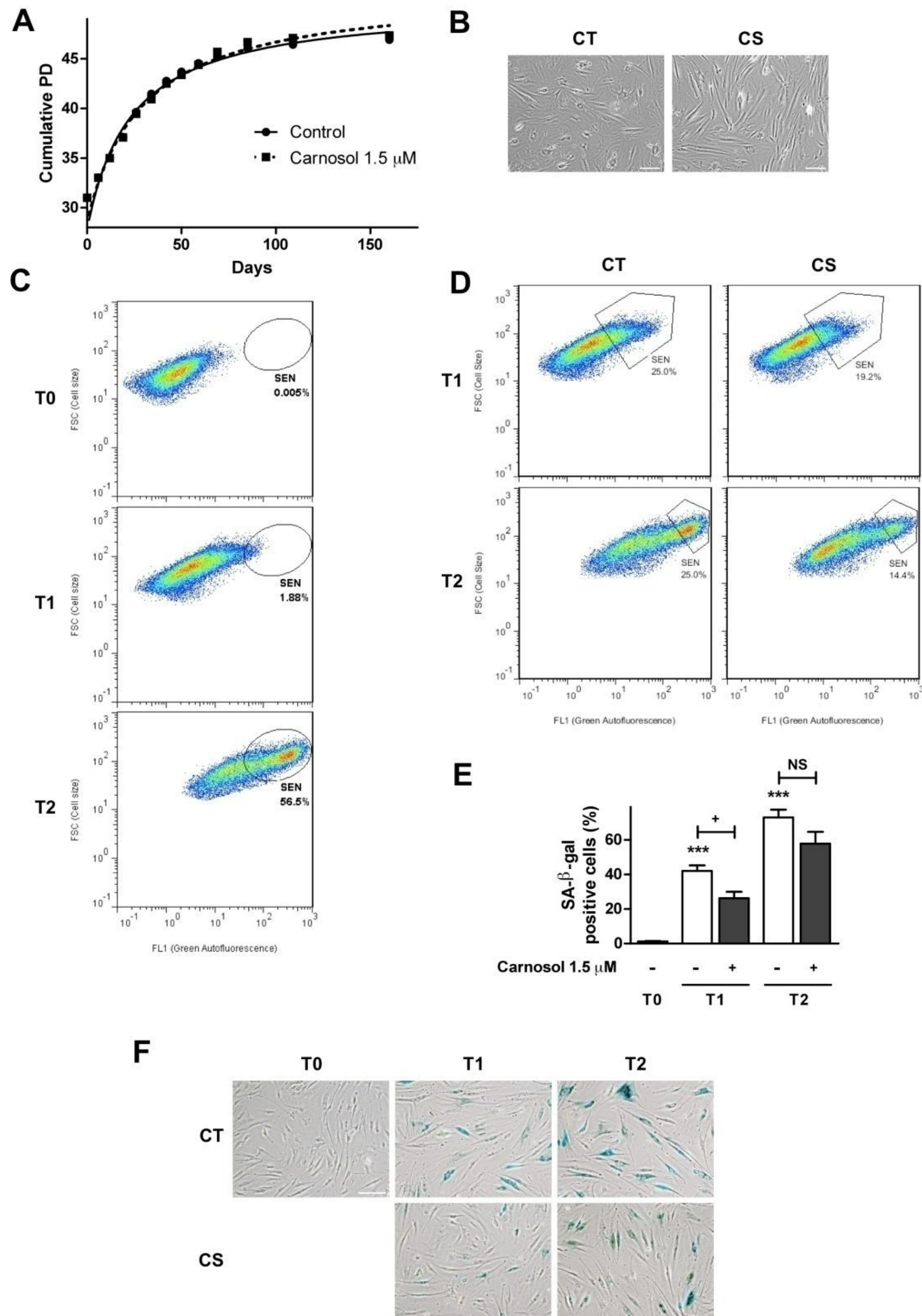

Fig. 6. Antiaging effect of carnosol (CS) on replicative senescence of ASF-2 cells. (A) Growth curves of cells serially cultivated with vehicle control (CT) and CS (1.5 µM) showing the increase in cumulative population doublings (CPDs) with time. (B) Representative images of cells serially cultivated with vehicle control and CS (1.5 µM) after 160 days in culture. Bar indicates 200 µm. (C) Flow cytometry analysis showing the senescence-associated increase in cell size and green autofluorescence with time in culture. (D) Flow cytometry analysis showing the protection conferred by CS (1.5 µM) against senescence-associated increase of cell size and green autofluorescence. (E) Percentage of SA-β-gal-positive cells serially cultivated with vehicle control and CS (1.5 µM). Values are mean ± SEM of three independent experiments. The effect of time in control was evaluated by one-way ANOVA: *** $P \leq 0.001$ when compared with T0. The effect of CS at T1 and T2 time points was evaluated by Student's *t* test: NS, not significant ($P > 0.05$); $^{+}$ $P \leq 0.05$ when compared with the respective time control. (F) Representative images showing the

increase of SA-β-gal-positive cells with time in culture and the protection conferred by CS (1.5 μM). Bar indicates 200 μm. (A, B, C, D, F) Results or images are representative of three independent experiments with similar results. (C, D, E, F) T0, 0 days; T1, 70 days; T2, 160 days of incubation.

4. Discussion

Mild stress-induced hormesis has been suggested as a promising strategy for targeting the age-related occurrence, removal, and accumulation of molecular damage and, therefore, for preventing diseases and promoting healthy aging [4–6]. In recent years, accumulating evidence suggests that fruits, vegetables, and spices contain hormetic phytochemicals capable of inducing mild cellular stress responses, which may explain, at least in part, the health benefits associated with their consumption [10–16]. Here, we tested two phenolic diterpenes as potential hormetins and inducers of intracellular antioxidant defenses with regard to stress tolerance and antiaging effects in human fibroblasts. Both CA and CS were tested in normal human diploid cells and shown to induce antioxidant and phase 2 enzymes without significant toxic effects. The use of normal cells is of the utmost importance when the aim is to search for hormetins for aging interventions [14]. The induction of cytoprotective enzymes such as HO-1 was shown to be redox dependent and associated with Nrf2 signaling. In addition, both compounds induced stress tolerance and protected significantly against H_2O_2-induced premature senescence. Carnosol, which was active at lower concentrations, also remarkably ameliorated some morphological and physiological features of fibroblasts undergoing replicative senescence, showing therefore its potential use toward the promotion of healthy aging.

Previous results from our group demonstrated that phytochemicals such as curcumin and rosmarinic acid also induced stress responses in normal human skin fibroblasts [12,44]. The stress responses elicited by curcumin led to increased protection against a further oxidant challenge [12], and rosmarinic acid reduced the number of cells expressing senescence-associated β-galactosidase and the proportion of enlarged cell population with high levels of green autofluorescence [44]. Induction of HSPs and the heat shock response was also previously shown to have a variety of antiaging effects [15,45,46]. Overall and together with our present results, these data support the view that mild stress-induced hormesis may be applied for the modulation of aging.

A stress response mediated by Nrf2/ARE signaling is triggered by a disturbance in the cellular balance between oxidation and reduction potentials, which leads to the induction of antioxidant and phase 2 enzymes in order to restore the cells' redox state

[18– 20]. Small changes in the reduction/oxidation balance toward the later inactivate Keap1 protein (Nrf2 inhibitor) that in turn activates Nrf2. On stimulation, this transcription factor plays a crucial role in the coordinated induction of several cytoprotective genes that contain at least one ARE within their promoters, including HO-1, GLC, NQO1, GST-P1, FTH1, and TXNRD1 [17,18]. Several studies have shown that, in fact, CA and CS activate Nrf2 signaling and trigger cytoprotective effects [28,30,42,47]. In normal human skin fibroblasts, we also observed that these phytochemicals increased the nuclear localization and transcriptional activity of Nrf2, confirming that the induction of cytoprotective proteins by CA and CS is associated with the activation of Nrf2/ARE signaling.

The tripeptide GSH is the main intracellular low molecular weight thiol and is central in the regulation of the cells' redox state. It has antioxidant activities and is the cosubstrate for a variety of antioxidant and phase 2 detoxifying enzymes [48]. During the aging process, it has been shown that the GSH:GSSG ratio progressively decreases due to an elevation in the GSSG content and a decline in the ability for de novo GSH biosynthesis [49]. The decrease of GSH levels adversely affects cellular thiol redox balance, leaving cells highly susceptible to different stresses [21]. In accordance with previous results in other cells [30,41,42], CA and CS also induced GSH levels in normal human skin fibroblasts, in a concentration- and time-dependent manner. In addition, CA and CS induced GCL, the rate-limiting enzyme in the de novo synthesis of GSH, and TXNRD1, the enzyme that catalyze the reduction of thioredoxin. The glutathione system (NADPH, glutathione reductase, glutathione, and glutaredoxin) and the thioredoxin system (NADPH, thioredoxin reductase, and thioredoxin) play a central role in maintaining cellular thiol-disulfide redox status and, therefore, in protecting human cells against different stresses [50]. The induction of GSH levels as well as GCL and TXNRD1 transcripts was probably through Nrf2 signaling after mild disturbance of cellular redox state by the phenolic diterpenes. This hypothesis was supported by the results obtained when cells were coincubated with NAC, which is an antioxidant and most importantly a cysteine precursor for GSH synthesis. Therefore, NAC is capable of increasing the cellular thiol-disulfide redox state, preventing the oxidant and redox cycling by potential electrophile compounds [12]. NAC inhibited the induction of HO-1 by CA or CS, corroborating that these phytochemicals induce antioxidant and cytoprotective defenses by disturbing the cellular redox state. This preventive effect of NAC was higher for the CS-mediated induction of HO-1. CS is an oxidation product of CA [51] and probably has

a most potent effect functioning as an electrophile in the cellular milieu. In fact, CS was active at lower concentrations, inducing several cytoprotective enzymes when compared with CA, although it was detrimental at higher concentrations as shown by the cells' growth inhibition capacity. In agreement with the previous statements, NAC was able to significantly prevent the protection conferred by CS against *tert*-BOOH but not by CA. The abolishment of this CS effect by NAC corroborates that the protective response induced by this phenolic diterpene is due to mild stress induction through redox imbalance, which signals for Nrf2 activation. Interestingly, contrary to what happened with curcumin [12], both CA and CS induced several cytoprotective enzymes without significantly decreasing GSH levels or induction of oxidative stress in the first hours of incubation. This probably explains their lower detrimental effects to human fibroblasts as compared with curcumin (comparison of present results with the ones in [12]). This is in accordance with the hypothesis raised very recently by Satoh and collaborators [52] that the lack of negative effects in GSH by CA and CS as compared with curcumin may decrease the clinical side effects and increase tolerability at therapeutic concentrations. During the aging process, there is a gradual decline of cellular antioxidant defenses and a reduced ability of stress responses to be induced by different stimuli [53–55]. For example, the age-associated decline in GSH synthesis has been found to be associated with the age-related loss of the transcriptional activity of Nrf2 [21]. Increasing experimental evidence shows that mild stress-induced protective stress responses (hormesis) at a young age can delay aging or protect from severe stress at an old age [6,14]. Here, the ability of CA and CS, that induce cellular antioxidant defenses, to lead to antiaging effects in human skin fibroblasts was tested in two *in vitro* models of senescence: the H_2O_2-induced premature senescence and the replicative senescence. Cellular senescence, originally defined as proliferative arrest that occurs after a limited number of cell divisions (cell aging, replicative senescence), has now become regarded more generally as a biological program of signal transduction leading to terminal, irreversible growth arrest, accompanied by a distinct set of alterations in the cellular phenotype [56]. Such alterations include enlargement and flattening of the cytoplasm, increased production of ROS, accumulation of lipofuscin, increased mitochondrial and lysosomal mass and their cellular contents, and loss of mitochondrial membrane potential [57]. Senescent cells also express cytosolic and nuclear markers such as senescence-associated β-galactosidase activity [39,58] and senescence-associated heterochromatin foci [59]. We demonstrated that preincubation of human fibroblasts with CA or CS

prevented significantly against H_2O_2-induced premature senescence, as shown by the increased cell growth and the fewer number of cells expressing senescence-associated β-galactosidase. Again, CS was more active than CA. Both phenolic diterpenes, however, did not extend the proliferative capacity of normal human fibroblasts. Nevertheless, CS but not CA was able to significantly ameliorate several features of cells undergoing aging, such as a decreased proportion of cells with enlarged morphology and with high levels of green autofluorescence (a reliable marker of cellular lipofuscin content), and fewer number of cells expressing senescence-associated β-galactosidase. Interestingly, curcumin tested at the same concentration range as CS did not show positive effects in human fibroblasts undergoing aging in vitro, but on the contrary it negatively affected morphological and physiological parameters of cells undergoing replicative senescence (our own not published data). As discussed above, these results highlight the importance of hormetins to not affect negatively GSH levels in order to possess cytoprotective and antiaging effects together with a high range of tolerability in normal cells.

Considering the positive effects of CS shown here in normal cells, it would be interesting to test in the near future its survival and life extension potential in animal models. Other phytochemicals such as resveratrol have been shown to extend the life span of worms (*C. elegans*), fruit flies (*D. melanogaster*), and a vertebrate fish (*N. furzeri*) [60,61]; a blueberry extract also increased the life span of *C. elegans* [62]. Until recently, caloric restriction was the only known intervention that successfully extended life span in mammals [63]. Niu et al. reported, however, that epigallocatechin gallate (EGCG) was able to extend the life span of rats (*R. norvegicus*) [64]. Therefore, it would be interesting to test whether compounds, such as CS, that increase antioxidant and cytoprotective defenses through redox signaling, would also increase the life span of mammals.

In conclusion, in this report we have shown that CA and CS increase the levels of GSH and antioxidant and phase 2 enzymes in normal human fibroblasts through induction of redox stress and associated with the activation of Nrf2/ARE signaling (Fig. 7). The stress responses elicited by CS, the most powerful compound, conferred a significant protection against a further oxidant challenge, demonstrating that this phytochemical work as hormetin. CS also protected against H_2O_2-induced premature senescence and ameliorated several features of cells undergoing replicative senescence in vitro (Fig. 7), such as the changes in cell size, morphological heterogeneity, loss of parallel cell arrangement, and senescence-associated β-galactosidase activity. Taken together, these

data support the view that mild stress-induced hormesis by CS may be applied for slowing down aging and preventing the onset of age-related diseases.

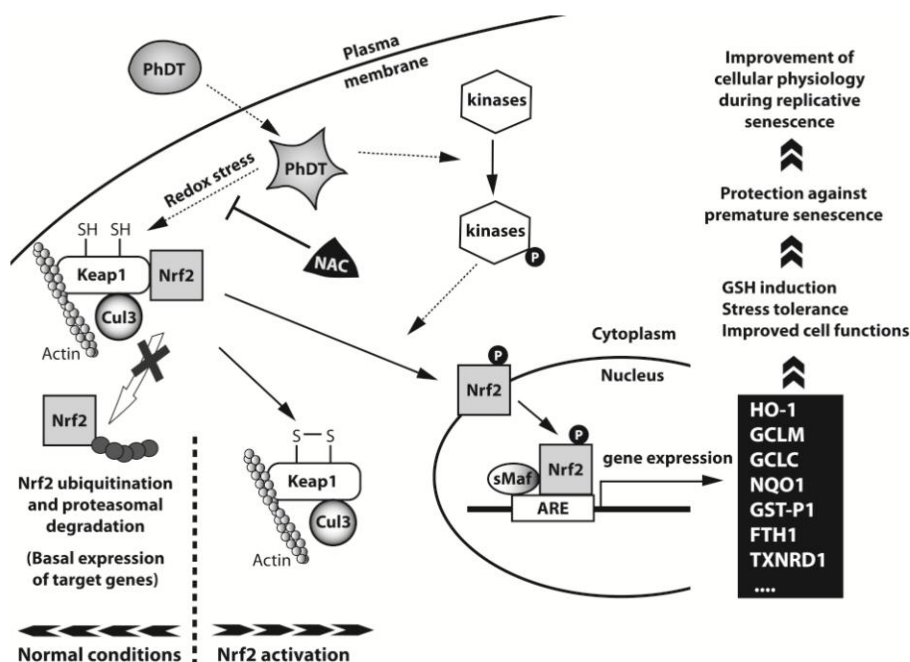

Fig. 7. Proposed mechanism of action of phenolic diterpenes (PhDT) in normal human skin fibroblasts. Under normal conditions, Keap1 forms a complex with Cul3 and targets Nrf2 for ubiquitination and proteasomal degradation. Incubation of cells with PhDT induces redox stress that may modify cysteine residues of Keap1, resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2. Under these conditions, Nrf2 accumulates in the nucleus, forms a heterodimer with small Maf proteins, which binds to ARE and activates transcription of genes encoding several cytoprotective enzymes, including HO-1 (heme oxygenase-1), GCLM (glutamate cysteine ligase modulatory subunit), GCLC (glutamate cysteine ligase catalytic subunit), NQO1 (NADP(H):quinone oxidoreductase-1), GST-P1 (glutathione S-transferase P1), FTH1 (ferritin heavy chain), and TXNRD1 (thioredoxin reductase 1). NAC (N-acetylcysteine) by abrogating redox stress prevents the hormetic induction of the cytoprotective action of PhDT. Additionally, phosphorylation of Nrf2 by upstream kinases may also contribute to its nuclear localization. The stress response elicited by PhDT increases stress tolerance, confers protection against stress-induced premature senescence, and improves the physiological state of cells during replicative senescence.

Acknowledgments

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Appendix A. Supplementary information

Supplementary Methods

ROS levels

The levels of reactive oxygen species (ROS) in ASF-2 cells treated with carnosic acid and carnosol were measured using the 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) probe, following the manufacturer's instructions. In the presence of ROS, $DCFH_2$-DA is oxidized to 2',7'-dichlorofluorescin (DCF), which can be detected by its fluorescence at 525 nm. In brief, 10,000 cells/well were seeded in a 96-well black cell culture treated plate and, one day after seeding, cells were washed with HBSS and then loaded with 10 μM $DCFH_2$-DA (or vehicle for control) for 30 min. Afterwards, cells were washed again with HBSS and incubated with carnosic acid (40μM) and carnosol (20μM) for 30 min (*tert*-BOOH 200 μM was used as positive control). Then, after washing cells with HBSS, DCF fluorescence was detected on a microplate fluorometer (Fluoroskan Ascent, Thermo Scientific, Waltham, MA, USA). The results were expressed as the DCF fluorescence increase relative to control condition.

Comet assay

To potential of carnosic acid and carnosol to induce DNA damage in ASF-2 cells was evaluated using the alkaline version of the single cell gel electrophoresis (comet) assay, as previously described[1]. Briefly, cells were embedded in low melting agarose (0.5% w/v) and spread on agarose coated slides. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH10 with NaOH, and 1% v/v triton X-100 added fresh) to expose DNA and then in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) to allow alkaline DNA unwinding. In the same solution, electrophoresis was carried out at 4°C for 20 min at 0.8 V/cm and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with SYBR Gold nucleic acid gel stain, and analyzed by fluorescence microscopy using the Comet Assay IV 4.3 software (Perceptive Instruments Ltd., Suffolk, UK). One hundred randomly selected nucleoids were evaluated from each sample and the tail intensity was determined.

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Wound healing assay

ASF-2 cells were seeded at a density of 50,000 cells per ml and grown until confluency. Then, two types of incubation regimes were used: 1) pre-incubation with carnosic acid (CA, 20 µM) and carnosol (CS, 10 µM) for 4h, followed by a recovery period with fresh medium (DMEM supplemented with 2.5% FBS); or 2) co-incubation with CA (2 µM) and CS (1 µM). A mechanical scratch-wound was made in the centre of the confluent layer using a sterile pipette tip. Scratched cells were removed from wells by replacing the culture medium with fresh medium or fresh medium containing CA and CS in pre- and co-incubation, respectively. Phase-contrast images of cells in a selected part of the scratched were captured using an inverted microscope equipped with a DP72 digital camera (Olympus IX70, Olympus, Hamburg, Germany). After 24 h, photos were again taken from exactly the same area as before and the extent of wound closure was calculated by subtracting the area not occupied by migrating cells (24 h) from the initial area of the wound (0 h).

Supplementary Table

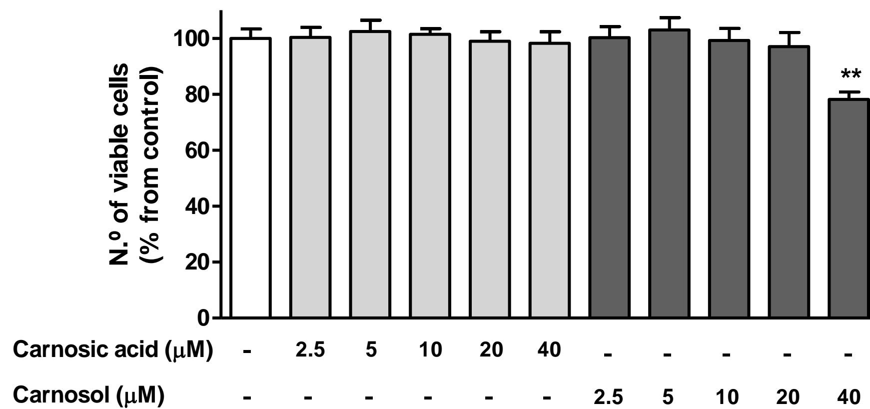
Suppl. Table 1

Effects of carnosic acid (CA) and carnosol (CS) on ROS levels and DNA damage in ASF-2 cells.

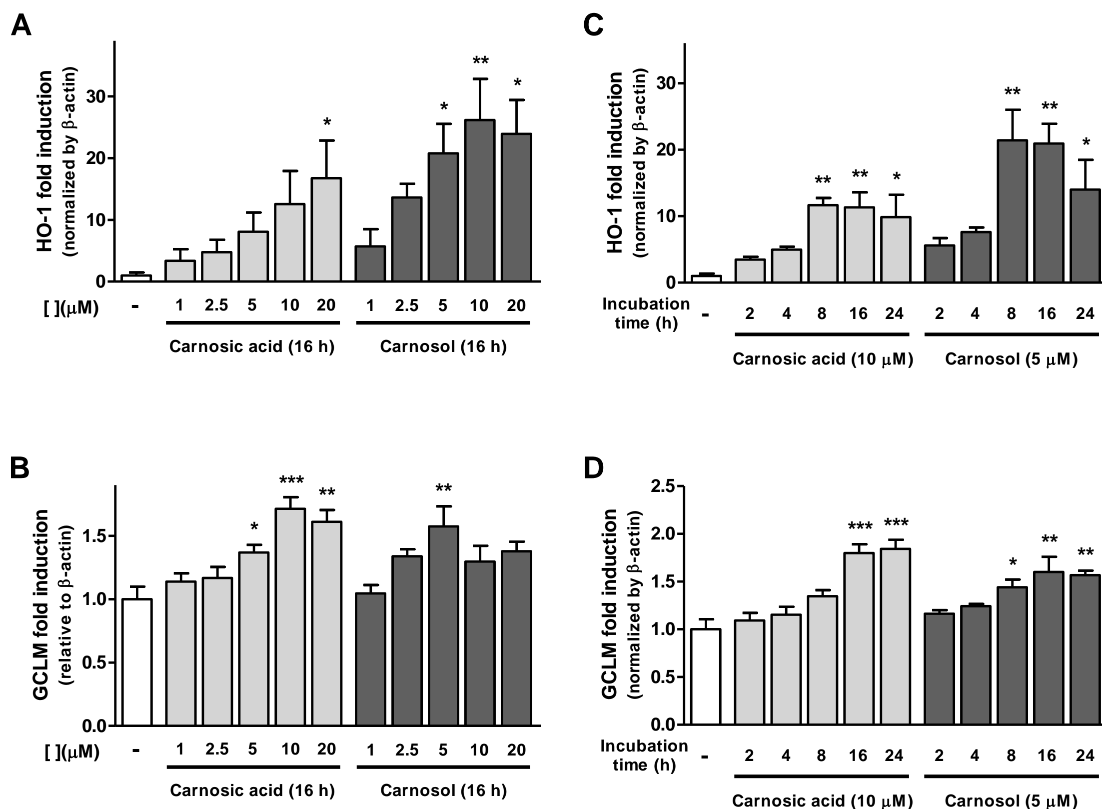
	ROS levels (relative to control)	**DNA damage (% of tail intensity)**
CT	1±0.04	4.37±0.74
CA 40 µM	1.24±0.23	5.46±0.77
CS 20 µM	1.01±0.38	5.43±0.70
***tert*-BOOH 200 µM**	33.05±1.78***	

ASF-2 cells were incubated with (40 µM) or CS (20 µM) for 30 min to measure ROS levels using the DCF probe and for 1h to evaluate DNA damage by the alkaline version of the comet assay. *tert*-BOOH 200 µM was used as a positive control. Values are mean ± SEM of three independent experiments. *** $P \leq 0.001$ when compared with control by the Student's *t*-test.

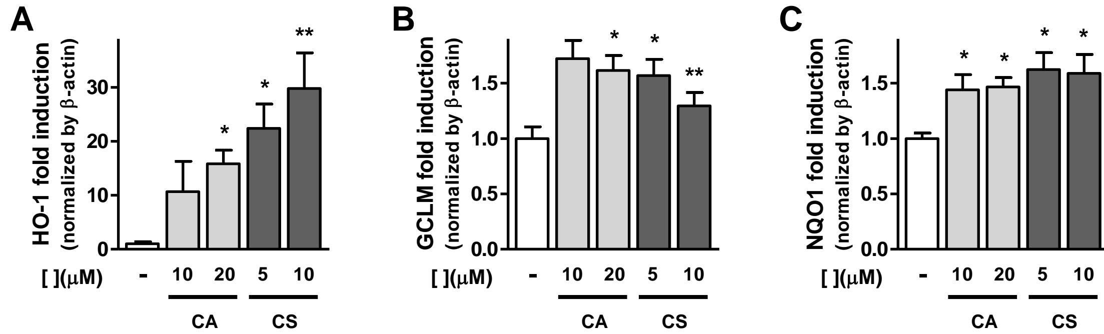
Supplementary Figures



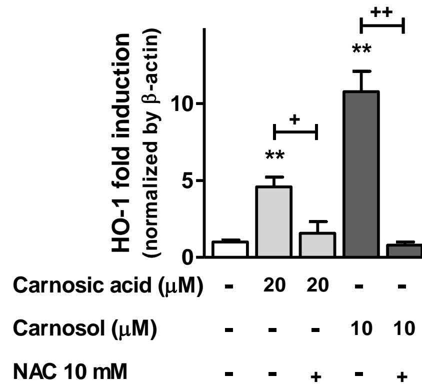
Suppl. Fig. 1. Viability of ASF-2 cells after 48 h of incubation with increasing concentrations of carnosic acid (CA) or carnosol (CS). Values are mean ± SEM of four independent experiments. ** $P \leq 0.01$ when compared with control by the one-way ANOVA.



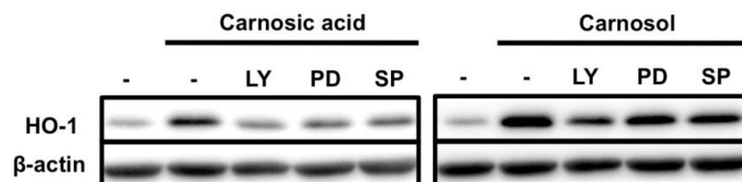
Suppl. Fig. 2. Effects of carnosic acid (CA) and carnosol (CS) on the levels of cytoprotective proteins in ASF-2 cells. Cells were incubated with CA and CS for 16 h **(A & B)** or with CA (10 μM) and CS (5 μM) for different incubation times **(C & D)**, and expression of heme oxygenase-1 (HO-1) **(A & C)** and glutamate cysteine ligase modulatory subunit (GCLM) **(B & D)** was analyzed by Western blot. The mean band intensity was quantified by the Quantity One software (Bio-Rad Laboratories) and the expression levels relative to that of β-actin were calculated. Values are mean ± SEM of three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ when compared with control by the one-way ANOVA.



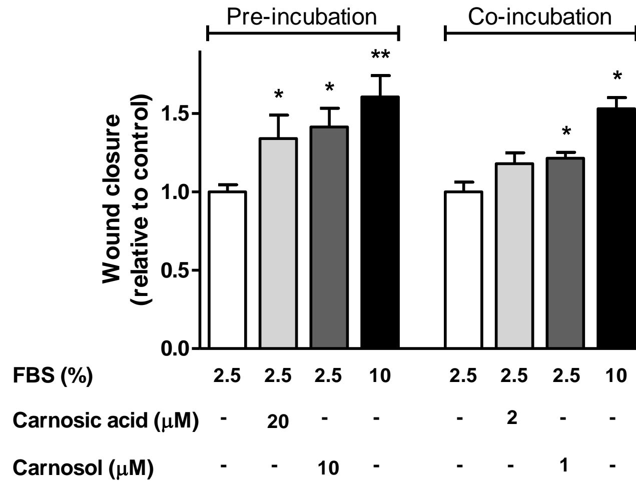
Suppl. Fig. 3. Effects of carnosic acid (CA) and carnosol (CS) on the levels of cytoprotective proteins in ASF-2 cells. Cells were incubated with CA (10 and 20 µM) and CS (5 and 10 µM) for 16 h, and the protein levels of heme oxygenase-1 (HO-1) **(A)**, glutamate cysteine ligase modulatory subunit (GCLM) **(B)** and NADP(H):quinone oxidoreductase-1 (NQO1) **(C)** were measured by Western blot. The mean band intensity was quantified by the Quantity One software (Bio-Rad Laboratories) and the expression levels relative to that of β-actin were calculated. Values are mean ± SEM of three independent experiments. [*] $P \leq 0.05$; [**] $P \leq 0.01$ when compared with control by the one-way ANOVA.



Suppl. Fig. 4. Effects of carnosic acid (CA) and carnosol (CS) on redox and Nrf2/ARE signaling. Cells were incubated with CA (20 µM) and CS (10 µM) with or without the antioxidant NAC (10 mM) for 8 h, and the levels of heme oxygenase-1 (HO-1) were measured by Western blot. The mean band intensity was quantified by the Quantity One software (Bio-Rad Laboratories) and the expression levels relative to that of β-actin were calculated. Values are mean ± SEM of three independent experiments. [**] $P \leq 0.01$ when compared with control and [+] $P \leq 0.05$; [++] $P \leq 0.01$ when compared when compared with each other by the Student's *t*-test.

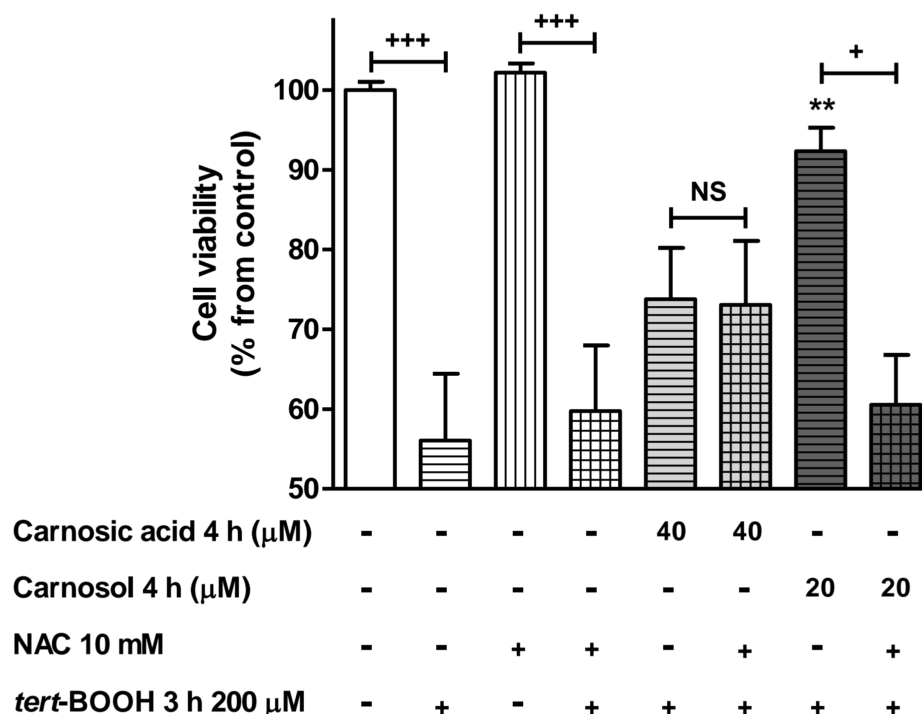


Suppl. Fig. 5. Involvement of PI3K/Akt, MAPK/ERK, and JNK signaling pathways in carnosic acid (CA)- and carnosol (CS)-induced HO-1 expression in ASF-2 cells. Cells were incubated with CA (20 µM) or CS (10 µM) for 8 h with or without theinhibitorsLY294002 (25 µM), PD98059 (25µM) or SP600125 (10µM), and the levels of heme oxygenase-1 (HO-1) were measured by Western blot. Inhibitors were added 30 min before CA and CS. Blots are representative of three independent experiments and β-actin was used as loading control.



Suppl. Fig. 6. Effect of carnosic acid (CA) and carnosol (CS) on the *in vitro* wound healing capacity of ASF-2 cells.Cells were pre-incubated with CA (20 μM) or CS (10 μM) for 4h, followed by a recovery period with fresh medium (DMEM supplemented with 2.5% FBS) or co-incubated with CA (2 μM) and CS (1 μM) for 24 h, and then wound closure was evaluated. Values are mean ± SEM of three independent experiments. * $P \leq 0.05$; $^{**}P \leq 0.01$ when compared with control by the Student's *t*-test.

Supplementary Scheme 1



Statistical analysis (two-way ANOVA):

1. Effect of NAC and *tert*-BOOH	√	√	√	√				
2. Effect of NAC and CA		√		√	√	√		
3. Effect of NAC and CS		√		√			√	√

Suppl. Scheme 1. Hormetic effects of carnosic acid (CA) and carnosol (CS) in ASF-2 cells. Cells were incubated with CA (40 μM) and CS (20 μM) with or without NAC for 4 h, followed by a recovery period of 6 h with fresh medium. Then, cells were incubated with *tert*-BOOH (200 μM) for 3 h and cell viability was measured by the LDH leakage method. Values are mean ± SEM of at four independent experiments. Results from Fig. 4C, shown here in **A**, were statistically evaluated using three independent two-way ANOVA as shown: the effects of NAC and *tert*-BOOH (1); NAC and CA (2), or NAC and CS (3). The results of the Bonferroni post-tests are shown in **B1**, **B2** and **B3,** respectively (see next page). ** $P \leq 0.01$ when compared with *tert*-BOOH alone. NS, not significant ($P > 0.05$); $^{+}$ $P \leq 0.05$; $^{+++}$ $P \leq 0.001$ when compared with each other.

Supplementary Scheme 1 (cont.)

B1. Effect of NAC and *tert*-BOOH

Effect of NAC: not significant

Effect of tert-*BOOH: P*≤0.001

Interaction: not significant

Bonferroni post-tests:	***tert*-BOOH (µM)**		**NAC (mM)**	
	0	**200**	**0**	**10**
Effect of NAC	**ns**	**ns**		
Effect of *tert*-BOOH			+++	+++

B2. Effect of NAC and CA (under *tert*-BOOH treatment)

Effect of NAC: not significant

Effect of carnosic acid: not significant

Interaction: not significant

Bonferroni post-tests:	**Carnosic acid (µM)**		**NAC (mM)**	
	0	**40**	**0**	**10**
Effect of NAC	**ns**	**ns**		
Effect of carnosic acid			**ns**	**ns**

B3. Effect of NAC and CS (under *tert*-BOOH treatment)

Effect of NAC: not significant

Effect of carnosol: P≤0.05

Interaction: P≤0.05

Bonferroni post-tests:	**Carnosol (µM)**		**NAC (mM)**	
	0	**20**	**0**	**10**
Effect of NAC	**ns**	+		
Effect of carnosol			**	**Ns**

CHAPTER 4

Methanolic extract of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens* provides protection against oxidative stress induced in human HepG2 cells

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Publication

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Methanolic extract of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens* provides protection against oxidative stress induced in human HepG2 cells

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Abstract

Hypericum perforatum L. (HP) is widely used in traditional medicine to treat several medical conditions since ancient times. In our group, this plant has been used for biotechnological production of bioactive phytochemicals. In the present study, the ability of a methanolic extract of HP cells elicited with *Agrobacterium tumefaciens* (AT) to induce intracellular antioxidant defenses of human HepG2 cells and to protect them against *tert*-butyl hydroperoxide-induced oxidative stress was tested. The elicited HP extract significantly prevented *tert*-butyl hydroperoxide-induced cell death, glutathione (GSH) depletion, and DNA damage, in both pre- and co-incubation regimes, while the extract from control HP did not. When incubated alone, none of the extracts were cytotoxic or genotoxic. Interestingly, contrary to control HP extract, incubation of HepG2 cells with extract from elicited HP cells induced significantly GSH levels and several cytoprotective enzymes. These effects were associated with an increase of Nrf2 levels in nucleus, which may explain the cytoprotective action of the elicited HP extract in the pre-incubation regime. Taken together, our results suggest that elicitation of HP cells with AT is an interesting biotechnological approach for the production of cytoprotective and antioxidant compounds for pharmaceutical applications.

Keywords: *Hypericum perforatum* cells; Plant elicitation; Human HepG2 cells; Antioxidant effects; Intracellular antioxidant defences stimulation; Cytoprotective effects.

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1. Introduction

Cells are continuously exposed to reactive oxygen species (ROS), of both endogenous and exogenous sources, and a complex antioxidant defense system protect them against toxic levels of ROS and to maintain cellular redox homeostasis. However, oxidative stress may arise when the antioxidant defense system is not able to properly respond to an overall increase of intracellular ROS. In this condition, damage to various cellular macromolecules, including lipids, proteins, and DNA can occur, inhibiting their normal function and ultimately causing cell death. Therefore, oxidative stress is thought to contribute to the pathogenesis of several diseases, including diabetes, liver, neurodegenerative, and cardiovascular diseases (Valko et al., 2007; Trachootham et al., 2008; Lima et al., 2011). Thus, much research have been focused in the search of antioxidants capable of reducing oxidative stress that, besides the ones produced by our cells (endogenous), are usually provided by our diet (exogenous) (Masella et al., 2005). Among these, plant bioactive compounds have been largely studied because they can act as direct antioxidants through scavenging ROS or inhibiting their formation and, also, as indirect antioxidants through upregulation of endogenous antioxidant defenses (Masella et al., 2005; Dinkova-Kostova and Talalay, 2008). This latter effect of phytochemicals is usually operated by activation of the nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) pathway, a stress response regulated by cell's redox state (Surh et al., 2008).

Hypericum perforatum L. (HP), commonly known as St. John's wort, is widely used in traditional medicine to treat several medical conditions since ancient times. Nowadays, it remains a popular plant in phytomedicine for treatment of anxiety, depression, cuts, and burns (Guedes et al., 2012). Recent research suggests that this herb is also effective in treating other ailments, including cancer, inflammation-related disorders, bacterial, and viral diseases, acting also as an antioxidant and neuroprotective agent (Silva et al., 2004, 2005, 2008; Franchi et al., 2011; Klemow et al., 2011). The pharmacological properties attributed to this species may derive from its wide variety of biologically active metabolites, including naphthodianthrones, phloroglucinolds, phenolic acids, flavonoids, and xanthones (Nahrstedt and Butterweck, 2010). Several studies suggest that xanthones belong to the defense arsenal employed by HP to combat biological stress factors, including infection by pathogens (Crockett et al., 2011). In particular, we have shown recently that both biosynthesis of previously present xanthones

and de novo production of new xanthones increased in HP cell suspension cultures elicited through co-cultivation with *Agrobacterium tumefaciens* (AT) (Franklin et al., 2009). Moreover, this rapid up-regulation of xanthone metabolism significantly increased the antiradical and antimicrobial properties of methanolic extracts from HP cells (Franklin et al., 2009).

Considering this metabolic shift of HP cells after elicitation with AT, in the present study, we evaluated the ability of methanolic extracts of control and elicited HP cells to protect human HepG2 cells against oxidative damage induced by *tert*-butyl hydroperoxide (*t*-BOOH), an organic peroxide widely used to induce oxidative stress (Buc-Calderon et al., 1991). *H. perforatum* extracts were pre- or co-incubated with the toxicant in HepG2 cells and cell viability, GSH levels, and DNA damage evaluated. In addition, the ability of HP extracts of induce intracellular antioxidant defenses of HepG2 cells was studied.

2 Material and methods

Chemicals and antibodies

Minimum essential medium eagle (MEM), antibiotic-antimycotic solution, HEPES, *tert*-butyl hydroperoxide (*t*-BOOH), anti-β-actin antibody, and all other not specified reagents were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). SYBR Gold nucleic acid gel stain was purchased from Invitrogen (Paisley, UK), and complete protease inhibitor cocktail from Roche (Penzberg, Germany).

Antibodies against NQO1, GCLC, Histone H1, and secondary anti-body goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GCLM and anti-PRDX4 antibodies developed by the Clinical Proteomics Technologies for Cancer were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa, Department of Biology, Iowa City, IA, USA. Anti-HO-1 antibody was from Enzo Life Sciences (Farming-dale, NY, USA), anti-Caspase-3 from EMD Millipore Corporation (Billerica, MA, USA), anti-Nrf2 from Novus Biologicals (Littleton, CO, USA), and secondary antibody goat anti-rabbit IgG-HRP from Cell Signaling Technology (Beverly, MA, USA).

Extracts from H. perforatum cells

The extracts from HP cells were the same used in the study by Franklin et al. (2009). In brief, HP cell suspension cultures (established in Dias et al., 2001) were maintained and elicited with AT. Control and elicited HP cells were harvested by vacuum filtration and freeze-dried in a lyophilizer (Alpha 2–4 LD plus, Christ, Osterode am Harz, Germany). Equal quantity of dry biomass from control and elicited cells were extracted in 90% MeOH under dark. Extracts were dried in a rotary evaporator and frieze-dried powders were redissolved in dimethyl sulfoxide (DMSO) at 20 mg/mL. Similarly, AT cells were harvested by centrifugation (10,000 × g), freeze-dried and extracted in methanol as above. The extracts' stock solutions were kept at −20°C in aliquots until use in the cell culture experiments. DMSO alone was used in control conditions at 0.5% (v/v).

The HP cells extracts were previously analyzed by HPLC (Franklin et al., 2009), and it was found that xanthone content increased significantly whereas flavonoids remained unchanged after HP elicitation with AT. In detail, flavonoids were present in about 0.9 mg/g of HP cells biomass dry weight with and without AT elicitation; xanthones were present at 0.3 mg/g in HP control cells and at 4.1 mg/g in elicited HP cells, being the major one the 1,3,6,7-tetrahydroxy-8-prenylxanthone representing 23% of the total xanthones (Franklin et al., 2009).

Cell culture and experimental conditions

The human hepatocellular carcinoma cells HepG2 (HB-8065) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in MEM supplemented with 10% FBS, 1% antibiotic antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES, and 1.5 g/L sodium bicarbonate, at 37°C in a humidified incubator containing 5% CO_2.

For experiments, cells were seeded at 2×10^5 cells/mL 48 h before incubation with HP extracts or AT extract with or without *t*-BOOH as specified below. The AT extract was used to control the possible presence of any bioactive compound present in this bacteria that would be extracted from the biomass of HP cells elicited with AT. The tested concentration of AT extract was normalized considering the extraction yield and the relative biomass of AT present in the elicited HP cells. Therefore, when the tested concentration for HP extracts was 100 µg/mL, for AT it was 25 µg/mL.

To study the effects of extracts against *t*-BOOH toxicity, two incubation regimes were used, described as follow. In the co-incubation regime, HepG2 cells were incubated

with extracts and 800 μM *t*-BOOH for 4 h. In the pre-incubation regime, HepG2 cells were incubated with extracts for 6 h, followed by a recovery period of 16 h in fresh medium without extracts to let antioxidant defenses to be synthesized, and, then, cells were incubated with 800 μM *t*-BOOH for 4 h. In the assessment of DNA damage induced by *t*-BOOH the concentration used of the toxicant was 200 μM for 1 h.

LDH leakage assay

In order to determine the effect of HP extracts and *t*-BOOH on cell viability the lactate dehydrogenase (LDH) leakage assay was used as previously described (Lima et al., 2005). Briefly, LDH activity was measured at 30°C by quantification of NADH (0.28 mM) consumption by continuous spectrophotometry (at 340 nm) on a microplate reader (SpectraMax 340pc, Molecular Devices, Sunnyvale, CA, USA) using pyruvate (0.32 mM) as substrate in 50 mM phosphate buffer (pH 7.4). LDH leakage was calculated using the following equation: LDH leakage (%) = 100 × extracellular LDH/total LDH.

Glutathione content

The effect of HP extracts and *t*-BOOH on glutathione (GSH) content was determined by the DTNB-GSSG reductase recycling assay, as previously described (Lima et al., 2004). Briefly, after protein precipitation with 5-sulfosalicylic acid, samples were centrifuged and supernatants were used for measurement of GSH following the DTNB oxidation at 415 nm and compared with a standard curve. The results were expressed as nmol GSH/mg of protein.

Comet assay

In order to assess the effects of HP extracts against *t*-BOOH-induced DNA damage, the alkaline version of the single cell gel electrophoresis (comet) assay was used as previously described (Lima et al., 2006). In brief, cells were embedded in 0.5% w/v low melting agarose and spread on agarose coated slides. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, and 1% v/v triton X-100 added fresh) to expose DNA and then in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) to allow alkaline DNA unwinding. In the same solution, electrophoresis was conducted at 4°C for 20 min at 0.8 V/cm and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with SYBR Gold nucleic acid gel stain, and analyzed by fluorescence microscopy, using the Comet Assay

IV 4.3 software (Perceptive Instruments Ltd., Suffolk, UK). One hundred randomly selected nucleoids were evaluated from each sample and the mean of the tail intensity was determined.

Protein extraction and Western blotting

To study the effects of HP extracts on the expression levels of proteins of interest, after incubation, HepG2 cells were rinsed with PBS and then lysed in ice-cold lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% v/v NP-40) containing 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF, and 1 × complete protease inhibitor cocktail for total protein extraction.

Protein concentration was quantified using DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and BSA used as protein standard.

For western blotting, 20 µg of protein was separated in a SDS polyacrylamide gel and then electroblotted to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% w/v non-fat dry milk, washed in TPBS and then, incubated with primary antibody. After washing, membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories). Band area intensity was quantified using the Quantity One software (Bio-Rad Laboratories). β-actin was used as loading control.

To study the effects of HP extracts on Nrf2 expression in HepG2 cells, protein levels were detected by western blot as above in protein nuclear extracts, which were isolated as previously described (Lima et al., 2011). Briefly, cells were incubated with ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5 mM DTT, 1 mM PMSF, and 1 × complete protease inhibitor cocktail, and then, NP-40 was added to a final concentration 0.7% (v/v). The homogenate was centrifuged and the cytosolic supernatant harvested. The nuclear pellet was resuspended in ice-cold nuclear buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA) containing 0.5 mM DTT, 1 mM PMSF, and 1 × complete protease inhibitor cocktail, and after centrifuging, the supernatant containing the nuclear proteins was harvested.

Statistical analysis

Data were expressed as mean ± SEM of at least three independent experiments. Statistical significances were assessed by the Student's *t*-test, using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences between groups were considered to be significant when $p \leq 0.05$.

3. Results

3.1. Extract from *H. perforatum* cells elicited with *A. tumefaciens* provides direct and indirect antioxidant effects in human HepG2 cells

The potential protective and antioxidant effects of methanolic extracts from HP cells cultured in suspension in normal conditions or elicited by AT were evaluated in HepG2 cells challenged with the oxidant *t*-BOOH. For that, two types of incubations regimes were used: co-incubation with extracts and *t*-BOOH, which mainly reflects the direct antioxidant effect of extracts on mediators of *t*-BOOH toxicity; or pre-incubation of extracts followed by a recovery period with fresh medium before addition of toxicant, which reflects the capacity of extracts to induce endogenous antioxidant defenses.

In both types of incubation, *t*-BOOH (800 µM for 4 h) significantly increased LDH leakage when compared with control (Fig. 1A). This increase, which is an indicator of cytotoxicity, was reverted by 35% when cells where pre-incubated with 100 µg/mL of elicited HP extract and totally abolished when co-incubated with the same extract. In contrast, extracts from control HP cells or AT alone did not protect against *t*-BOOH-induced cell death (Fig. 1A). Incubation of cells with each extract alone did not induce cell death (as measured by LDH leakage) both when tested in the same conditions of the pre- or co-incubation regimes and when incubated continuously for 24 h (Fig. 1B).

As shown in Fig. 2A, induction of cell death by *t*-BOOH treatment (800 µM for 4 h) was accompanied with a significant GSH depletion. This was in turn significantly prevented by the elicited HP extract by 65% and 100%, in pre- and co-incubation regimes, respectively (Fig. 2A). On the other hand, HP control extract was able to inhibit GSH depletion only in the co-incubation experiment for about 35%. The extract of AT did not show any significant effect against *t-BOOH*-induced depletion of GSH levels in HepG2 cells (Fig. 2A).

Similarly, DNA damage significantly increased with *t*-BOOH treatment (200 µM for 1 h; Fig. 2B). From all the three tested extracts, only the elicited HP extract was able to significantly inhibit *t*-BOOH-induced DNA damage by 40% and 55% in the pre- and

co-incubation regimes, respectively. The extracts alone did not induce DNA damage when incubated for 1 h (Fig. 2B).

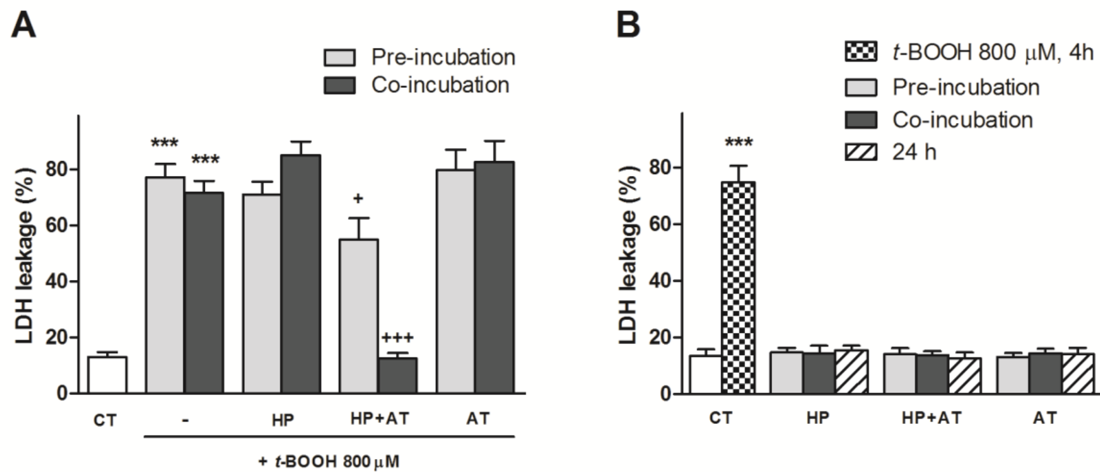

Fig. 1. Effect of methanolic extracts from control *H. perforatum* cells (HP), HP cells elicited with *A. tumefaciens* (HP + AT), and *A. tumefaciens* (AT) against *t*-BOOH-induced toxicity in HepG2 cells (A), as measured by LDH leakage. In the pre-incubation regime, cells were incubated with HP (100 μg/mL), HP + AT (100 μg/mL) or AT (25 μg/mL) for 6 h, followed by a recovery period of 16 h in fresh medium, before incubation with *t*-BOOH 800 μM for 4 h. Alternatively, cells were co-incubated with extracts and *t*-BOOH. Values are mean ± SEM of at least three independent experiments. *** $p \leq 0.001$, when compared with control (CT). $^{+}$ $p \leq 0.05$; $^{+++}$ $p \leq 0.001$, when compared with the respective *t*-BOOH alone by the Student's *t*-test. In (B), it is present the effect of extracts alone in cell viability in the same conditions of pre- and co-incubation regimes, as well as after a continuous incubation for 24 h.

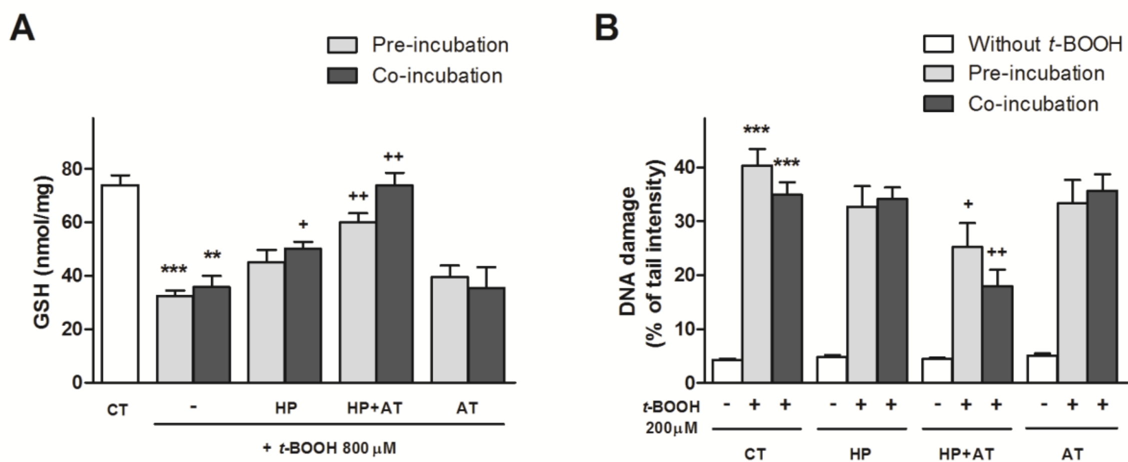

Fig. 2. Effect of methanolic extracts from control *H. perforatum* cells (HP), HP cells elicited with *A. tumefaciens* (HP + AT), and *A. tumefaciens* (AT) against *t*-BOOH-induced GSH depletion (A) and DNA damage (B) in HepG2 cells. In the pre-incubation regime, cells were incubated with HP (100 μg/mL), HP + AT (100 μg/mL) or AT (25 μg/mL) for 6 h, followed by a recovery period of 16 h in fresh medium, before incubation with *t*-BOOH 800 μM for 4 h (glutathione depletion) or 200 μM for 1 h (DNA damage). Alternatively, cells were co-incubated with extracts and *t*-BOOH for 1 h. In the DNA damage assay, the extracts were incubated also for 1 h without *t*-BOOH (white bars in (B)). Values are mean ± SEM of at least three independent experiments. ** $p \leq 0.01$; *** $p \leq 0.001$, when compared with control (CT). $^{+}$ $p \leq 0.05$; $^{++}$ $p \leq 0.01$, when compared with the respective *t*-BOOH alone by the Student's *t*-test.

3.2. Indirect antioxidant effects from the extract of elicited *H. perforatum* cells are associated with induction of endogenous antioxidant defenses in HepG2 cells

The observed protective effect of the extract of elicited HP cells against *t*-BOOH-induced oxidative damage in the pre-incubation regime suggests that this extract induces endogenous antioxidant defenses in HepG2 cells. To confirm this hypothesis, the effect of extracts for 24 h on intracellular antioxidant defenses was tested. As shown in Fig. 3A, the extract of elicited HP cells significantly induced the levels of the important intracellular antioxidant GSH by around 40%, whereas control HP and AT extracts did not show significant effects. As well, only the extract from elicited HP cells was able to induce the levels of the cytoprotective enzymes heme oxygenase-1 (HO-1), peroxiredoxin 4 (PRDX4), the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM, respectively), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Fig. 3B).

To test if these antioxidant defenses were also induced in same experimental conditions used in the above pre-incubation regime, before *t*-BOOH addition, the levels of GSH and protein expression of HO-1 and PRDX4 were also tested after 6 h incubation with extracts followed by a recovery period of 16 h in fresh medium. As shown in Fig. 4A, only the extract of elicited HP cells (100 μg/mL) significantly induced the levels of GSH (by ~20%). In addition, the same extract was the only one capable of inducing the cytoprotective enzymes HO-1 and PRDX4 (Fig. 4B).

In order to determine whether Nrf2 could play a role in the induction of GSH and cytoprotective enzymes by the extract of elicited HP cells, HepG2 cells were incubated with extracts from control HP (100 μg/mL), elicited HP (100 μg/mL) or AT (25 μg/mL) for 6 h, and nuclear extracts were obtained. The nuclear levels of Nrf2 were measured by western blot and, as shown in Fig. 5, only the extracts from elicited HP cells resulted in increased nuclear localization of Nrf2.

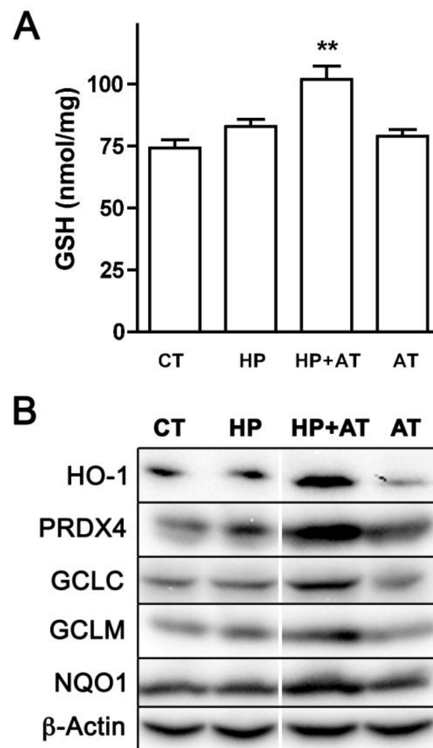

Fig. 3. Effect of methanolic extracts from control *H. perforatum* cells (HP, 100 µg/mL), HP cells elicited with *A. tumefaciens* (HP + AT, 100 µg/mL), and *A. tumefaciens* (AT, 25 µg/mL) for 24 h on GSH levels (A) and protein expression of the cytoprotective enzymes heme oxygenase-1 (HO-1), peroxiredoxin 4 (PRDX4), the catalytic and modifier subunits of glutamate cysteine ligase (GCLC and GCLM), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (B) in HepG2 cells. In (A), values are mean ± SEM of at least three independent experiments; ** $p \leq 0.01$ when compared to control (CT) by the Student's *t*-test. In (B), blots are representative of three independent experiments with similar results; β-actin was used as loading control.

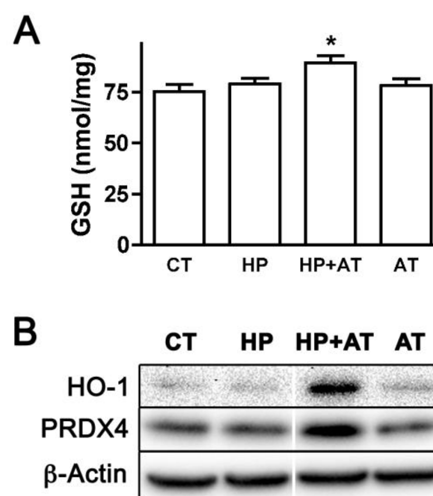

Fig. 4. Effect of methanolic extracts from control *H. perforatum* cells (HP, 100 µg/mL), HP cells elicited with *A. tumefaciens* (HP + AT, 100 µg/mL), and *A. tumefaciens* (AT, 25 µg/mL) for 6 h, followed by a recovery period of 16 h in fresh medium, on GSH levels (A) and protein expression of the cytoprotective enzymes heme oxygenase-1 (HO-1) and peroxiredoxin 4 (PRDX4) (B) in HepG2 cells. In (A), values are mean ± SEM of three independent experiments; * $p \leq 0.05$ when compared to control (CT) by the Student's *t*-test. In (B), blots are representative of two independent experiments with similar results; β-actin was used as loading control.

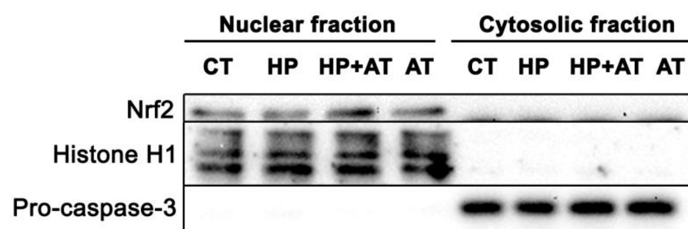

Fig. 5. Effect of methanolic extracts from control *H. perforatum* cells (HP, 100 µg/mL), HP cells elicited with *A. tumefaciens* (HP + AT, 100 µg/mL), and *A. tumefaciens* (AT, 25 µg/mL) for 6 h on Nrf2 expression in HepG2 cells. Blots are representative of two independent experiments. Histone H1 was used as loading control for nuclear fraction and pro-caspase-3 as loading control for cytosolic fraction.

4. Discussion

Since oxidative stress has been implicated in the pathogenesis and etiology of several diseases, natural antioxidants and plant extracts have been proposed as possible preventive and therapeutic agents. In our group, cell cultures of *H. perforatum* have been exploited for the biotechnological production of interesting bioactive phytochemicals. In a previous work, we demonstrated that the methanolic extract from HP suspension cultures elicited with AT has significantly increased antiradical scavenging activity and stronger protection against synaptosomal lipid peroxidation as compared with the extract from control HP cultures (Franklin et al., 2009). In this work, we evaluated the antioxidant effect of extracts of HP cells (control HP) and HP cells elicited with AT (elicited HP) directly in human HepG2 cells, and their ability to protect against *t*-BOOH-induced oxidative damage. This human hepatoma cell line retains many of the specialized functions of normal hepatocytes, including the activity of many phase I, phase II, and antioxidant enzymes, which ensures that they constitute a good tool to evaluate cytoprotective effects of natural compounds and plant extracts (Knasmuller et al., 2004). Here, oxidative damage was inflicted to HepG2 cells with *t*-BOOH, a toxicant widely used to induce oxidative stress and damage to cells, which mechanisms of action are well known (Lima et al., 2006).

The extract of elicited HP significantly protected HepG2 cells against *t*-BOOH-induced cell death, while the extracts of control HP and AT did not, in both pre- and co-incubation regimes. This shows that the production of a higher amount of xanthones and the presence of new ones in the extract of HP cells due to AT elicitation confer much higher antioxidant potential to the extract due to both direct and indirect effects. This demonstrates that HP elicitation produces bioavailable antioxidant compounds with cytoprotective effects, which may be interesting for healthcare and cosmetic applications.

The protection afforded by the extract of elicited HP cells was accompanied by prevention of DNA damage and GSH depletion, important cellular oxidative stress markers. In the co-incubation regime where cell death induced by *t*-BOOH was totally abolished by the extract of elicited HP, GSH depletion was reverted by almost 100% while DNA damage was inhibited by 55%. This suggests that GSH depletion is more relevant for the *t*-BOOH-induced cell death than DNA damage. GSH depletion is indeed one of the primary mechanisms of *t*-BOOH-induced loss of cell viability (Buc-Calderon et al., 1991; Martin et al., 2001). Previously, using the same experimental model, we also showed that prevention of GSH depletion was more essential than preventing lipid peroxidation or DNA damage in the ability of phenolic compounds to protect against *t*-BOOH-induced cell death (Lima et al., 2006). This may also explain why the small protection in GSH depletion afforded by the extract of control HP cells was not associated with protection against cell death induced by *t*-BOOH.

When compared with control extracts, the remarkable protection of the methanolic extract from elicited HP cells in the co-incubation regime indicates strong direct antioxidant effects of their constituents against *t*-BOOH toxicity. In this extract total xanthones increases 12 times, as well new xanthones are present (Franklin et al., 2009). The direct antioxidant effects of the xanthones may include their known high antiradical scavenging activity and the ability to inhibit lipid peroxidation (Franklin et al., 2009). In addition, their potential capacity to chelate metal ions and to inhibit enzymes involved in the activation of *t*-BOOH, such as peroxidases and cytochromes P450 (Lin et al., 2000; Lima et al., 2006), may also be potential mechanisms of action. Other reports have also shown the antioxidant effects in HepG2 cells of xanthones, such as for mangiferin against cadmium chloride- and mercury-induced oxidative stress (Satish Rao et al., 2009; Agarwala et al., 2012).

The observed protective effect of the extract of elicited HP cells against *t*-BOOH-induced oxidative damage in the pre-incubation regime suggests that this extract possess indirect antioxidant effects. This incubation regime with a washout period for recovery with fresh medium and without extracts ensures no direct interaction of extracts' components with toxicant at the time of its incubation. Protection against *t*-BOOH toxicity in these conditions may indicate dowregulation of proteins involved in the activation of this toxicant (not studied here) or induction of endogenous antioxidant defenses. Indeed, our results show that the extract from elicited HP cells but not from control cells increases significantly GSH levels and several cytoprotective enzymes, such

as the sub-units of the rate-limiting enzyme of GSH synthesis GCLC and GCLM, as well as the antioxidant and drug-metabolizing enzymes HO-1, PRDX4, and NQO1. All of transcripts of these enzymes are part of a set of genes that are under the influence of a redox-sensitive transcription factor–Nrf2 (McMahon et al., 2001; Surh et al., 2008).

Several studies have shown that Nrf2 plays a key role in orchestrating endogenous antioxidant defenses and therefore, the activation of this transcription factor is considered to be crucial for the protection against oxidative stress. Under normal physiologic conditions, the Kelch-like ECH-associated protein (Keap1) forms a complex with cullin 3 (Cul3) and represses Nrf2 by presenting it for ubiquitination and subsequent proteasomal degradation. Upon stimulation, the highly reactive cysteine residues of Keap1 are modified resulting in conformational changes that abrogate the capacity of Keap1 to repress Nrf2. The transcription factor undergoes nuclear translocation, and in heterodimeric combination with small Maf transcription factors, binds to the ARE and recruits the basal transcriptional machinery to activate transcription of cytoprotective genes (Motohashi and Yamamoto, 2004; Kobayashi and Yamamoto, 2006). In this work, we demonstrated that HP cells elicited by AT produce new compounds that are able to increase the nuclear levels of Nrf2 in HepG2 cells, which indicates its activation and may be associated with the observed induction of endogenous antioxidant defenses. The Nrf2 induction was probably through inhibition of Keap1 by modification of its cysteine thiols as previously described for other plant extracts and phenolic compounds (Surh et al., 2008; Lima et al., 2011). In fact, it is known that phenolic compounds in the cellular milieu, in the absence of a higher oxidant compound (for example *t*-BOOH), have predisposition for autoxidation (Lima et al., 2006, 2011). Therefore, a mild stress inflicted to the cells by compounds present in the elicited HP extract could have inhibited Keap1 and induced cytoprotective enzymes through Nrf2 signaling. Considering that xanthones total content increased 12-fold after elicitation of HP cells with AT (Franklin et al., 2009), it would be interesting to test in future experiments their role, namely the major one (1,3,6,7-tetrahydroxy-8-prenylxanthone), in the coordinated induction of Nrf2-mediated enzymes and their involvement in the regulation of cellular redox state and stress tolerance.

In conclusion, we demonstrate that the methanolic extract of HP cells elicited with AT have improved bioavailable antioxidants when compared with control HP cells, as shown by its ability to protect HepG2 cells against oxidative stress and to induce endogenous antioxidant defenses in human cells. Therefore, elicitation of HP cells with

biotic or abiotic stresses can be viewed as a biotechnological approach for the production of bioactive compounds with cytoprotective and antioxidant effects, interesting for pharmaceutical applications, without the use of genetic means for the modulation of HP secondary metabolism.

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CHAPTER 5

Conclusions and Future Perspectives

CHAPTER 5: Conclusions and Future Perspectives

1. Conclusions

People around the world are living longer although in poorer health, which generates an urgent need to identify strategies to improve human healthspan. Consumption of fruits and vegetables has been strongly associated with reduced risk of age-related diseases but the scientific basis for the strategy to improve health through diet is largely missing. The main objective of this doctoral thesis was to clarify if the hormetic induction of cellular stress responses by phytochemicals, in particular the Nrf2/ARE signaling pathway, can yield anti-aging effects and therefore explain the health beneficial effects of consumption of fruits and vegetables. To achieve this, different natural compounds and plant extracts were studied in human cells in culture.

In chapter 2, the extensive available literature on curcumin was reviewed to clarify if this phytochemical can be useful for healthspan-extending interventions. Indeed, the ancestral use of turmeric in traditional medicine and the extensive research over the last decades suggest that curcumin can be exploited for nutritional and pharmacological purposes. The numerous biological activities associated to curcumin are related with its peculiar chemical structure and its ability to modulate multiple molecular targets. Many of them impact in the recently proposed hallmarks of aging. Curcumin has been increasingly recognized as a useful phytochemical for aging interventions as shown by its actions on healthspan and longevity in different model organisms. This compound has also been associated with health promoting effects because of its potential in the prevention and treatment of aging-related diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases. Nevertheless, there is a lack of epidemiological and clinical evidences with human subjects of health and longevity promotion by curcumin. The medicinal application of curcumin is restricted by its poor solubility and low bioavailability and, therefore, new delivery systems are being developed to envisage its use for nutritional and pharmacological applications. The development of these new curcumin formulations may lead in the near future to the safe use of this phytochemical in strategies to promote healthy aging.

In chapter 3, the ability of the phenolic diterpenes carnosic acid (CA) and carnosol (CS) to induce stress responses in normal human skin fibroblasts was evaluated and related with hormetic effects. Additionally, the anti-aging effects of CA and CS were studied in a stress-induced premature senescence model as well as during replicative

senescence of normal fibroblasts. CA and CS significantly induced the levels of glutathione and several cytoprotective proteins in normal human fibroblasts through redox stress induction and associated with Nrf2/ARE signaling activation. The stress responses elicited by CS, the compound that elicited the most powerful responses, conferred significant protection against an oxidant challenge with *tert*-butyl hydroperoxide, demonstrating that this compound works as a hormetin. CS also protected against H_2O_2-induced premature senescence and ameliorated several features in cells undergoing replicative senescence *in vitro*, such as the changes in cell size, morphological heterogeneity, loss of parallel cell arrangement and senescence-associated β-galactosidase activity. Taken together, these data support the view that the hormetic induction of stress responses by CS may be applied for slowing down aging and preventing the onset of age-related diseases.

In chapter 4, the ability of methanolic extracts of HP cells (control HP) and of HP cells elicited with AT (elicited HP) to protect against oxidative stress induced in human HepG2 cells was evaluated. The extract from elicited HP, in contrast to the one from control HP, significantly inhibited *tert*-butyl hydroperoxide-induced cell death, GSH depletion and DNA damage, in pre- and co- incubation regimes in HepG2 cells. The extracts alone were not cytotoxic or genotoxic. Incubation of HepG2 cells with the extract from elicited HP cells also significantly induced the levels of glutathione and several cytoprotective enzymes, whereas the extract of control HP did not. These effects were associated with an increase of Nrf2 levels in the nucleus, which may explain the cytoprotective action of the elicited HP extract in the pre-incubation regime. Altogether, these results indicate that the elicitation of HP cells with biotic or abiotic stresses can be viewed as a biotechnological approach for the production of bioactive compounds (e.g. xanthones) with cytoprotective and antioxidant effects, of interest for pharmaceutical applications.

Overall, these findings support that phytochemicals can be viewed as a mean to promote healthy aging. In particular, the hormetic induction of cellular stress responses by carnosol can support anti-aging effects in normal human fibroblasts. That said, it is important to emphasize that even though this compound has shown promising anti-aging effects, more studies are needed to recommend its safe use in interventions to promote human healthspan.

2. Future perspectives

Some of the compounds and extracts addressed in this doctoral thesis have shown promising results regarding their future application in interventions to promote healthy aging although further studies are required.

Given that the induction of endogenous antioxidant defenses by the compounds and extracts was associated with the Nrf2/ARE signaling, it would be interesting to confirm the involvement of this pathway. To directly demonstrate the role of Nrf2 in ARE activation by phytochemicals, cells can be transfected with a construct containing the ARE consensus sequence fused to a reporter gene (e.g. firefly luciferase). To further confirm this, cells can be co-transfected with a dominant negative Nrf2 construct or an Nrf2 siRNA. In addition, it is possible that redox stress is involved in the induction of antioxidant defenses by the compounds and extracts. As previously mentioned, the modification of critical cysteine residues of Keap1 is important to Nrf2 activation. Therefore, it would be useful to assess thiol-disulfide redox status by measuring the oxidation of the pair cysteine/cystine by diagonal electrophoresis coupled with Western blot.

Considering the hormetic effects of curcumin and potential to induce senescence at high doses, appropriate tissue exposure needs to be established with proper experimental design in order to foresee the use of curcumin in interventions for improving healthspan and longevity.

Regarding the anti-aging effects of carnosol in normal human cells, the next logical step would be to validate these results *in vivo*. Lifespan assays can be performed in the presence of carnosol, and mean and maximum lifespan calculated from the survival curves of model organisms. The nematode *Caenorhabditis elegans* is a good model for this type of assays because there are mutant strains for skn-1 (Nrf2 homologue) and daf-16 (FOXO3a homologue) that can be used to test their involvement in lifespan extension.

Considering that xanthones total content drastically increased after elicitation of *H. perforatum* cells with *A. tumefaciens*, it would be interesting to test their role in the coordinated induction of Nrf2-mediated enzymes and their involvement in the regulation of cellular redox state and stress tolerance. Additionally, it would also be relevant to study this extract and its major xanthone (1,3,6,7-tetrahydroxy-8-prenylxanthone) in normal human cells, to better link their effects to the aging process.

Finally, it would be interesting to produce chemical modifications and/or new formulations of active compounds in order to improve its activity and safety.