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ANTIOXIDANT PROTECTION OF TUSCAN TOMATO PEEL POLYPHENOLS IN A CELLULAR MODEL OF SARCOPENIA

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Alla mia famiglia

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// ABSTRACT

Background: Tomato by-products contain a great variety of biologically active substances and might represent a significant source of natural antioxidant supplements of the human diet. The preliminary studies were carried out on two ancient Tuscan tomato peel varieties, Rosso di Pitigliano (RED) and Perina a Punta della Valtiberina (PER), obtained by growing plants in normal (-Ctr) or in drought stress conditions (-Ds) present in the Regional Bank of the Germplasm of Tuscany. The variety chosen was Rosso di Pitigliano for the best beneficial effects on vascular related dysfunction. The preliminary aim of the thesis was to create an *in vitro* model of sarcopenia, induced by dexamethasone using human skeletal muscle myoblasts (HSMM). Sarcopenia is a disease that affects athletes who practice endurance physical activity. In these, an excessive exercise increased reactive oxygen species (ROS) levels, that, if not properly balanced by the endogenous antioxidant system, can compromise the performance of the athletes. Furthermore, in controlling muscle mass an important role is played by serine/threonine kinase and a decreased activation of the Akt-mTOR pathway by sarcopenia contributes to protein synthesis reduction. The main aim of study was to evaluate the cytoprotective properties of tomato peel polyphenols from Rosso di Pitigliano, cultivated in normal or in drought stress conditions, on an *in vitro* model of sarcopenia.

Methods: The antioxidant activity and total polyphenol content (TPC) were measured. The identification of bioactive compounds of several tomato peel was performed by HPLC. HUVEC were pre-treated with different TPC of RED-Ctr or RED-Ds, then stressed with H_2O_2. Cell viability, ROS production and CAT, SOD and GPx activities were evaluated. Permeation of antioxidant molecules contained in RED across excised rat intestine was also studied. The phenol content of both peel extracts was investigated by Ultra High-Performance Liquid Chromatography (UHPLC) analyses coupled to

electrospray ionization high-resolution mass spectrometry (ESI-HR-MS). Morphological sarcopenia induction and treatment with tomato peels extracts were performed. The effector's expression was evaluated by Real-Time PCR reactions after setting the optimal reaction conditions. Myotubes-differentiated were examined for the expression of Myosin heavy chain-2 (MYH2), Troponin T type 1 (TNNT) and Myogenin (MYOG). Furthermore, Protein kinase B (AKT1) and Forkhead Box O1 (FOXO1) mRNA expression was evaluated. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were performed.

Results: RED-Ds tomato peel extract possessed higher TPC than compared to RED-Ctr (361.32 ± 7.204 mg vs. 152.46 ± 1.568 mg GAE/100 g fresh weight). All extracts were non-cytotoxic. Two hours pre-treatment with 5 µg GAE/mL from RED-Ctr or RED-Ds showed protection from H_2O_2-induced oxidative stress and significantly reduced ROS production raising SOD and CAT activity (* $p < 0.05$ and ** $p < 0.005$ vs. H_2O_2, respectively). The permeation of antioxidant molecules contained in RED-Ctr or RED-Ds across excised rat intestine was high with non-significant difference between the two RED types (41.9 ± 9.6% vs. 26.6 ± 7.8%).

Phenolic acids increase in the stressed tomato peel extract, while flavonoids decrease. Data shows a protective effect of 5µg GAE/ml TPC of Red DS extract on the sarcopenia. FOXO1 mRNA expression was significantly increased when cells treated with Dexa, but this expression was significantly decreased in Red Ds+Dexa ($p <0.0001$ vs control). AKT1 mRNA expression was increased in myotubes pre-treated with Red Ds and Dexa ($p <0.0001$ vs control). Myosin heavy chain 2 (MYH2), troponin T (TNNT1), miogenin (MYOG), were express in myotubes differentiated ($p<0.001$ vs Control). DEXA significantly reduces the antioxidant enzyme activity of SOD compared with untreated cells ($p < 0.0001$), but RED-Ds increased SOD activity.

Conclusions: The final results show that the tomato peel extract of Rosso di Pitigliano, grown in conditions of drought stress, represents a good source of bioactive molecules, which protects the endothelium from oxidative stress even at low concentrations. Furthermore, the polyphenols from tomato peel show a cytoprotective effect in the *in vitro* model of sarcopenia without the use of vehicles for absorption.

Keywords: tomato peel extract; tomato by-products; oxidative stress; drought stress; polyphenols; sarcopenia; human skeletal muscle myoblasts; human vascular endothelial cells

RIASSUNTO

Introduzione: I sottoprodotti del pomodoro contengono una grande varietà di sostanze biologicamente attive e rappresentano una fonte significativa di integratori antiossidanti naturali della dieta umana. Gli studi preliminari sono stati condotti su due antiche varietà toscane di bucce di pomodoro, Rosso di Pitigliano (RED) e Perina a Punta della Valtiberina (PER), ottenute coltivando piante in condizioni normali (-Ctr) o di stress da siccità (-Ds) presenti nella Banca Regionale del Germoplasma della Toscana. La varietà scelta è stata il Rosso di Pitigliano per i migliori effetti benefici sulla disfunzione vascolare. Lo scopo preliminare della tesi è stato realizzare un modello *in vitro* di sarcopenia, indotta da desametasone sui mioblasti del muscolo scheletrico umano (HSMM). La sarcopenia è una patologia che colpisce gli atleti che praticano attività fisica di endurance. In questi, un esercizio eccessivo aumenta i livelli di specie reattive dell'ossigeno (ROS), che, se non adeguatamente bilanciate dal sistema antiossidante endogeno, possono compromettere le prestazioni degli atleti. Inoltre, nel controllo della massa muscolare un ruolo importante è svolto dalla serina/treonina chinasi e una ridotta attivazione della via Akt-mTOR da parte della sarcopenia contribuisce alla riduzione della sintesi proteica. Lo scopo principale dello studio è stato valutare le proprietà citoprotettive dei polifenoli della buccia del pomodoro, Rosso di Pitigliano, coltivato in condizioni normali o di stress da siccità, in un modello *in vitro* di sarcopenia.

Metodi: L'attività antiossidante e il contenuto totale di polifenoli (TPC) sono stati misurati. L'identificazione dei composti bioattivi di diverse bucce di pomodoro è stata eseguita mediante HPLC. HUVEC sono state pretrattate con differenti TPC di RED-Ctr o RED-Ds, quindi stressate con H_2O_2. Sono state valutate la vitalità cellulare, la produzione di ROS e le attività CAT, SOD e GPx. È stata anche studiata la permeazione delle molecole antiossidanti contenute in RED attraverso l'intestino di ratto asportato. Il

contenuto di fenolo di entrambi gli estratti di buccia è stato approfondito mediante analisi di cromatografia liquida ad altissima prestazione (UHPLC) accoppiate a spettrometria di massa ad alta risoluzione a ionizzazione elettrospray (ESI-HR-MS). Sono stati eseguiti l'induzione morfologica della sarcopenia e il trattamento con estratti di bucce di pomodoro. L'espressione degli effettori è stata valutata mediante Real Time PCR dopo aver impostato le condizioni di reazione ottimali. I miotubi differenziati sono stati esaminati per l'espressione della catena pesante della miosina-2 (MYH2), della troponina T tipo 1 (TNNT) e della miogenina (MYOG). Inoltre, è stata valutata l'espressione dell'mRNA della proteina chinasi B (AKT1) e della Forkhead Box O1 (FOXO1). Sono state valutate le attività di superossido dismutasi (SOD), catalasi (CAT) e glutatione perossidasi (GPx).

Risultati: l'estratto di buccia di pomodoro RED-Ds possedeva un TPC più alto rispetto a RED-Ctr (361,32 ± 7,204 mg vs. 152,46 ± 1,568 mg GAE/100 g di peso fresco). Tutti gli estratti erano non citotossici. Il pre-trattamento di due ore con 5 µg di GAE/mL da RED-Ctr o RED-Ds ha mostrato protezione dallo stress ossidativo indotto da H_2O_2 e ha ridotto significativamente la produzione di ROS aumentando l'attività di SOD e CAT (* $p < 0.05$ e ** $p < 0.005$ vs. H2O2, rispettivamente). La permeazione delle molecole antiossidanti contenute in RED-Ctr o RED-Ds attraverso l'intestino di ratto asportato era elevata con una differenza non significativa tra i due tipi RED (41,9 ± 9,6% vs 26,6 ± 7,8%).

Gli acidi fenolici aumentano nell'estratto di buccia di pomodoro stressato, mentre i flavonoidi diminuiscono. I dati mostrano un effetto protettivo di 5µg GAE/ml TPC di estratto di Red DS sulla sarcopenia. L'espressione dell'mRNA di FOXO1 era significativamente aumentata quando le cellule venivano trattate con Dexa, ma questa espressione era significativamente ridotta in Red Ds+Dexa ($p < 0,0001$ vs controllo). L'espressione dell'mRNA di AKT1 era aumentata nei miotubi pretrattati con Red Ds e

Dexa ($p < 0,0001$ vs controllo). La catena pesante della miosina 2 (MYH2), la troponina T (TNNT1), la miogenina (MYOG), sono state espresse in miotubi differenziati ($p<0,001$ vs controllo). DEXA riduce significativamente l'attività enzimatica antiossidante della SOD rispetto alle cellule non trattate ($p < 0,0001$), ma RED-Ds ha aumentato l'attività di SOD.

Conclusioni: I risultati finali dimostrano che l'estratto di buccia di pomodoro del Rosso di Pitigliano, coltivato in condizioni di stress da siccità, rappresenta una buona fonte di molecole bioattive, che protegge l'endotelio dallo stress ossidativo anche a basse concentrazioni. Inoltre, i polifenoli della buccia di pomodoro, mostrano un effetto citoprotettivo nel modello *in vitro* di sarcopenia senza l'utilizzo di veicoli per l'assorbimento.

Parole chiave: estratto di buccia di pomodoro; sottoprodotti del pomodoro; buccia di pomodoro; stress ossidativo; stress da siccità; polifenoli; sarcopenia; mioblasti del muscolo scheletrico umano; cellule endoteliali vascolari umane

ABBREVIATIONS

AKT1= protein kinase B

B2M= beta-2 microglobulin

BSA= bovine serum albumin

CAT= catalase

COVID-19= coronavirus disease 19

CVD = cardiovascular disease

DEXA= dexamethasone

eEF1A= eukaryotic translation elongation factor 1 alpha

FBS= fetal bovine serum

FOXO1= Forkhead box O1

GAE= gallic acid equivalent

GPx= glutathione peroxidase

GSH = glutathione transferase

H_2O_2= hydrogen peroxide

HEPES= 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC= high-performance liquid chromatography

HUVEC= Human Umbilical Vein Endothelial Cells

HSMM= Human Skeletal Muscle Myoblasts

M199= medium 199

MAFbx= muscle atrophy F-box

mTOR= mammalian target of Rapamycin

MuRF1= muscle ring-finger 1

MYH2= myosin heavy chain 2

MYOG= myogenin

MY-32= anti-skeletal myosin fast primary antibody

RED= Rosso di Pitigliano

RNS= Reactive Nitrogen Species

ROS= Reactive Oxygen Species

RPL13A= ribosomal protein L13a

PER= Perina a Punta della Valtiberina

SKGM-2= Skeletal Muscle Cell Growth Medium-2

SOD= superoxide dismutase

TNNT1= Troponin T

TPC= Total Polyphenol Content

UHPLC= ultra-high performance liquid chromatography

WST-1= 4-[3-(4-iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolium]-1,3 benzenedisulfonate

CHAPTER 1 Introduction

1.1 Tomato Production and Crops

The tomato (*Solanum lycopersicum L.*) is an edible Mediterranean plant already known for its beneficial properties. Whether it is fresh or processed, it is one of the most consumed vegetables worldwide [1,2] and a key component of the daily meals in many countries. The tomato is the most widely consumed vegetable in the world and hence is the major contributor of dietary nutrients and antioxidants. Their average daily consumption is higher than that of all other fruits and vegetables because of their year-round availability. During the 13th World Processing Tomato Congress, held in June 2018, it has been shown that since 1994 the tomato production and processing has grown worldwide by 59%. From the data reported by Ismea in 2017 in Rome, is shown that California, Italy, and China are the biggest tomato producers of the world followed by Spain, Turkey and Portugal [3]. The cultivation of industrial tomatoes in Italy is concentrated in two geographically well-defined basins: the northern basin and the southern basin. The two-production district, as reported in **Figure 1**, are characterised by a different organization and structure of production [4].

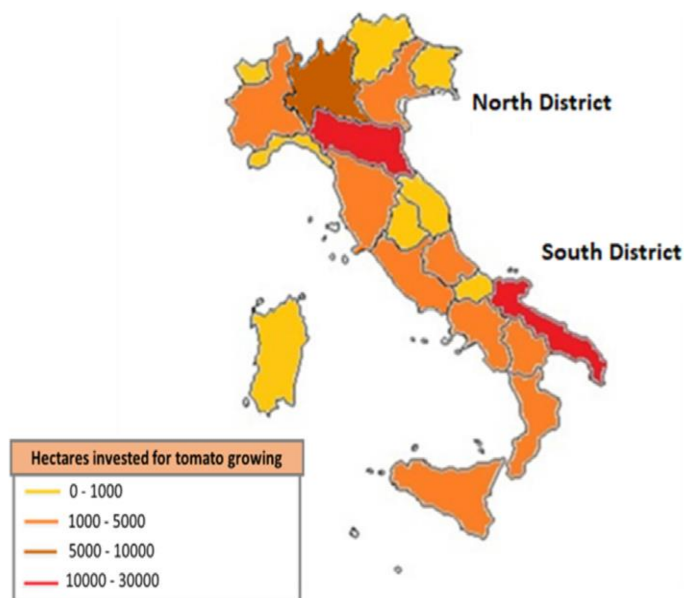


Figure 1:The figure shows tomato production by country (Cuna D. et al., 2018).

Furthermore, tomato plant is susceptible to abiotic and biotic stresses [5,6]. Drought stress is one of the most severe conditions for plants, especially in the face of the emerging problem of global warming. Given the increased frequency of acute drought conditions due to global warming, its severity will most likely be exacerbated by future climate change [7]. Tomato plants adapt to different climatic conditions and to abiotic stress, such as the shortage of water which is one of the most serious problems concerning crops [8]. Unfortunately, tomato plants are highly sensitive to drought stress. The lack or shortage of water is the most common environmental factor that influences plant growth and productivity/yield [9]. In the future, water will become a strategic resource and, therefore, industrial, and agricultural processes requiring low water consumption must be developed. Plants have adopted various strategies to cope with water deficiency, including changes in growth and development [10,11]. In normal conditions, antioxidant defense systems are in a dynamic balance in plant cells, whereas stress conditions may result in an increase in antioxidant compounds that are either enzymatic or non-enzymatic in nature [12,13]. Water shortage stress can affect the yield of tomato crops, the volume, diameter and composition of the fruits, e.g., lycopene and total soluble sugars content [14].

In **Figure 2** shows tomato plants of Rosso Di Pitigliano growing in normal or drought stress conditions.

Figure 2: Tomato plants of Rosso di Pitigliano cultivated in greenhouse (Botanical Garden, University of Siena, Siena, Italy).

1.2 Recovery of By-Products

In addition to the bioactive components that could give rise to the production of health foods, equally interesting substances can be recovered from the by-products. The industrial transformation of vegetables and fruits generates large quantities of by-products, i.e., peels and seeds, rich in bioactive compounds. The recycling or reusage of by-products accumulated during processing and are available in high amounts can reduce treatment costs. These natural compounds could be used in foods, pharmaceuticals, and cosmetics. In **Figure 3**, tomato by-products are shown [15].

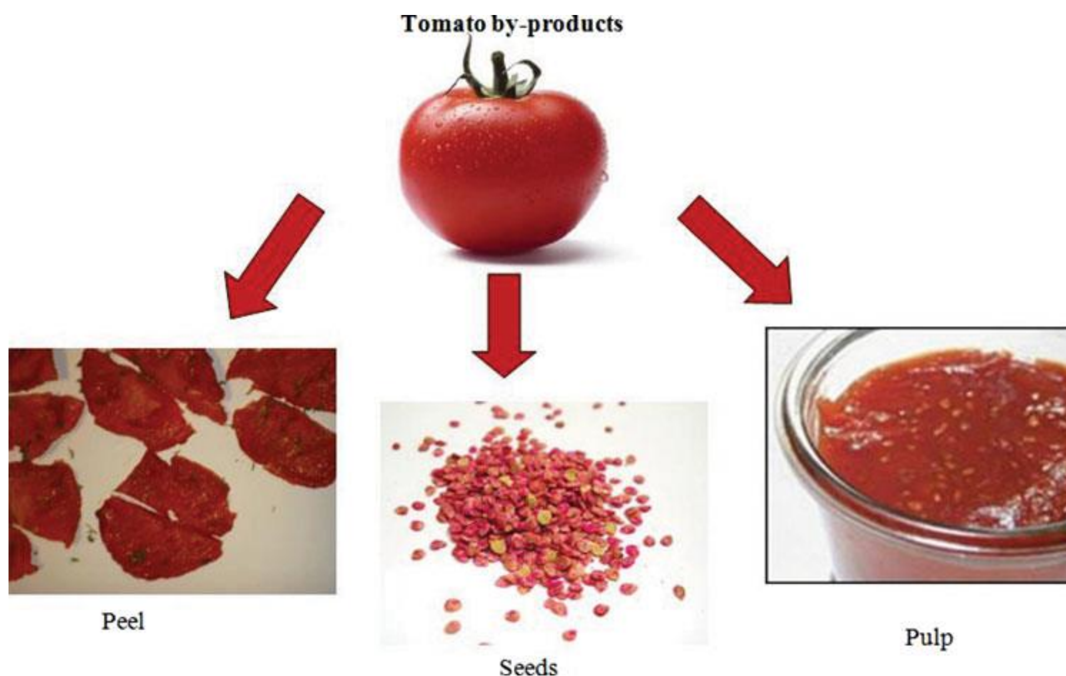


Figure 3: The by-products obtained during tomato processing (Viuda-Martos M. et al, 2014).

Moreover, what is remarkable is the amount of various kinds of useful waste products, such as peels (about 60%) and seeds (around 40%). During the industrial processing of tomatoes, large quantities of by-products are generated, such as peels, which have high nutritional value [16]. Many studies have tested their potential beneficial effects on health [17-19] and optimized the extraction process of carotenoids from tomato peels [20]. Moreover, it is important to recycle industrial tomato processing waste to reduce the environmental impact of agro-industrial transformation processes. Hydroxycinnamic acids, such as chlorogenic, caffeic, ferulic and p-coumaric, contribute to the antioxidant ability of tomatoes and their derivatives.

1.3 Regional Bank of the Germplasm of Tuscany

The Regional Bank of the Germplasm of Tuscany is aimed at guaranteeing the protection, through ex situ conservation, of the regional autochthonous genetic resources (seed banks, collection fields, etc.). The Bank carries out all operations aimed at safeguarding

the genetic material stored therein, from any form of contamination, alteration, and destruction.

The tomato varieties were chosen from nine local Tuscan varieties (*Solanum lycopersicum L.*) registered at the Tuscan Regional Germplasm Bank and characterized by different morphological and agronomic features. Two tomato varieties, Rosso di Pitigliano (RED) and Perina a Punta della Valtiberina (PER) were chosen in virtue of its highest concentration of antioxidants. In **Figure 4 a, b**, Rosso di Pitigliano tomatoes are shown.

(a)

(b)

Figure 4: Tomato Rosso di Pitigliano (a) Incomplete ripening tomato (b) Ripe tomato from plants grown under normal (CTR) or drought stress (DS) conditions.

1.4 Role of Tomato Extracts on Vascular Diseases

Cardiovascular disease (CVD) is the leading cause of death in the world. The World Health Organization defines CVD as a group of disorders of the heart and blood vessels

that includes numerous problems, many of which are related to a process called atherosclerosis. These disorders originate from a chronic inflammatory vascular process that affects the wall of medium-sized arteries and ends up producing endothelial dysfunction, arteriosclerosis. Several studies suggest that bioactive compounds present in foods as tomato products have potential benefits against CVD [21,22]. Such potential benefits have been ascribed in part to high concentrations of lycopene in the tomatoes. Epidemiological studies have revealed a strong association between tomato consumption and decreased risk of chronic degenerative diseases such as cardiovascular and neurodegenerative pathologies, and cancer [22-26]. To reduce cardiovascular risk, it is important to use strategies that cover the entire population, acting on risk factors such as an unhealthy diet.

In virtue of their high consumption, tomatoes and tomato products represent an important source of diet [27-29]. Tomatoes are well known for their content in carotenoids such as lycopene as well as ascorbic acid, vitamin E and phenol compounds, in particular, flavonoids and hydroxycinnamic acids [30,31].

Lycopene is one of the main antioxidants to be found in fresh tomatoes and processed tomato products. Because of the presence of long-chain conjugated double bonds, lycopene has been reported to possess higher antioxidative activity than luteolin or β-carotene. Lycopene content also accounts for the redness of the fruit [32]. Colour also serves as a measure of total quality for tomato and tomato products. Among the different antioxidant compounds present in tomato fruits, phenols are the major contributors for the higher antioxidant capacity of the fruit. Along with phenols, higher intake of flavonoids, vitamin C and carotenoids are also known to contribute to the antioxidant capacity of fruits and vegetables. Lycopene and β-carotene are the main C40 carotenoids found in tomatoes [33], whilst the chemical class of polyphenols is represented by rutin

(quercetin 3-O-rutinoside), quercetin, naringenin and chalconaringenin, which are just a few of the various flavonoids found in this fruit, together with organic acids such as benzoic, protocatechuic and cinnamic [34,35]. Many articles have reported the presence of flavonoids and hydroxycinnamic acids in tomatoes [36,37]. This group of polyphenols includes a variety of chemical structures that act on antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), resulting in very efficient peroxyl radicals scavenger activity [38]. The above are three key enzymes in the first line defense. They are very fast in neutralizing any potentially oxidative molecules in the cells [39].

HUVEC is an excellent model to study a broad array of diseases, including cardiovascular and metabolic diseases [40] so several studies have tested antioxidant molecules derived from natural products on HUVEC by *in vitro* experiments related to vascular dysfunction [17, 41-43].

1.5 Sport Sarcopenia

The decline of skeletal muscle mass and strength that leads to sarcopenia is a pathology that might represent an emergency healthcare issue in future years. Decreased muscle mass is also a condition that mainly affects master athletes involved in endurance physical activities. As first introduced by Rosenberg in 1988, to indicate "muscle loss" [44], sarcopenia is defined by structural, biochemical, molecular, and age-associated functional muscle changes. Over time, the definition has been revisited in order to take into account the fact that a loss of strength and muscle power is also associated with aging.

The physical activity induces a whole-body physiological response at the systemic and cellular level, but an excessive exercise can elevate stress, thus increasing reactive oxygen species (ROS) levels, which, if not properly balanced by the endogenous antioxidant

system, can compromise the performance of athletes. The decrease in muscle mass can take on pathological connotations, up to sarcopenia. This pathology is characterized by age-related loss of muscle mass and function; it represents one of the first implications of old age. The pathogenesis of sarcopenia is the consequence of multifactorial processes including intrinsic and extrinsic factors that reduce the regeneration capacity of damaged muscle fibers. Sarcopenia favors oxidative stress and show a decline in antioxidant protection of antioxidants enzymes activities [45]. Skeletal muscle atrophy, characterized by mass loss and muscle function decline, is due to an increase in muscle protein degradation and reduced protein synthesis [46,47]. Muscle mass reduction is the consequence of various conditions, such as aging, fasting, denervation, and disease [48-51]. In controlling muscle mass an important role is showed a serine/threonine kinase, the mammalian target of rapamycin (mTOR) activated by various intracellular and environmental changes [52,53]. A decreased activation of the Akt-mTOR pathway contributes to protein synthesis reduction [54,55].

Numerous studies have shown that glucocorticoids decrease muscle size, largely due to accelerated muscle protein breakdown, a mechanism linked to an increased catabolism of muscle proteins by the ubiquitin-proteasome pathway [56,57]. In a recent study, Klein [58] reported the effect of both endogenous and exogenous glucocorticoids on bone and muscle, demonstrating that the supraphysiologic loads of glucocorticoids are similar, regardless of whether they enter the body as medication, or are produced by the body in response to stimuli, such as inflammation. In particular, protein degradation occurs by the covalent attachment of the protein ubiquitin, by ubiquitin E3 ligases, namely muscle atrophy F-box (MAFbx, also called atrogine-1) and muscle ring-finger 1 (MuRF1). MAFbx and MuRF1 are two muscle-specific ubiquitin ligases that have been linked to muscle atrophy when upregulated [59-61]. In addition to regulating protein breakdown,

both MuRF1 and MAFbx may also contribute to a decrease in protein synthesis in response to glucocorticoid administration [62]. Thus, to mimic *in vitro* sarcopenia, treatment with dexamethasone, a synthetic glucocorticoid, is used. The phosphoinositide 3-kinase/protein kinase B signaling pathway (PI3K/Akt) is involved in myotube atrophy induced by glucocorticoids. As shown in **Figure 5**, the Akt-mediated inhibition of the FoxO family of transcription factors is reduced by dexamethasone, which prevents Akt phosphorylation and leads to activation of FoxO transcription factors and MuRF1 and MAFbx upregulation [63-65]. FoxO1 and FoxO3 have been implicated in muscle wasting. Due to the critical role of FoxO transcription factors in the development of muscle atrophy, therapeutic approaches to inhibit FoxO phosphorylation are of clinical interest to counteract muscle wasting.

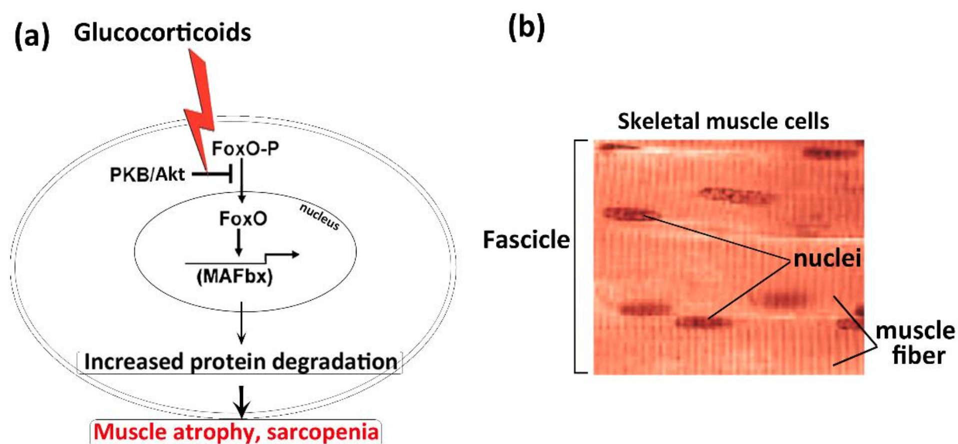


*Figure 5: Model of sarcopenia induced by dexamethasone (**a**)Protein kinase B (Akt) causes phosphorylation and nuclear exclusion of Forkhead box protein O (FoxO) family, which suppresses atrogene (muscle atrophy F-box, MAFbx) expression and proteolysis. Dexamethasone prevents Akt-mediated phosphorylation of FoxO (FoxO-P), inducing its transfer to the nucleus and the consequent MAFbx upregulation and protein degradation. (**b**) The skeletal muscle structure.*

The use of antioxidants has been shown to be efficient against sarcopenia. Polyphenols are divided into four classes: phenolic acids, flavonoids, stilbenes, and lignans. These antioxidants are classified based on the number of phenol rings and of the structural elements that bind these rings to one another. Phenolic acids are non-flavonoid

polyphenolic compounds which can be further divided into two main types, benzoic acid (gallic acid) and cinnamic acid (caffeic acid, p-coumaric acid) derivatives. Polyphenols are involved in the prevention or delay of muscle disorders correlated to muscle mass loss [66].

1.6 Antioxidants in the Prevention of Sarcopenia

1.6.1 Exercise and ROS Production: How to Prevent Oxidative Damage

As first reported by Davies et al. in 1982 [67], numerous studies have subsequently reported that strenuous exercise and endurance training causes ROS and RNS accumulation in skeletal muscle, which promote oxidative stress [68-70]. Oxidative stress is the result of an imbalance between the physiological production of free radicals and the cells' ability to scavenge them. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, are constantly produced by muscle cells, both in resting conditions and during contraction, controlling force production [71]. The consequent pro-oxidant state causes the alteration of the mitochondrial DNA and some anomalies in the electron transport system, leading to a reduction in the absorption of calcium by the sarcoplasmic reticulum and irreversible damage to the cell and its consequent death [72].

A moderate increase in the levels of ROS, which can be observed from light to moderate exercise, causes an increase in the development of muscle strength up to a maximum peak; however, a further increase induces a drastic decline in strength [73,74].

ROS are produced in muscle cells by various fonts in different cell compartments and through different pathways. During exercise, the mitochondrial electron transport chain is one of the main sites of ROS production [75]. Due to high oxygen consumption by increased mitochondrial activity, the transfer of a single electron to molecular oxygen gives rise to a monovalent reduction of oxygen, which leads to the formation of

superoxide ions. The enzymatic process can also promote superoxide production through NADPH oxidase enzymes or the xanthine/xanthine oxidase system [76]. Moreover, in the presence of transition metal ions (e.g., $Fe^{2+\3+}$, $Cu^{+\2+}$), hydrogen peroxide (H_2O_2) produces the highly reactive hydroxyl radical ($OH^{\cdot}$) and hydroxyl ion (OH^-), according to the Fenton reaction [77] (**Figure 6**).

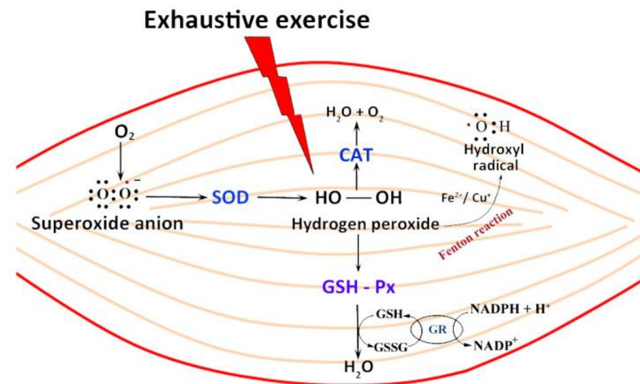


Figure 6: Antioxidant enzyme activity to counteract reactive oxygen species (ROS) production and accumulation in skeletal muscle cells after exercise. Exhaustive exercise induces an increased production of reactive oxygen species: superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH.) according to the Fenton reaction. Catalase (CAT), glutathione transferase (GSH), glutathione peroxidase (Px), and superoxide dismutase (SOD), work to maintain a state of oxidative balance, producing water and oxygen. Glutathione disulfide (GSSG), Glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and nicotinamide adenine dinucleotide phosphate (NADP).

To prevent oxidative cell damage, a well-organized system of antioxidants acts in a synchronized fashion. Cells, including muscle fibers, contain a network of antioxidant defense mechanisms to reduce the potential of oxidative damage during periods of increased ROS production. The enzymatic and non-enzymatic (i.e., vitamin A, vitamin C, vitamin E, β-Carotene) systems regulate the homeostasis redox status of muscle cells. Catalase (CAT), glutathione transferase (GSH), glutathione peroxidase (GPx), and superoxide dismutase (SOD), are some of the components of the enzyme elimination system that are significantly depressed in elderly muscle. Generally, antioxidant enzymes (e.g., CAT, SOD, GPx, or GSH) work to maintain a state of oxidative balance, converting ROS into more stable molecules, such as water and molecular oxygen (**Figure 6**). Most studies investigating the adaptive responses to exercise-induced ROS/RNS generation

have shown that both acute and regular exercise [78] induce increased activities of antioxidant defense enzymes in skeletal muscle, in particular SOD [79]. Existing in the cytoplasm of the eukaryotic cells and in the mitochondria (as Cu/ZnSOD and MnSOD, respectively), SOD is involved in the dismutation of superoxide anion in molecular oxygen and hydrogen peroxide [80], which is converted into water by catalase or GPx. In particular, an increase in SOD protein content has been observed during repeated bouts of aerobic exercise in mice muscle [78].

In the reaction catalyzed by GPx, glutathione is oxidized to glutathione disulfate, which can be converted to glutathione by glutathione reductase in an "NADPH-consuming" process [81]. Catalase (CAT) is a high molecular weight tetrameric enzyme containing porphyrin in the active site. It is one of the fundamental antioxidant enzymes that mitigates oxidative stress to a significant extent by destroying cellular hydrogen peroxide to produce water and oxygen [82]. Antioxidants play important roles in regulating ROS levels through direct free radical scavenging mechanisms, through regulation of ROS/RSN- producing enzymes, and/or via adaptive electrophilic-like mechanisms. However, the relationship between free radical generation, antioxidant enzymes, and exercise in skeletal muscle remains controversial, due to differences in exercise intensity, mode, duration of training program, and muscle fiber type. In fact, each muscle fiber type has distinct oxidative potential and metabolic characteristics as well as antioxidant defense capacity [83]. As reported by Gonchar [84], in a study carried out on rats, after endurance swimming training, a significant decrease in GPx, glutathione reductase activities and GSH content in both fast- and slow-twitch muscles was observed, confirming that antioxidant enzyme response to chronic exercise is highly muscle fiber specific.

To reinforce the natural endogenous protection, nutritional supplements may represent a good strategy, contributing to reducing the indices of oxidative stress. Nutritional antioxidants act indifferent mechanisms and compartments: (1) neutralize free radicals; (2) repair oxidized membranes; (3) decrease ROS production; and (4) neutralize ROS via lipid metabolism, short-chain free fatty acids, and cholesteryl esters [85]. Moreover, dietary antioxidant vitamins (such as C, E, and carotenoids) are auspicious candidates for the prevention of age-related loss of mass and function.

1.6.2 Exogenous Antioxidants

Exogenous antioxidants have generated growing interest in preventing oxidative stress, in decreasing muscle pain and physical stress, and in improving sport performance. It is important to note that higher dosages of antioxidants may not necessarily be beneficial for athletes training for and competing in different sporting events but can also elicit detrimental effects by interfering with performance-enhancing and health-promoting training adaptations [86].

Exogenous antioxidants act in addition to the endogenous ones. Tocopherols or vitamin E, L-ascorbic acid or vitamin C, carotenoids, ubiquinone, and polyphenols are the most well-known exogenous antioxidants [87]. Although natural compounds with antioxidant activity are numerous, we reported the most known nutritional antioxidants, mainly vitamin C, vitamin E, carotenoids, flavonoids, and polyphenols [88-91], whose functions on muscles have been more extensively studied. According to the hydrophobicity of the administered molecule, the supplementation of different antioxidants will provide different effects on oxidation. For example, fat-soluble antioxidants, such as vitamin E, are mostly effective in inhibiting lipoprotein peroxidation, while water-soluble

antioxidants, such as vitamin C, are better able to protect the aqueous phase. On the other hand, these antioxidants also act cooperatively and sometimes even synergistically.

Vitamin C, an electron donor (reducing agent), whose antioxidant function derives from its ability to reduce oxidized species or oxidant radicals [92], is an indispensable nutrient, which plays a critical role in multiple hydroxylation reactions, maintaining redox homeostasis in organelles, such as mitochondria and the endoplasmic reticulum [93] and is one of the most important antioxidants in human plasma [92]. The effect of vitamin C supplements on antioxidant defense in human skeletal muscle during training has been widely studied. Khassaf et al. [79] observed that antioxidant defense mechanisms of vitamin C were more specifically on SOD and CAT activity; thus, improving skeletal muscle antioxidant defense. Very recently, Welch et al. [94] studied the effect of antioxidants, particularly vitamin C, on loss of skeletal muscle mass and power during the aging process. The authors investigated the associations between an extensive range of sarcopenic indices, including DXA-measured fat-free mass, grip strength, and leg explosive power, and a range of antioxidant vitamins (A, C, E, and carotenoids), observing a significant improvement of sarcopenic indices of skeletal muscle after antioxidant intake.

Unlike most mammals, and other animals, humans cannot synthesize vitamin C because they lack the enzyme l-gulonolactone oxidase in the biosynthetic process. Therefore, it must be obtained by dietary intake, particularly from fruits and vegetables. The principal sources of vitamin C are kiwifruit, broccoli, kale, strawberries, tomatoes, and sweet red pepper [95]. Neubauer et al. [96] discussed the findings of a number of key studies and their implications for defining guidelines for the intake of vitamin C and E in athletes.

The most common isoform of vitamin E in the human body is α-tocopherol, although vitamin E includes eight fat-soluble isoforms. Rich sources of α-tocopherol are sunflower

seeds, almonds, and hazelnuts, as well as many vegetable oils (e.g., olive oil and canola oil) and vegetables (e.g., tomato, spinach, and asparagus). The number of methyl groups on the chroman ring is responsible for the antioxidative effects of vitamin E isomers. In particular, three methyl groups make up α-tocopherol, while one methyl group in the chroman ring organizes δ-tocopherol. The order of $\alpha > \beta > \gamma > \delta$ indicates the strength of antioxidative activity between vitamins E isomers, and α-tocopherol is stronger than tocotrienol [97].

Peroxyl radical are intercepted by α-tocopherol, preventing lipid peroxidation and the negative effects of free radicals in membranes and plasma lipoproteins [98]. Recently, Miyazawa et al. [99] reviewed the role of vitamin E in redox interaction, reporting its relationship with ROS and the antioxidative mechanism of action. However, the antioxidant effects exerted by vitamin E are complicated; furthermore, the mechanisms are not yet well understood.

Rokitzki et al. [100] reported that administration of combined vitamins C and E for 1 month prior to a marathon race decreased the indices of muscle damage after the race. However, consuming high doses of single antioxidants (such as vitamins C and E) may inhibit the signaling pathways normally triggered by the oxidative stress of exercise during training [101].

Recent human studies have investigated the interrelation of antioxidant supplementation and exercise training, using a more sophisticated design, methodologies, and techniques, and focusing not only on performance, but also on the health aspects of endurance training.

Carotenoids, a terpenoid-based compound, are organic pigments present in fruits and vegetables, such as pumpkins, carrots, corn, and tomatoes, representing important dietary antioxidants. Few carotenoids (about a dozen) are known to be healthy components for

the human diet. Among these, β-carotene and several xanthophylls, such as lutein, without or with oxygen in the molecule, respectively, are included. Recently, Bohn et al. [102] reviewed the benefits of carotenoids in humans. They evaluated the role of carotenoids in human chronic diseases characterized by oxidative stress and reported that the beneficial health effects of carotenoids shown in small-scale studies and in subjects suffering from chronic oxidative stress are not evident in large-scale trials with carotenoid supplements. Finally, as recently evidenced by Sandmann et al. [103], carotenoids can play a role as direct antioxidants, by quenching singlet oxygen and peroxide radicals.

Polyphenols are a group of water-soluble, plant-derived substances, characterized by the presence of more than one phenolic group [90]. These molecules are divided into two sub-categories: flavonoids and phenolic acids. There are at least six subclasses of flavonoids, often referred to together, based on distinct differences in chemical structure. Flavonols are present in a wide variety of fruits and vegetables. Quercetin and kaempferol are the most common flavonols in food [104].

Polyphenols have potent free radical scavenging abilities. However, the in vivo relevance of these mechanisms is debatable due to their generally poor oral bioavailability with transient low accumulation [105], relatively poor uptake into peripheral tissues, such as skeletal muscle [106], and poor competition for ROS/RNS with endogenous antioxidants. Ingested polyphenols undergo extensive metabolism and modification in the liver and intestines. Resveratrol, a natural polyphenol found in grapes, peanuts, and berries, has shown a protective effect against oxidative stress in skeletal muscle through the expression of antioxidant enzymes [107]. Moreover, evidence in rodent skeletal muscle has shown improvements in oxidative stress and induction of endogenous antioxidant enzymes after resveratrol supplementation when combined with exercise [108-110].

Recently, Myburgh K.H. [111], reviewed the benefits of polyphenol supplementation for exercise performance, paying attention to the details of study design, subject population, supplementation regimen, and testing protocol aspects (and not only the outcomes of the various studies). The author affirmed that "although it is clear that polyphenol supplementation in a variety of forms and doses is able to increase the capacity to quench free radicals, at least in the circulation, it is not yet clear whether it holds beneficial effects for athletes" [111].

CHAPTER 2 First Experimental Section: EFFECT OF TOMATO PEELS EXTRACTS ON VASCULAR RELATED DYSFUNCTION

The first section presents the ability of Tuscan tomato peel extracts, obtained by growing plants in normal or in drought stress conditions, to protect human endothelial cells from oxidative stress, antioxidant activity, antioxidant enzymes activity and tomato peel extracts polyphenols intestinal permeation. To this purpose an in *vitro* model of human endothelial vascular cells, the HUVEC, was used. Furthermore, the extracts were evaluated both in vitro and *ex vivo* in order to compare their beneficial effects on vascular related dysfunction following oral intake.

In this chapter each section will be divided into materials and methods, results and discussion, tables, and figures. The future perspectives will be discussed in Chapter 4.

2.1 Materials and Methods

2.1.1 Materials

Hexane, acetone and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich (Milan, Italy). H_2O_2 was bought from Farmac-Zabban S.p.a. (Calderara di Reno, BO, Italy); gelatin was obtained from Sigma-Aldrich (Milan, Italy). Medium 199 (M199), fetal bovine serum (FBS), penicillin-streptomycin solution, l-glutamine and HEPES buffer were supplied by Hospira S.r.l. (Naples, Italy). 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzenedisulfonate (WST-1 assay) was purchased from Roche Applied Science (Mannheim, Germany); 5-(and-6)-chloromethyl-2′,7′-dichloro-di-hydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA) was supplied by Invitrogen (Thermo Fisher Scientific).

2.2 Sample Preparation

2.2.1 Fruit Harvesting and Stress Condition

The tomato varieties used for the study were chosen from nine local Tuscan varieties (*Solanum lycopersicum L.*) registered at the Tuscan Regional Germplasm Bank and characterized by different morphological and agronomic features. The preliminary studies were carried out on two tomato varieties, Rosso di Pitigliano (RED) and Perina a Punta della Valtiberina (PER), obtained by growing plants in normal or in drought stress conditions. Then to continue the study, the tomato variety chosen was Rosso di Pitigliano obtained by growing plants in normal or in drought stress conditions. Four plants were used for the control and four for the stress condition, which are cultivated in a greenhouse (Botanical Garden, University of Siena, Siena, Italy) and all plants were well-watered until the beginning of the water stress treatment. The drought stress conditions began when the first fruits started to grow. The plants were around 120 cm high at the beginning of the stress test and the waterless treatment lasted for 20 days [112,113]. Ripe tomatoes (*Solanum lycopersicum L.*) were harvested in the period between 1 August to 31 August 2019 and fruits were refrigerated at - 80 °C to stop their normal biological processes.

2.2.2 Tomato Peel Extracts Preparation

Lyophilized tomato peel sample was prepared according to Beconcini et al. [42], with some modifications. Briefly, frozen tomatoes were hand peeled and weighed to obtain 2 g of tomato peel and then 6 mL of water was added. The mixture was homogenized (Ultra-Turrax® T25 based IKA, Saint Louis, MS, USA) for about 3 min and sonicated (Elma Transsonic T 460/H, Wetzikon, Switzerland) for 20 min to guarantee complete cellular decomposition. The mix was again homogenized one minute and centrifuged 5 min at

13,000 rpm (Eppendorf® 5415D centrifuge, Hamburg, Germany) to separate the biomolecules from the pellet. The supernatant was filtered through a 0.45 μm cellulose acetate membrane filter (Sartorius, Göttingen, Germany). Finally, the tomato peel extracts were freeze-dried 48 h (freeze dryer LIO 5P, 5pascal, Italy). The freeze-dried tomato peel extracts were transferred into airtight containers and stored at -20 ° C. The freeze-dried tomato peel extracts were diluted in water for further analysis.

2.3 HPLC Characterization

2.3.1 Antioxidant Molecules

Rutin, quercetin, naringenin and caffeic acid were determined by HPLC (Perkin Elmer Nelson 3200 Series) with an RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm). Extraction was conducted in agreement with Tokusoglu et al. [114], although with some differences. One gram of each fruit peel was added to 1 mL of 70% acetone containing 1% (v/v) of HCl and 0.02 mg/mL of TBHQ (tert-Butylhydroquinone). Then, the mix was homogenized by Ultra-Turrax (IKA®) and 0.2 mL of HCl (1.2 M) was added. The mixture was incubated 2 h at 90 °C under continuous stirring. The sample was then cooled to room temperature and sonicated for 3 min. Finally, the extract was centrifuged 5 min at 3000 × g and filtered through a 0.45 μm membrane filter. The HPLC measurements were performed in agreement with Kumar et al. [115], with slight differences. The mobile phase was composed of 2 solvents: water (A) and acetonitrile with 0.02% trifluoracetic acid (TFA) (B), with a linear gradient elution of 80% A and 20% B (0–5 min), 60% A and 40% B (5–8 min), 50% A and 50% B (8–12 min), 60% A and 40% B (12–17 min) and 80% A and 20% B (17–21 min). The flow rate was 1 mL/min and the absorbance was set at 365 nm for rutin and quercetin, 325 nm for caffeic acid and 280 nm for naringenin in a run time of 21 min. For the quantification of rutin, quercetin,

naringenin and caffeic acid, respective standard calibration curves were used with each consisting of five points obtained from standards in the 5–80 µg/mL concentration range (Sigma Chemical, St. Louis, MI, USA).

2.3.2 Lycopene

The extraction of lycopene was conducted according to Olives Barba et al. [116]; 0.3 g of tomato peel was added to 10 mL of hexane/acetone/ethanol (50:25:25 v/v/v) and the mix homogenized with Ultra-Turrax (IKA®). Then, 1.5 mL of distilled water was added followed by vortexing. One ml of the upper layer was dried under vacuum and the dry extract was resuspended in 0.4 mL of solution tetrahydrofuran (THF)/acetonitrile (ACN)/methanol (15:30:55 v/v/v). The mobile phase for HPLC consisted of methanol/CAN (90:10 v/v) and triethanolamine (TEA) 9 mM at a flow rate of 0.9 mL/min in a RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm) at an absorbance of 475 nm and in a run time of 20 min. For quantification, a standard calibration curve consisting of five points at the increasing concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL of lycopene standard (Sigma Chemical, St. Louis, MI, USA) was used.

2.3.3 Vitamin C

Ascorbic acid was extracted from 1 g of tomato peel dispersed in 2 mL of distilled water followed by homogenization with Ultra-Turrax (IKA®) and filtration through a 0.45 µm membrane filter [117]. For the HPLC method, an RP-C18 column (SUPELCO Kromasil 100A-5u-C18 4.6 mm × 250 mm) was used. The mobile phase consisted of 0.01 mol/L KH2PO4 buffer solution (pH = 2.6 with o-phosphoric acid), with a flow rate of 0.5 mL/min and a detection absorbance set at 250

nm. For quantification, a standard calibration curve consisting of five points at the increasing concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL of ascorbic acid standard (Sigma Chemical, St. Louis, MI, USA) was used.

2.4 Antioxidant Activity

The total antioxidant potential of tomato peel extracts was determined using the FRAP assay reported by Benzie and Strain [118]. This is based on the reduction in Fe^{3+}-2,4,6-Tri(2- pyridyl)-s-triazine (TPTZ) to a blue-colored Fe^{2+}-TPTZ. Briefly, FRAP reagent was freshly prepared and the solution was heated at 37 ° C for one hour. The absorbance was read at 593 nm using an UV-Vis spectrophotometer (Perkin Elmer, Lamba 25 spectrophotometer, Waltham, MA, USA). The FRAP values of the samples, which were expressed as µmol of Fe^{2+} per g of fresh weight (FW), were determined from a standards curve built up using ferrous sulphate.

2.5 Total Polyphenolic Content

The TPC in tomato peel extracts was determined in fresh fruit by the spectrophotometric method of Folin-Ciocalteau [119]. The absorbance was read at 765 nm. The results were obtained from a standard curve built up using gallic acid (GA) (Sigma-Aldrich) and were expressed as mg gallic acid equivalent (GAE) per 100 g FW.

2.6 HUVEC Isolation and Culture

HUVEC was harvested and isolated by enzymatic digestion as described by Jaffe et al. [120]. Human cells were obtained from discarded umbilical cords and treated anonymously; as such, approval from the University Ethics Review Board was not necessary. Isolated cells were centrifuged, and the cell pellet was plated on gelatin pre-coated flasks and incubated for 24 h at 37 ° C, 5% CO_2 in Medium 199 (Life Sciences,

Grand Island, NY, USA), which contained 10% fetal bovine serum (FBS), antibiotics (penicillin-streptomycin), growth factors (heparin, 50U/mL and endothelial cell growth factor, 10 mg/mL) (all from Sigma-Aldrich, St. Louis, MO, USA), L-glutamine and HEPES buffer. After 24 h, the growth medium was replaced to remove the excess red blood cells.

2.7 Cell Treatment

The HUVEC between passage P2–P4 was treated as previously described [17]. Briefly, cells were treated for 2 h and 24 h with different concentrations of TPC (5, 20, 50 or 100 µg GAE/mL) of RED tomato peel extracts and PER tomato peel extracts (Perina a Punta della Valtiberina), obtained from plants grown under normal or drought stress conditions, in growth medium with 5% FBS. Cells without treatment were used as a positive control. Then, the cells were washed with PBS and treated with 100 µM H_2O_2 for 1 h to induce oxidative stress. The concentration of H_2O_2 and the time of treatments were chosen as reported [18]. At the end of each treatment, the cells were analysed for viability and ROS production.

2.8 Cell Viability

After treating the HUVEC with the test samples or with H_2O_2 (100 µM), cell viability was assessed by WST-1 colorimetric assay, as previously described [17]. Briefly, at the end of the treatment, the cells were incubated with tetrazolium salt (10 µL/well) for 3 h at 37 °C in 5% CO_2. Formazan dye formation was quantified by measuring the absorbance at 450 nm, with a multiplatform reader (Thermo Scientific Multiskan FC photometer for microplates, Thermo Fisher Scientific Oy, Vantaa, Finland). Absorbance was directly related to the number of metabolically active cells and viability was expressed as a percentage of viable cells.

2.9 ROS Production

ROS fluorescent probe CM-H2DCFDA, which is a cell permeable indicator for these compounds, was used to evaluate the intracellular production of ROS. As previously described [17], during the last 15 min of treatment with RED-Ctr, RED-Ds or H_2O_2, the HUVEC and CM-H2DCFDA (10 μM/well) dissolved in PBS and were co-incubated in the dark at room temperature. ROS production was detected by measuring the increase in fluorescence over time by the microplate reader (Fluorometer Thermo Scientific Fluoroskan Ascent Microplate). Fluorescence was measured by excitation at 488 nm and emission at 510 nm.

2.10 SOD, CAT and GPx Activities

After pre-treatment with 5 μg GAE/mL of RED-Ctr and RED-Ds for 2 h and 24 h with H_2O_2 (100 μM), the cells were scraped and lysated. The supernatant was collected and the CAT, SOD and GPx enzymatic activities were determined by commercially available kits, in accordance with the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, Michigan, MI, USA). The values were expressed in U/mL for SOD activity and nmol/min/mL for CAT and GPx activity.

2.11 RED Stability Studies

The stability of RED-Ctr and RED-Ds at pH 1.2 was assessed according to the procedure described in a previous study [42]. A solution of RED-Ctr or RED-Ds in simulated gastric fluid pH 1.2 (SGF) was equilibrated at 37 °C in a water bath under continuous stirring. At 30 min intervals over a total of 240 min, samples of 50 μL were withdrawn and analyzed for content in antioxidant molecules by the Folin-Ciocalteau method [119].

2.12 Studies of Permeation

These studies were carried out as described in previous papers [121,122]. Briefly, the intestinal mucosa was excised from non-fasted male Witstar rats weighing 250–300 g. All experiments were conducted under veterinary supervision and the protocols were approved by the scientific-ethical committee of the Italian University and Ministry of University and Research. The intestine was longitudinally cut into strips, rinsed free of luminal content, and mounted in Ussing type cells (0.78 cm^2 exposed surface) without removing the underlying muscle layer. After 20 min equilibration at 37 °C, RED-Ctr or RED-Ds in a concentration equivalent to 110 µg GAE /mL in phosphate buffer pH 7.4 (0.13 M) was added to the apical chamber. Three ml of fresh phosphate buffer pH 7.4 (0.13 M) was inserted in the acceptor compartment. Clioxicarb (95% O_2 plus 5% CO_2 mix) was bubbled in both compartments to ensure oxygenation of tissue and stirring. The apical to basolateral transport of tomato extracts was studied. At 30 min intervals of a total of 150 min, 100 µL samples were withdrawn from the acceptor and replaced with fresh pre-thermostated medium. The amount of antioxidant molecules permeated was determined by analyzing the withdrawn samples by the Folin-Ciocalteau method, as described previously [123]. The mean cumulative percentage permeated in a given time was calculated to plot each permeation profile.

2.13 Statistical Analysis

All results were presented as means ± standard deviation (SD) of at least three independent experiments. One-sample Kolmogorov–Smirnov test was used to evaluate distributions of data. Data showed normal distribution. The difference among groups of values was evaluated by a one-way ANOVA and a post hoc analysis was performed by Turkey's or Bonferroni's multiple comparison test. In ex vivo experiments the

significance of the difference between two values was evaluated by the Student's t-test. Differences were considered significant, i.e., the null hypothesis was rejected for p values lower than 0.05. The GraphPad Prism Software v. 7.0 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analysis of the data.

2.14 Results

2.14.1 Characterization of Bioactive Compounds

Antioxidant activity, which is measured by the ferric-reducing antioxidant power (FRAP) assay, and TPC, which is measured by Folin-Ciocalteu assay, are reported in **Table 1**. Although reference is made to TPC, it should be kept in mind that the Folin-Ciocalteau reagent is not specific for phenolic compounds and could also react with other molecules present in the extract with reducing characteristics. The data revealed that plants grown in drought stress condition had a higher TPC than plants grown in normal conditions, while no significant difference was found between the two extracts in the antioxidant activity as determined with the FRAP assay.

Table 1: Rosso di Pitigliano (RED) tomato peel extract characterization.

Plant Growth Condition	Antioxidant Activity (µmol Fe^{2+}/g Fresh Weight) ± SD	Total Polyphenols Content (mg GAE/100 g Fresh Weight) ± SD
Ctr	23.885 ± 0.375	152.46 ± 1.568
Ds	26.052 ± 0.556	361.32 ± 7.204*

Plants were obtained under normal growth (Ctr) or under drought stress (Ds) conditions.
* Significantly different from each other ($p < 0.05$).

The identification of bioactive compounds of tomato peel was performed by high performance liquid chromatography (HPLC) analysis. The content of lycopene, vitamin C, rutin, caffeic acid, naringenin and quercetin are reported in **Table 2**.

Table 2: Bioactive compounds profile of the tomato peel of RED varieties grown in different conditions.

Tomato Variety	Condition	Lycopene	Vitamin C	Rutin	Caffeic Acid	Naringenin	Quercetin
PER	Ctr	115.4 ± 5.78	37 ± 4.24	28.74 ± 0.74*	2.68 ± 0.29*	13.03 ± 0.42*	/
	Ds	91.8 ± 3.11	39.1 ± 0.14	24.86 ± 0.25*	3.83 ± 0.06*	14.25 ± 0.29*	3.99 ± 0.12
RED	Ctr	171.0 ± 1.4*	30.5 ± 8.81	11.60 ± 0.33	0.83 ± 0.08	1.13 ± 0.08	/
	Ds	95.48 ± 6.39	39.6 ± 0	12.59 ± 0.14	1.19 ± 0.08	1.32 ± 0.03	/

Data are expressed in mg/100 g of fresh fruit (FW) ± SD. *Significantly different from each other ($p < 0.05$). PER: Perina a Punta della Valtiberina; RED: Rosso di Pitigliano; Ctr: normal plant growth conditions; Ds: drought stress condition.

No significant differences were observed between the peel of the Rosso di Pitigliano (RED) growth in normal condition (-Ctr) and that of the RED growth in drought stress condition (-Ds) fruits for all compounds, except for lycopene. However, the contents in rutin, caffeic acid and naringenin were moderately increased in RED-Ds with respect to RED-Ctr peel extracts. Neither quercetin nor chlorogenic acid could be detected. Perina a Punta della Valtiberina (PER) resulted richer in rutin, caffeic acid and naringenin, whereas vitamin C is in the same range for both varieties and conditions. Quercetin is found only in PER-Ds.

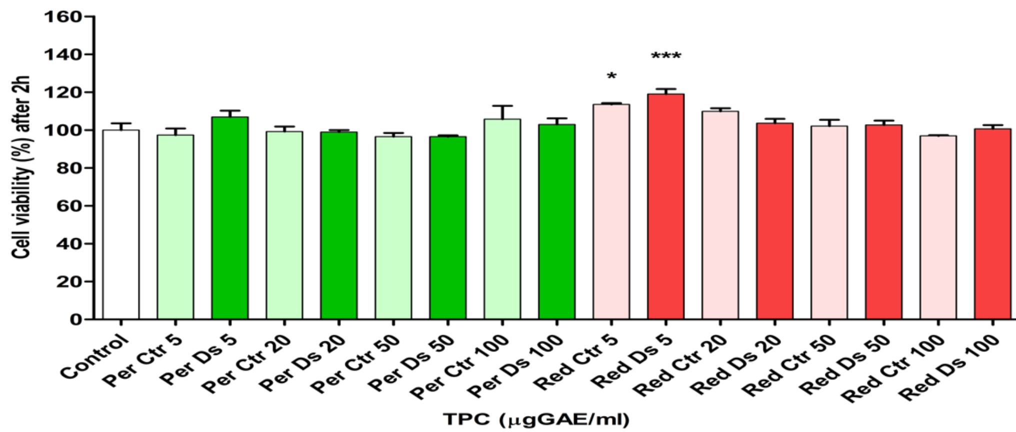
2.14.2 Dose and Time Dependent Response of Cell Viability

Cell viability was evaluated by the WST-1 colorimetric assay. HUVECs were treated for 2 h and 24 h with different concentrations of TPC of PER or RED tomato peels extracts (5-20-50-100µg GAE/ml), obtained in plants grown in normal (Ctr) or in drought stress (Ds) conditions. The time of treatments was chosen as reported [17, 18, 42]. The data obtained after 2 h or 24 h of treatment (**Figure 7a,b** and **Figure 8a,b**) show the non-toxicity of extracts at all concentrations tested. Significant support to cell viability was observed after 24 h of treatment with 5 µg GAE/mL of RED-Ds extracts compared to the

control (p < 0.0001), demonstrating that the presence of the extracts prevents the decay of cell viability which occurs after 24 h of culture.

The preliminary studies were carried out on the two tomato varieties, but tomato peel extract of Perina a Punta della Valtiberina did not give protection from oxidative stress, and we chose to continue the studies on tomato peel extract of Rosso di Pitigliano.

(a)



(b)

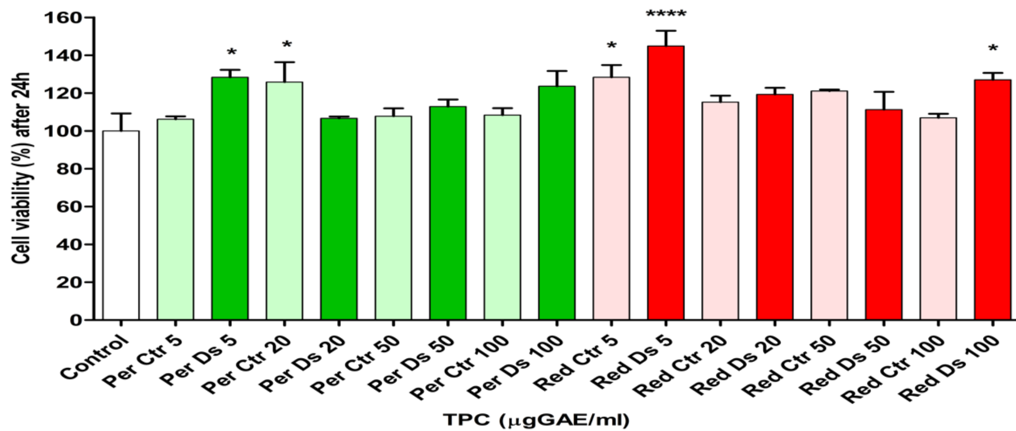
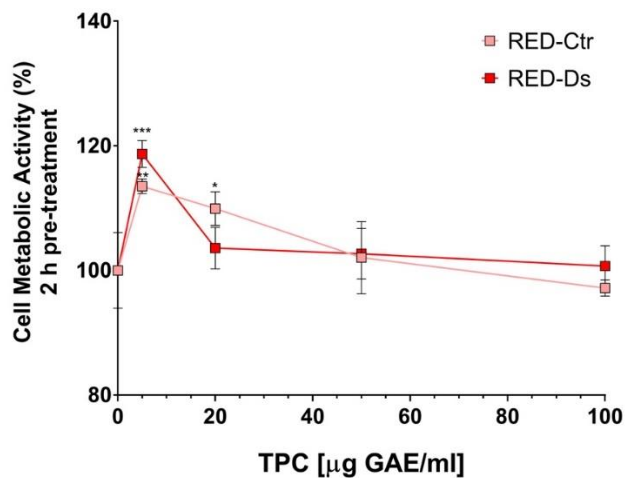


*Figure 7: Dose- and time-dependent cell metabolic activity. HUVECs were cultured for 2 h (**a**) and 24 h (**b**) with different concentrations of total polyphenol content (TPC) of Perina a Punta della Valtiberina (Per) and Rosso di Pitigliano (Red) tomato extracts (5-20-50-100 µg GAE/ml), obtained under normal growing conditions for the plant (Ctr) or in drought stress conditions (Ds). Data are expressed as % of metabolically active cells compared to control (untreated cells). Graphical data are represented as mean ± SD of three separate experiments run in triplicate. * p < 0.05, ** p < 0.01 and *** p <0.0001 vs Control (Untreated cells).*

(a)



(b)

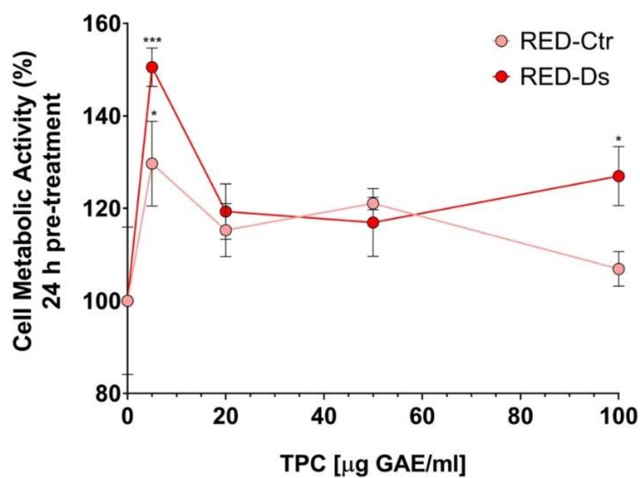
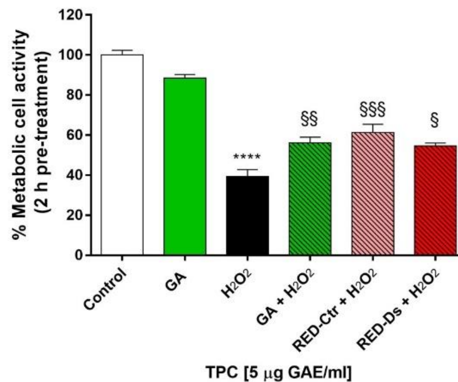


*Figure 8: Dose- and time-dependence of cell metabolic activity. HUVEC were cultured 2 h (**a**) or 24 h (**b**) with different concentrations of total polyphenols contained (TPC) in Rosso di Pitigliano (RED) tomato peel extracts (5-20-50-100 µg GAE/mL), obtained under normal plant growing conditions (Ctr) or in drought stress conditions (Ds). Data are expressed as % of metabolically active cells on an untreated cell basis (control). Graphical data are represented as mean ± SD of three separate experiments run in triplicate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ vs Control (untreated cells).*

2.14.3 Effect against Oxidative Stress

In order to evaluate the protective effect of tomato peel extracts against oxidative stress, we used the active antioxidants concentration of 5 µg/mL GAE. The same concentration of standard gallic acid (GA) was used as positive control. The data show that HUVEC treatment with H_2O_2 significantly reduced the viable cell number, expressed as % of metabolic cell activity on an untreated cells basis. The results showed a protective effect after 2 h pre-treatment with both RED-Ctr and RED-Ds tomato peel extracts ($p < 0.0005$ and $p < 0.05$ vs. H_2O_2 treated cells, respectively), but no protective effect after 24 h pre-treatment (**Figure 9a,b**). The same concentrations of standard GA protect the cell after 2 h ($p < 0.005$ vs. H_2O_2), but not after 24 h pre-treatment.

(a)



(b)

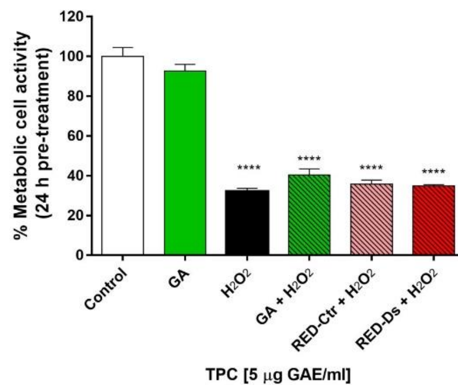


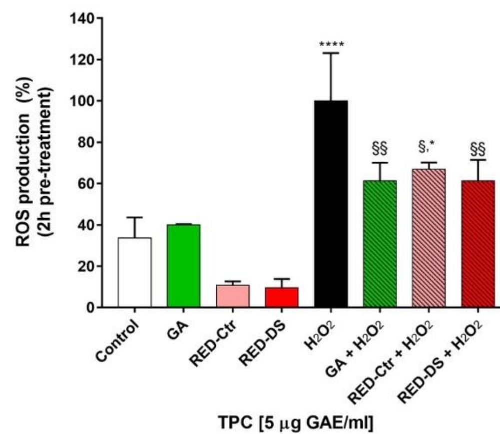
*Figure 9: Cell viability after (**a**) 2 h or (**b**) 24 h pre-treatment with 5 µg GAE/mL TPC from Rosso di Pitigliano peel extracts or with 5 µg/mL gallic acid (GA) as positive control and the subsequent 1 h treatment with H_2O_2 (100 µM). RED-Ctr: plants grown in normal condition; RED-Ds: plants grown in drought stress condition. **** $p < 0.0001$ vs. Control (untreated cells); § $p < 0.05$, §§ $p < 0.005$ and §§§ $p < 0.0005$ vs. H_2O_2.*

2.14.4 Reactive Oxygen Species Production

The antioxidant activity of the bioactive molecules in the tomato peel was assessed through the evaluation of ROS production, both with and without H_2O_2-stress induction on HUVEC.

ROS accumulation in HUVEC was evaluated after 2 h or 24 h pre-treatment with 5 µg GAE/mL of tomato peel extracts. The same concentration of standard GA was used as the positive control. Treatment of HUVEC with H_2O_2 significantly increased intracellular ROS production compared with untreated cells ($p < 0.0001$). As shown in **Figure 10a**, after 2 h pre-incubation, RED-Ds reduced ROS production significantly than compared with H_2O_2 treatment ($p < 0.005$). On the contrary, the 24 h pre-treatment with either RED-Ctr or RED-Ds did not prevent the increase in ROS production induced by H_2O_2, **Figure 10b**. On its part, 5 µg/mL GA exerted a protective effect only after 2 h pre-treatment.

(a)



b)

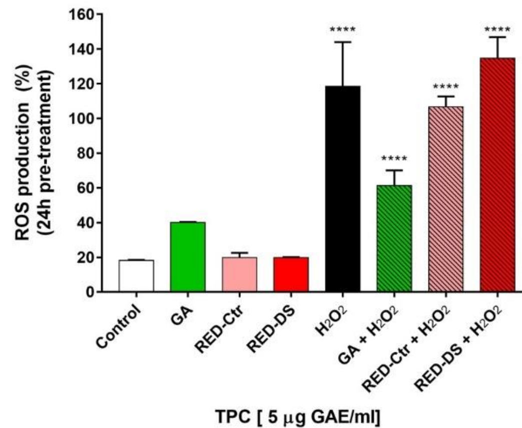
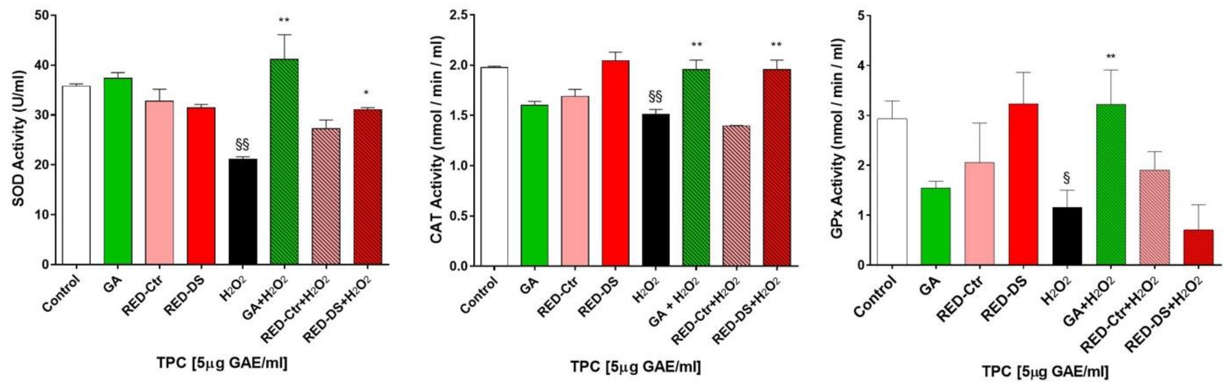


*Figure 10: Reactive Oxygen Species (ROS) production in HUVEC pre-treated with 5 µg GAE/mL of total polyphenol content (TPC) of Rosso di Pitigliano peel extracts or with 5 µg/mL gallic acid (GA) as positive control for 2 h (a) and 24 h (b) and with H_2O_2 (100 µM) for 1 h. RED-Ctr: plants grown in normal condition; RED-Ds: plants grown in drought stress condition. Data are expressed as % ROS production and are representative of three separate experiments run in triplicate. * $p < 0.05$ and **** $p < 0.0001$ vs. Control (untreated cells); § $p < 0.05$, §§ $p < 0.005$ vs. H_2O_2*

2.14.5 Antioxidant Enzymes Activity

The antioxidant activity was evaluated by measuring SOD, CAT and GPx enzymes activity in HUVEC pre-treated for 2 h (**Figure 11a**) or 24 h (**Figure 11b**) with 5 µg GAE/mL of tomato peel extracts or with 5 µg/mL GA as positive control and with H_2O_2 (100 µM) for 1 h. In **Figure 11a**, treatment of HUVEC with H_2O_2 appears to significantly reduce the antioxidant enzymes activity compared with untreated cells ($p < 0.005$). Nevertheless, 2 h pre-treatment of cells with RED-Ds prevented the negative effect of H_2O_2 by acting on SOD and CAT enzymes, which are known to be involved in the first line defense system against ROS. No effect of RED-Ds is observed in **Figure 11b** on CAT and GPx activity after 24 h pre-treatment, whereas RED-Ctr was observed to act on SOD activity. The GA positive control showed a protective effect after either 2 h or 24 h pre-treatment by acting on CAT, SOD and GPx after 2 h and on SOD after 24 h pre-treatment.

(a)



(b)

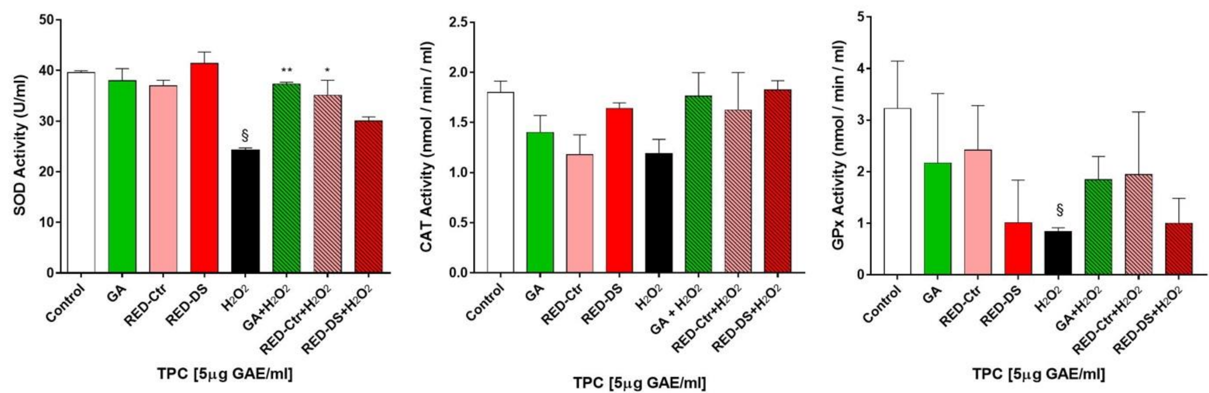


*Figure 11: Activity of SOD, CAT and GPx enzymes in HUVEC pre-treated for 2 h (**a**) and 24 h (**b**) with 5µg GAE/mL of tomato peel extracts, or with 5 µg/mL gallic acid (GA) as positive control, and with H_2O_2 (100µM) for 1 h. RED-Ctr: plants grown in normal condition; RED-Ds: plants grown in drought stress condition. *$p < 0.05$, **$p < 0.005$ vs H_2O_2 ; § $p < 0.05$, §§ $p < 0.005$ vs Control.*

2.14.6 Effect on Permeation of Antioxidant Molecules

The excised jejunal rat epithelium was chosen among the ex vivo intestinal models for the studies of antioxidant molecules permeability because its tight junctions are similar in tightness and number to those of the human jejunum [124]. The data on the permeation of antioxidant molecules contained in either RED-Ctr or RED-Ds across the excised jejunal rat epithelium, reported in **Figure 12**, show a non-significant difference in antioxidant molecules fraction permeated between the two extracts being compared. However, it appears from comparing the mean RED-Ctr and RED-Ds cumulative permeated fractions (41.9 ± 9.6 vs. $26.6 \pm 7.8\%$) that the antioxidant from RED-Ctr are more permeable. It is observed in **Figure 12** that with RED-Ctr the antioxidant molecules fraction permeated gradually increases over time up to 150 min, whereas with RED-Ds a maximum in permeation is reached after 120 min. These trends might not be ascribed to the difference in the antioxidant molecule's ability to permeate, but rather, to a difference in the molecules contained in the extracts. Indeed, the Clioxicarb that was bubbled in the two hemi-cells during the permeation experiment made the environment strongly oxidizing and this could cause antioxidant molecules degradation, as we had al-ready observed with grape seed extracts [123]. A more marked oxidation of the antioxidant contained in RED-Ds than those contained in RED-Ctr was observed, which implies a higher antioxidant potential of the former extract. At any rate the data in **Figure 12** shows an ability of the antioxidants contained in the extracts under study to permeate across the intestinal epithelium also in the free form, not encapsulated in release systems able to protect them from degradation [42]. By the way the stability study, contrary to what observed with cherry extracts, had shown that at the end of the experiment, i.e., after 4 h, no degradation of the antioxidant molecules contained in either RED-Ctr or RED-Ds was

observed. It should be considered, though, that, unlike the ex vivo experiments, no Clioxicarb was used in this experiment.

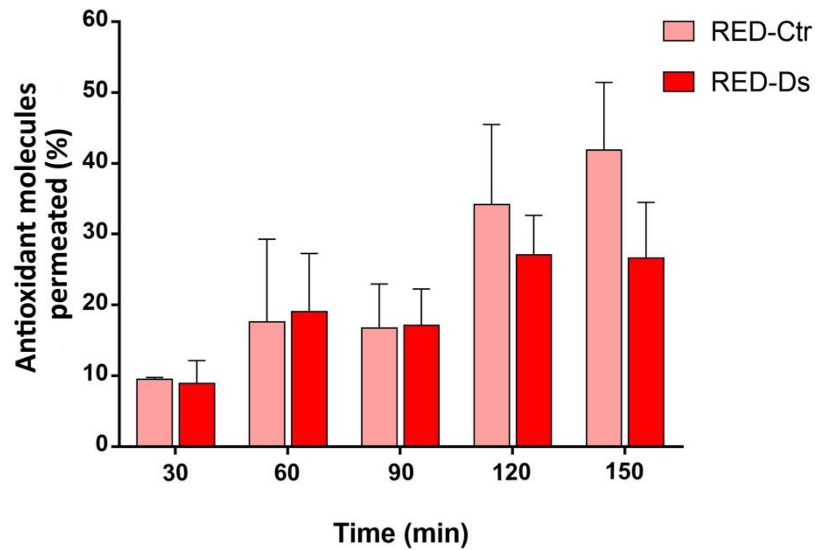


Figure 12: Data on the permeation of antioxidant molecules contained in RED-Ctr or RED-Ds in phosphate buffer, pH 7.4, 0.13 M, at the equivalent gallic acid concentration (GAE) of 110 µg/mL across the excised jejunal rat epithelium (n = 4).

2.15 Discussion

Tomato is an important component of the Mediterranean diet. Its cultivation is largely focused in semi-arid Mediterranean zones, where it needs to be cultivated under irrigation and where drought events related to climate change are generally more frequent [125]. Moreover, the management of by-products obtained during tomato processing is one of the most important issues related to environmental sustainability. If these by-products remain unused, there is a problem with their disposal and also it is an expensive process for companies. One way to avoid this problem is to reuse tomato processing by-products. Tomato by-products contain a great variety of biologically active substances, which have been demonstrated by in vitro and in vivo studies to possess antioxidant, hypolipidemic

and anticarcinogenic properties [15]. Most of the functional compounds are derived from the secondary metabolism of plants and are synthesized in response to biotic stresses, as well as during normal physiological processes. Drought stress is one of the most impacting factors that seriously alters the plant physiology [126]. One of the first relevant reactions observed under drought is the enhancement of the antioxidant apparatus of the plant [127]. Some works have described an increase in biomolecules as well as in both antioxidant activity and total polyphenols in tomato plants subjected to water stress [128,129]. The present investigation is the first study where the antioxidant effects of the molecules contained in the peels of a variety of Tuscan tomatoes grown under normal conditions are compared with those obtained from peels of the same tomato variety grown under drought stress conditions.

In line with other studies [126-128, 130,131], we observed the presence of a significantly higher content in bioactive compounds in the peel extracts of plants grown under water stress conditions compared to plants grown in normal conditions (361.32 mg GAE/100 g FW vs. 152.46 mg GAE/100 g FW). Moreover, a slight increase in vitamin C, rutin, caffeic acid and naringenin was observed in stressed plants. Our results suggest that water stress can affect peel TPC. The increase in TPC in RED-Ds peel extracts may suggest that plants are counteracting stress by synthesizing antioxidant molecules [130].

Lycopene is the main antioxidant in tomatoes for it is responsible for the red color [132] and represents more than 80% of total tomato carotenoid content in the fully ripened fruit. Lycopene is the major compound present in RED tomato peel. This bioactive compound has notable biological properties related to its antioxidant activity [133,134]. However, our data indicate that lycopene does not increase in plants subjected to drought stress but rather decreases. This is confirmed in other papers where drought stress was found to be capable of lowering the lycopene content compared to well irrigated plants [135,136].

Indeed, irrigation seems to have a pronounced effect on the biosynthesis of lycopene [137] and the differences may be due to the contrast in the responsive capacity of the genotypes to the different hydric conditions. In general, we agree that the various tomato crops respond differently and therefore generates a different concentration of metabolites when subjected to abiotic or biotic stress.

Several foods and dietary supplements have been shown to exert a protection against cardiovascular disease (CVD) as well as to the disorders related to oxidative stress, including allergy, cancer, diabetes, immune, inflammatory obesity, and Parkinson's diseases [138].

In this study, we have evaluated the protective effect of Tuscan tomato peel extracts on endothelial cells. Our results show that lower concentrations of RED significantly prevents the decay of cell viability after 24 h of treatment compared to untreated cells. Oxidative stress induced cell apoptosis when the endogenous antioxidant factors were decreased [139]. In particular, vascular oxidative stress contributes to the mechanisms of vascular dysfunction and has been stated to play an important role in a number of cardiovascular pathologies. In our study, we observed that low concentrations of TPC and the short-time of treatment of RED-Ds were sufficient to significantly reduce the effect of oxidative stress induced by hydrogen peroxide and to produce cytoprotective effects in HUVEC, as well as a significant reduction in ROS production, when compared with untreated cells. Our results demonstrate a direct antioxidant potential of RED-Ds peel extract, and this is in agreement with the well described general quenching activity of carotenoids against hydrogen peroxide [140]. Moreover, our data show that the antioxidant properties of RED-Ds are due to its effect on SOD and CAT enzyme activity. In this work the extract obtained under water-shortage stress conditions, RED-Ds, has shown to possess antioxidant activity on HUVEC, i.e., on the cells of the endothelium of

blood vessels and thus could be potential candidate compounds for the prevention of CVD. Furthermore, the protective effects are mainly due to the presence of bioactive compounds such as carotenoids, vitamins and polyphenols, which probably interacts synergistically [141]. Various studies have demonstrated that both concentrations and combinations of antioxidants can influence the synergistic antioxidant ability. Indeed, the biological activity of lycopene can be enhanced by the presence of other active antioxidant compounds such as vitamin C, β-carotene or vitamin E [142-144]. Nevertheless, particular concentrations and combinations of antioxidants can also exert antagonistic effects [145]. Thus, the cellular protective effect of the RED-Ds peel extracts may be due to proper concentrations and combinations of antioxidants found in the Ds peels. Furthermore, since the antioxidant molecules permeation was followed in the permeation experiments, we can hypothesize that these bioactive molecules can reach the target site in sufficient concentration to carry out their protective action on endothelial cells. In fact, in the in vitro model both in the RED-Ctr and RED-Ds cases, a permeation of the total polyphenols is observed at about 30% of the applied dose (110 µg GAE/mL) and the experiments on HUVEC showed that the polyphenols are already active at a concentration of 5 µg GAE/mL.

Natural extracts have shown their effects against chronic diseases, including CVD. For the treatment of CVD, prevention plays an important role and the introduction of nutraceuticals into the diet could represent the first defense mechanism of the body from oxidative stress. Nevertheless, the current coronavirus (COVID-19) pandemic has highlighted the importance of an appropriate nutrition with functional compounds that can offer further antiviral approaches to public health, optimizing the ability of the immune system to prevent and control pathogenic viral infections, as highlighted in recent works [146].

CHAPTER 3 Second Experimental Section: PROPERTY OF TOMATO PEEL POLYPHENOLS AGAINST SARCOPENIA

The second section presents the comparation of cytoprotective properties between tomato peel extracts cultivated in normal or in drought stress conditions, on in vitro model of sarcopenia. The capacity of primary human skeletal muscle cells to reproducibly differentiate into multinucleated myotubes was evaluated. Myoblasts differentiated into myotubes were examined for the expression of various markers of skeletal muscle cells such as and Myosin heavy chain-2 (MYH2), Troponin T type 1 (TNNT) and Myogenin (MYOG).

In this chapter each section will be divided into materials and methods, results and discussion, tables and figures. The future perspectives will be discussed in Chapter 4.

3.1 Materials and Methods

3.1.1 Cells, Media, and Supplement

Human skeletal muscle myoblasts (HSMM, Catalog #CC-2580, lot # 0000655307, Lonza) isolated from the quadriceps muscle of an 18-year-old male and Skeletal Muscle Cell Growth Medium-2 (SKGM-2) with single quots kit were purchased from Lonza (Walkersville, MD).

HSMM differentiation medium was prepared by adding 2% horse serum to DMEM-F12 medium (both from Lonza, Walkersville, MD).

Anti-skeletal myosin Fast primary antibody (MY-32); Dexamethasone (DEXA); bovine serum albumin (BSA); formalin, Triton X-100 and Hoechst nuclear stain (#33342) were purchased from Sigma-Aldrich (Milan, Italy). Alexa 488 conjugated anti-mouse IgG secondary antibody was purchased from Invitrogen (Thermo Fisher Scientific, Italy).

Methanol, formic acid, and water for UHPLC analyses were purchased from Romil-Deltek (Pozzuoli, Italy). Chlorogenic acid (purity ≥95%), rutin (purity ≥95%), and

naringenin (purity ≥95%), used as reference standards were purchased from Sigma-Aldrich (Milano, Italy).

3.1.2 UHPLC-HR-ESI-MS Analyses

Tomato peel extracts were analysed by means of ultra-high-performance liquid chromatography (UHPLC) using a Vanquish Flex Binary pump LC system coupled with an electrospray source-high resolution-mass spectrometer (ESI-HR-MS), Q Exactive Plus MS, Orbitrap-based FT-MS system (Thermo Fischer Scientific Inc., Bremem, Germany). Each sample was injected (5 µL) on a C-18 Kinetex® Biphenyl column (100 x 2.1 mm, 2.6 µm particle size) provided of a Security Guard TM Ultra Cartridge (Phenomenex, Bologna, Italy). A linear solvent gradient was developed for elution using formic acid in MeOH 0.1% v/v (solvent A) and formic acid in H_2O 0.1% v/v (solvent B), from 5 to 55% A within 14 min, at a flow rate 0.5 mL/min. The autosampler and column oven temperatures were maintained at 4 and 35 °C, respectively. HR mass spectra were acquired in a scan range of m/z 250-1200 in ESI negative ion mode, by using ionization parameters as previously reported [147] operating in full (70000 resolution, 220 ms maximum injection time) and data dependent-MS/MS scan (17500 resolution, 60 ms maximum injection time).

The quantitative analysis of phenols was performed by constructing calibration curves based on rutin (concentration range 0.1-50 µg/mL), naringenin (concentration range 0.1-100 µg/mL), and chlorogenic acid (concentration range 5-500 µg/mL), as external standards for flavonol glycosides, flavones, and phenolic acids, respectively. Standard methanol solutions were prepared in triplicate at different concentrations by serial dilution starting from a stock solution of 1.0 mg/mL. The calibration curve was obtained

by using concentration (µg/mL) with respect to the areas obtained by integration of full MS peaks of the reference standards. Linear simple correlation was used to analyze the relation between variables (R^2 = 1 for rutin and naringenin; R^2 = 0.9989 for chlorogenic acid). The phenol amount was obtained by using a Microsoft® Office Excel and expressed as µg of 100 g of fresh weight (FW).

3.1.3 HSMM Differentiation

HSMM were expanded in complete growth medium and incubated in a humidified incubator at 37 °C, 5% CO_2. Culture medium was changed every three days. Cells were passaged when they reached 50–70% confluency.

To induce differentiation, cells were plated at 20,000- cells/cm^2 in 12-well polystyrene cell culture plates and incubated overnight in growth medium in a cell culture incubator (37 °C, 5% CO_2). The following morning, the growth medium was replaced with differentiation medium and the cultures were incubated for 3 days, during which time myotube differentiation occurred.

3.1.4 Sarcopenia Model

The cellular model of sarcopenia was reproduced by treating human skeletal myotubes with dexamethasone (DEXA). In fact, to examine the effects on differentiated myotubes, DEXA (50µM), Red Ctr (5µg GAE/ml TPC), Red Ds (5µg GAE/ml TPC), ascorbic acid (5µg) were added on day 3 of differentiation and cultures were incubated for another 2 days. Cells were then treated for immunostaining measurement. Moreover, the cell pellet was stored at -80 °C in a local refrigerator for gene expression studies.

3.1.5 Immunostaining and Fluorescence

The HSMM differentiation into Myosin Heavy Chain-2 (MYH2)-positive multinucleated myotubes was assessed by immunofluorescent techniques. Briefly, cells were fixed with 10% formalin for 20 min at room temperature (RT). Cells were washed three times with PBS, and then permeabilized with 0.5% Triton X-100 at RT for 15 min. Non-specific binding sites were blocked by incubation with 2% BSA, 0.25% Triton X-100 in PBS at RT for 30 min. Cells were then incubated with anti-skeletal myosin FAST primary antibody, MY-32, diluted 1:500 in blocking buffer at RT for 2 h. After rinsing with blocking buffer without Triton X-100 for 10 min, cells were incubated with Alexa 488 (green) conjugated anti-Mouse IgG secondary antibodies at 1:400 dilution along with Hoechst nuclear stain at 1:5,000 dilution in blocking buffer at RT for 1h. Cells were washed with PBS three times, 5 min each, and viewed under a fluorescence microscope (Nikon Eclipse Ti, Amsterdam, The Netherlands) equipped with a digital CCD camera and 20× objective. Images were acquired using 20× magnification and are representative of three different experiments in duplicate. The most representative images were shown. Images analyses were obtained from the mean of five acquisition fields per sample, in duplicate. Images were recorded using AxioVision (Carl Zeiss MicroImaging, GmbH) software. All images (micro and macroscopic) were analyzed using FIJI Software by Image J (NIH, Bethesda, MD, United States.

3.1.6 RNA extraction, Reverse Transcription and Real-Time PCR

Total RNA was extracted from cultured cells with High Pure RNA Isolation Kit (Roche Diagnostics Indianapolis, USA). RNA concentration was determined by Nanodrop 1000 (Thermo Fisher Scientific) at 260 nm. The ratio of readings at 260 nm and 280 nm (A260/A280) provided an estimate of the purity of RNA and only samples that showed

OD 260/280 ratios of 1.9-2.1 were used. The RNA samples were stored at -80 °C for use in gene expression studies. First strand cDNA was synthesized with iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA) using about 200 ng of total RNA as template. The cDNA samples obtained were placed on ice and stored at 4 °C until further use. Real-Time PCR reactions were performed in duplicate in the Bio-Rad CFX Connect Thermal Cycler (CFX-Connect Real-Time PCR detection systems, Bio-Rad). EvaGreen (SsoFAST EvaGreen Supermix, Bio-Rad) was used for monitoring cDNA amplification. PCR was performed in a volume of 20 μL per reaction, including 0.2 μM of each primer (Merck Life Science S.r.l., Italy), samples, reagent, and sterile H_2O. Primer pairs of both interested markers and reference genes were designed with Primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

All reactions were performed in duplicate. The conditions for the Real-Time PCR reaction were optimized in the samples for all genes. In **Table 3** were reported the primer sequence details of the analyzed genes.

Table 3: Beta-2 Microglobulin (B2M); Eukaryotic translation elongation factor 1 alpha (eEF1A); Forkhead Box O1 (FOXO1); Myogenin (MYOG); Myosin heavy chain 2 (MYH2); Protein kinase B (AKT1); Ribosomal protein L13a (RPL13A); Troponin T (TNNT1).

PRIMER SEQUENCE DETAILS OF THE ANALYZED GENES							
Gene	**Sequence**		**GenBank, accession**	**Length (bp)**	**Temperature (°C)**	**Efficiency (%)**	**R^2**
AKT1	Forward	CTGCACAAACGAGGGGAGTA	NM_001014431.2	142	60	87.4	0.999
	Reverse	GCGCCACAGAGAAGTTGTTG					
B2M	Forward	CACTGAATTCACCCCCACTGA	NM_004048.4	102	60	91.2	0.999
	Reverse	GCTTACATGTCTCGATCCCAC					
eEF1A	Forward	CTTTGGGTCGCTTTGCTGTT	NM_001402	183	60	90.3	0.999
	Reverse	CCGTTCTTCCACCACTGATT					
FOXO1	Forward	GGGTTAGTGAGCAGGTTACAC	NM_002015.4	170	60	106.8	0.997
	Reverse	CTTTGCTGCCAAGTCTGACG					
MYH2	Forward	CTCAAAGCTCTCTGCTACCCC	NM_017534.6	88	60	83.7	0.992
	Reverse	CTACTGCGTTGGACACCTGTTCT					
MYOG	Forward	AGATTGTCTTCCAAGCCGGG	NM_002479.6	112	60	88	0.999
	Reverse	CTGGCTTCCTAGCATCAGGG					
RPL13A	Forward	CGCCCTACGACAAGAAAAAG	NM_012423	206	60	87.6	0.996
	Reverse	CCGTAGCCTCATGAGCTGTT					
TNNT1	Forward	GTCAGAGAGAGCCGAGCAAC	NM_001126133.3	197	60	90.2	0.995
	Reverse	CACGCTTCTGTTCTGCCTTG					

3.1.7 SOD, CAT and GPx Activities

After pre-treatment with Red Ctr (5µg GAE/ml TPC), Red Ds (5µg GAE/ml TPC), ascorbic acid (5µg) and DEXA (50µM), the cells were scraped and lysated. Then the supernatant was collected, and the CAT, SOD and GPx enzymatic activities were determined by commercially available kits, in accordance with the manufacturer's instructions (Cayman Chemical, USA). The values were expressed in U/ml for SOD activity and nmol/min/ml for CAT and GPx activity.

3.1.8 Statistical Analysis

Real-Time PCR data were normalized with three most stably reference genes, eEF1a, RPL13a, B2M, (Avg M value=0.269) and then the $2^{-\Delta\Delta Ct}$ algorithm was applied for relative quantification Bio-Rad's CFX Maestro software (CFX Real-Time PCR detection systems, Bio-Rad Laboratories Inc., Hercules, CA, USA).

The GraphPad Prism Software vs. 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. One-sample Kolmogorov–Smirnov test was used to evaluate distributions of data. Data showed normal distribution. The significant difference (p-value < 0.05) between groups of values was evaluated by a one-way ANOVA or Turkey's or Bonferroni's multiple comparisons. All results were presented as means ± standard deviation (SD) of at least three independent experiments.

3.2 Results

3.2.1 Phenolic Profile of Tomato Peel Extracts

To get more accurate information on the compounds of interest furthermore, the phenol content of both peel extracts was investigated by UHPLC analyses coupled to ESI-HR-MS. The chromatograms shown in **Figure 13** evidenced a very similar profiles with 15 identified phenol derivatives. Both extracts are rich in phenolic acids (compound 1-5, 11, and 13) and flavonoids (compounds 6-10, 12, 14, and 15). All compounds were identified by comparison of full MS and fragmentation patterns with literature data, except for chlorogenic acid (3a and 3b isomers), rutin (7), and naringenin (9) confirmed by injection of reference standards. Results are in agreement with phenol composition of tomato peels previously reported [148,149,34]. Phenolic acids are caffeoyl/*p*-coumaroyl glucosides and caffeoylquinic derivatives, all revealed as cis/trans isomers. Among flavonoids,

flavonol glycosides having quercetin and kaempferol as aglycones were found, as well as flavones derivatives with naringenin derivatives. Results from quantitative analyses (**Table 5**) showed a higher total phenol content in the control extract (1201 ± 28 µg/100 g FW) compared to that derived from plants grown in drought stress conditions (1073 ± 9 µg/100 g FW). Interestingly, even though the total phenols decreased in RED-Ds extract, the total content of phenolic acids is higher compared to the control. Indeed, the reduction seem to be due to the variation especially in naringenin content (556 ± 2 vs 793 ± 19 µg/100 g FW) and its derivatives.

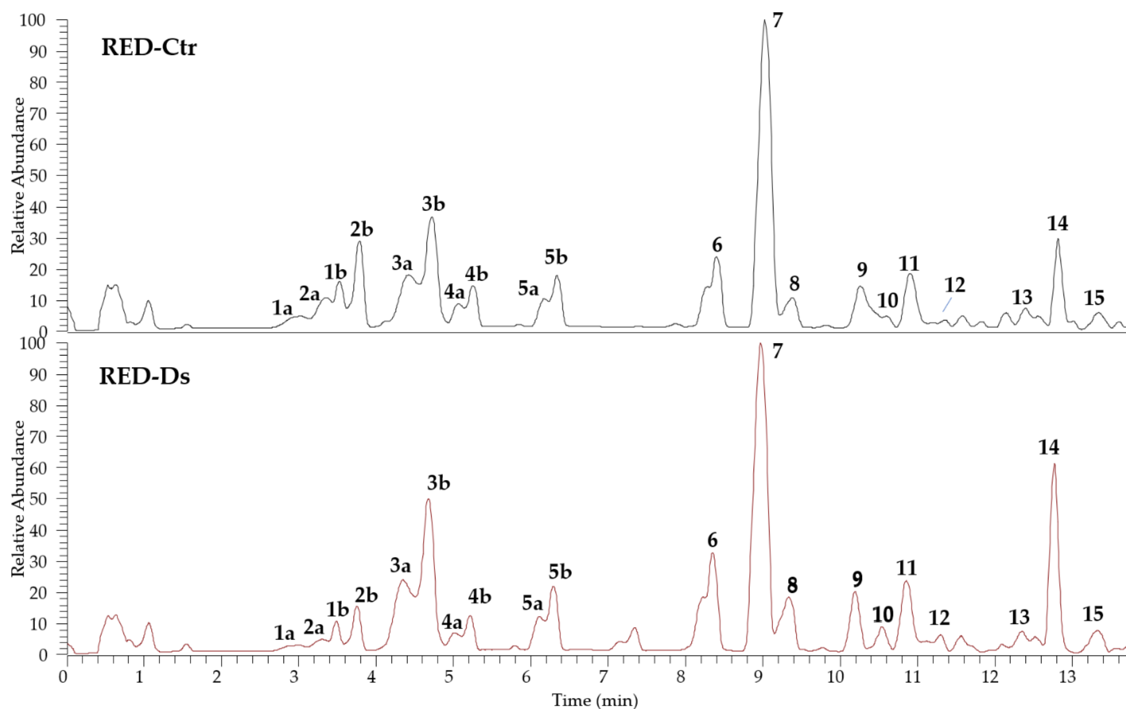


Figure 13: UHPLC-HR-ESI-MS profiles of tomato peel extracts of plants grown in normal (RED-Ctr) and in drought stress (RED-Ds) conditions.

Table 4: UHPLC-HR-ESI-MS/MS data of phenols detected in peel extracts of plants grown in normal (RED-Ctr) and in drought stress (RED-Ds) conditions. Peak data are shown in Table 4.

[a]Compound numbers correspond with peak numbers in Figure 1. [b]Tentatively identified based on MS/MS and literature data. [c]The ion base peaks are shown in bold. [d]Confirmed by reference standard.

Peak[a]	Compound[b]	t_R (min)	HR-[M−H]⁻ (*m/z*)	HR-MS/MS Product Ions (*m/z*) [c]	Molecular formula	Error (ppm)	RED Extract
	Phenolic acids						
1a	Caffeic acid glucoside isomer I	3.0	341.0875	**179.03**, 145.03, 161.02, 135.04	$C_{15}H_{18}O_9$	-0.73	Ctr, Ds
2a	*p*-Coumaric acid glucoside isomer I	3.3	325.0927	**163.04**, 119.05, 93.03	$C_{15}H_{18}O_8$	-0.31	Ctr, Ds
1b	Caffeic acid glucoside isomer II	3.5	341.0875	**179.03**, 145.03, 161.02, 135.04	$C_{15}H_{18}O_9$	-0.73	Ctr, Ds
2b	*p*-Coumaric acid glucoside isomer II	3.8	325.0927	**163.04**, 145.03, 119.05, 93.03	$C_{15}H_{18}O_8$	-0.31	Ctr, Ds
3a	Chlorogenic acid isomer I[d] (3-*O*-caffeoylquinic acid)	4.4	353.0878	**191.06**, 179.03, 173.04, 135.04	$C_{16}H_{18}O_9$	0.00	Ctr, Ds
3b	Chlorogenic acid isomer II[d]	4.7	353.0878	**191.06**, 179.03, 173.04, 135.04	$C_{16}H_{18}O_9$	0.00	Ctr, Ds
4a	*p*-Coumaric acid glucoside isomer III	5.1	325.0927	**163.04**, 145.03, 119.05, 93.03	$C_{15}H_{18}O_9$	-0.31	Ctr, Ds
4b	*p*-Coumaric acid glucoside isomer IV	5.2	325.0927	**163.04**, 145.03, 119.05, 93.03	$C_{15}H_{18}O_9$	-0.31	Ctr, Ds
5a	Caffeoylquinic acid isomer I	6.2	353.0878	**191.06**, 179.03, 135.04	$C_{16}H_{18}O_9$	0.00	Ctr, Ds
5b	Caffeoylquinic acid isomer II	6.3	353.0878	**191.06**, 179.03, 135.04	$C_{16}H_{18}O_9$	0.00	Ctr, Ds
11	Dicaffeoylquinic acid isomer I	10.8	515.1191	**353.09**, 191.06, 179.03, 173.04, 135.04	$C_{25}H_{24}O_{12}$	-0.78	Ctr, Ds
13	Dicaffeoylquinic acid isomer II	12.4	515.1191	**353.09**, 191.06, 179.03, 173.04, 135.04	$C_{25}H_{24}O_{12}$	-0.78	Ctr, Ds
	Flavonoids						
6	Quercetin 3-*O*-(2″-*O*-apiofuranosyl-6″-*O*-rhamnopyranosyl-glucopyranoside)	7.3	741.1885	**300.03**, 301.02, 271.03	$C_{32}H_{40}O_{21}$	+0.13	Ctr, Ds
7	Rutin [d]	9.0	609.1461	**300.03**, 301.02, 271.03	$C_{27}H_{30}O_{16}$	0.00	Ctr, Ds

Peak[a]	Compound[b]	t_R (min)	HR-[M−H]⁻ (*m/z*)	HR-MS/MS Product Ions (*m/z*) [c]	Molecular formula	Error (ppm)	RED Extract
8	Kaempferol rutinoside-pentoside	9.3	725.1932	**284.03**, 285.04, 255.03	$C_{32}H_{38}O_{19}$	-0.28	Ctr, Ds
9	Kaempferol 3-*O*-rutinoside	10.2	593.1509	284.03, **285.04**, 255.03	$C_{27}H_{30}O_{15}$	-0.34	Ctr, Ds
10	Naringenin 7-*O*-glucoside	10.5	433.1138	**271.06**, 151.00, 119.05	$C_{21}H_{22}O_{10}$	-0.46	Ctr, Ds
12	Naringenin chalcone glucoside	11.3	433.1138	**271.06**, 151.00, 119.05	$C_{21}H_{22}O_{10}$	-0.46	Ctr, Ds
14	Naringenin [d]	12.8	271.0611	151.00, 119.05	$C_{15}H_{12}O_5$	-0.37	Ctr, Ds
15	Naringenin calchone	13.4	271.0611	151.00, 119.05	$C_{15}H_{12}O_5$	-0.37	Ctr, Ds

Table 5: Amount (µg/100 g ± standard deviation of fresh weight) of constituents detected in peel extracts of plants grown in normal (RED-Ctr) and in drought stress (RED-Ds) conditions. Compound numbers correspond to the peak numbers in Figure 13 and Table 4.

Peak	Compound	RED-Ctr (µg/100 g ± SD)	RED-Ds (µg/100 g ± SD)
	Phenolic acids		
1a+1b	Caffeic acid glucoside (isomers I and II)	8.18 ± 0.1	18.6 ± 0.1
2a+2b	*p*-Coumaric acid glucoside (isomers I and II)	12.8 ± 0.1	37.8 ± 0.1
3a+3b	Chlorogenic acid (isomers I and II)	46.9 ± 0.5	51.9 ± 0.9
4a+4b	*p*-Coumaric acid glucoside (isomers III and IV)	9.13 ± 0.1	16.3 ± 0.2
5a+5b	Caffeoylquinic acid (isomers I and II)	15.0 ± 0.2	18.3 ± 0.2
11	Dicaffeoylquinic acid (isomer I)	16.5 ± 0.4	20.5 ± 0.4
13	Dicaffeoylquinic acid (isomer II)	3.47 ± 0.06	5.03 ± 0.1
	Flavonoids		
6	Quercetin 3-*O*-(2″-*O*-apiofuranosyl-6″-*O*-rhamnopyranosyl-glucopyranoside)	16.1 ± 0.4	16.7 ± 0.4
7	Rutin [d]	45.5 ± 0.5	62.3 ± 0.9
8	Kaempferol rutinoside-pentoside	4.88 ± 0.3	5.04 ± 0.2
9	Kaempferol 3-*O*-rutinoside	7.23 ± 0.3	8.17 ± 0.2
10	Naringenin 7-*O*-glucoside	103 ± 4	90.0 ± 2
12	Naringenin chalcone glucoside	119 ± 1	109 ± 1
14	Naringenin [d]	793 ± 19	556 ± 2
15	Naringenin chalcone	77.4 ± 2	58.0 ± 0.6
	Total phenolic acids	112 ± 1	168 ± 2
	Total flavonoids	1089 ± 27	905 ± 7
	Total phenols	1201 ± 28	1073 ± 9

3.2.2 Human Skeletal Muscle Myoblasts Differentiation

HSMM demonstrated robust myotube differentiation, moreover can readily cultured as undifferentiated cells in growth medium on polystyrene cell culture plates. Cells were used at passage 5 for differentiation. Results showed that HSMM differentiated into myosin heavy chain (MYH2)-positive multinucleated myotubes (green) within 3 days in differentiation medium (**Figure 14**).

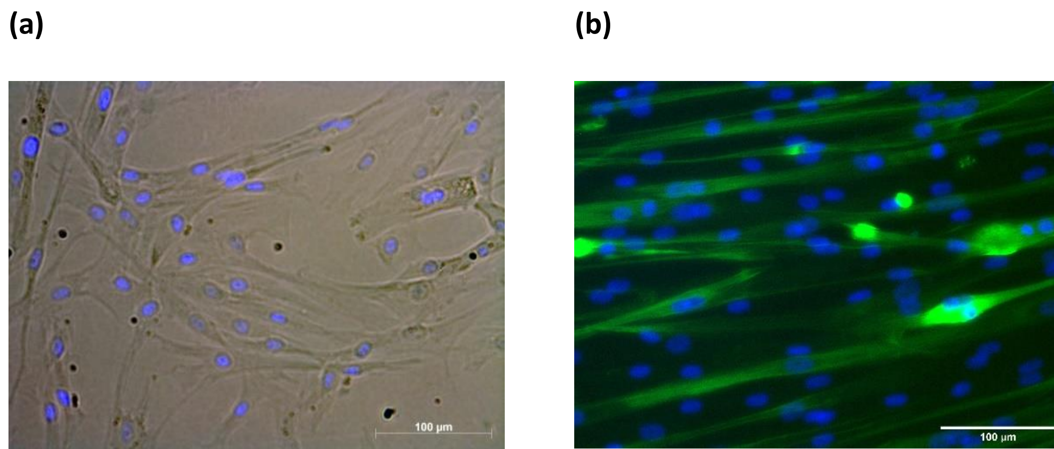


*Figure 14: HSMM differentiated into Myosin Heavy Chain-2 (MYH2)-positive myotubes. Representative and dark-field images of multinucleated myotube after 1 day growth medium (phase contrast image) (**a**) and 3 days in the differentiation medium (**b**), labelled with MYH2-immunostain (green). Nuclei were stained with Hoechst (blue). Magnification 20×; scale bar = 100 µm).*

3.2.3 Induction of Sarcopenia

Dexamethasone (DEXA) was used to induces myotubes atrophy [150]. In particular, myotubes derived from HSMM were treated with DEXA (50 µM) for 48 h and then fixed and immuno-stained for MYH2. Nuclei were visualized by Hoechst-staining. Representative images of untreated myotubes or those treated with DEXA were shown in **Figure 15**. HSMM undergo an atrophy-like response. Total myotube areas were

measured as described by Lecomte [151] and results were plotted as the percent decrease in myotube area compared to untreated control cultures.

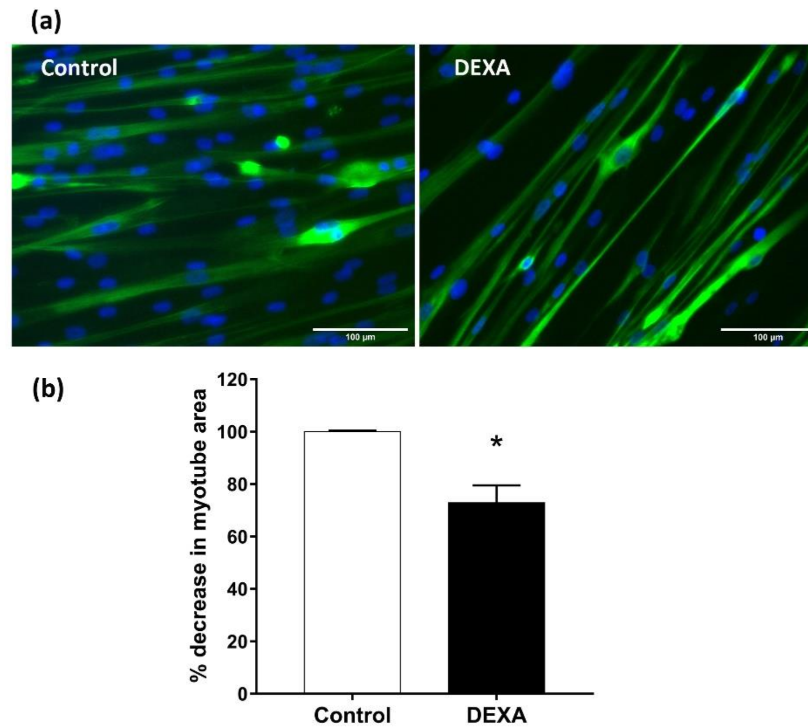
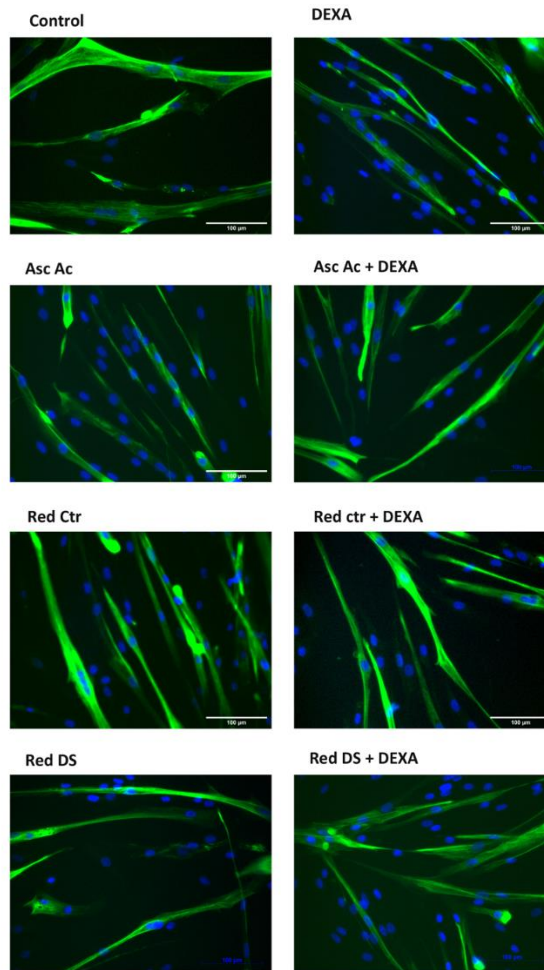


*Figure 15: HSMM immuno-stained for MYH2. Nuclei were visualized by Hoechst-staining. (**a**) Representative images of untreated myotubes or treated with DEXA (50µM) for 48 h. (b)Total myotube areas were measured and plotted as the percent decrease in myotube area compared to untreated control cultures. Images were acquired using 20× magnification and are representative of three different experiments in duplicate. The most representative images were shown. Images analyses were obtained from the mean of five acquisition fields per sample, in duplicate.*

3.2.4 Tomato Peel Extracts Effect On Sarcopenia

Differentiated myotubes were pre-treated with tomato peel extracts obtained by plants grown in normal (Red Ctr) or in drought stress condition (Red Ds). Ascorbic Acid (Asc) was used as positive control. Total myotube areas were measured as percent decrease of untreated myotube area. Results are shown in **Figure 16**. Data shows a protective effect of 5µg GAE/ml TPC of Red DS extract on the sarcopenia induced by DEXA treatment.



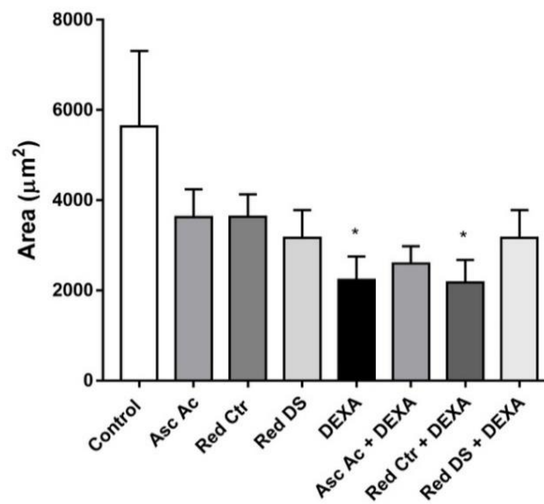
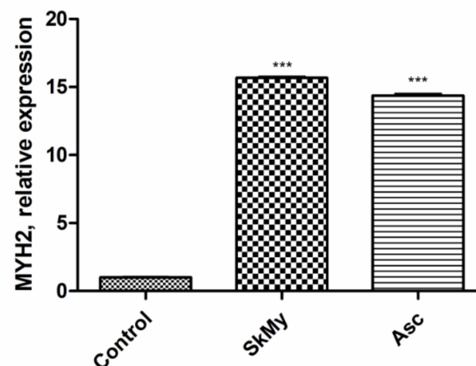


Figure 16: Tomato peel extracts effect on cellular model of sarcopenia. Representative images of MYH2/Hoechst-labelled myotubes treated with 50µM DEXA for 48h or 5 µgGAE/ml TPC of tomato peel extracts of plants grown in normal (Ctr) o drought stress (DS) condition. Plots of area, from untreated cells, in myotube area at each test agent are shown. Images were acquired using 20× magnification and are representative of three different experiments in duplicate. The most representative images were shown. Images analyses were obtained from the mean of five acquisition fields per sample, in duplicate.

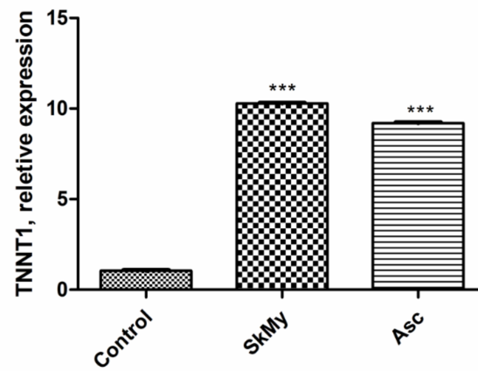
3.2.5 Gene Expression Profile

To evaluate the efficiency and linearity of the amplification for each gene analyzed, Real Time PCR reactions were carried out by varying the annealing temperature and the concentration of RNA. The expression of the reference genes studied was detected in all samples. In order to dissect the best group of reference genes for the normalization of Real Time PCR data, the stability was evaluated. The three most stable genes are: eEF1a, RPL13a, B2M, (Avg M value=0.269). Myosin heavy chain 2 (MYH2), troponin T (TNNT1), miogenin (MYOG) were late markers of muscle cell differentiation [150, 152, 153]. After 6 days of treatment, gene expression of later markers of muscle cell differentiation, Myosin heavy chain 2 (MYH2), troponin T (TNNT1), miogenin (MYOG), was evaluated in human skeletal muscle myoblasts and myotubes, as reported in **Figure 17**. MYH2 mRNA expression was resulted significantly higher in SkMy ($p<0.001$ vs Control) as shown in **Figure 17 a**. Similarly, TNNT1 and MYOG were highly expressed in SkMy ($p<0.001$ vs Control), Figure **17 b** and **17 c**. Therefore, human skeletal muscle myoblasts (HSMM) were differentiated in myotubes.

(a)



(b)



(c)

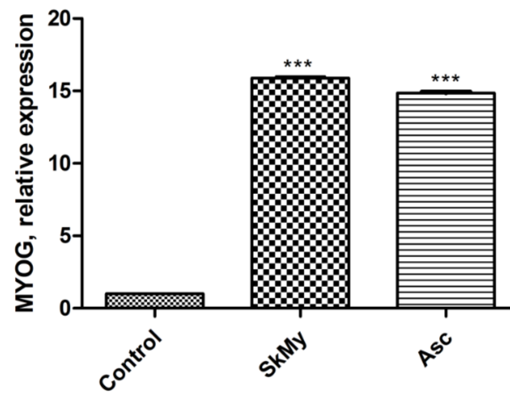
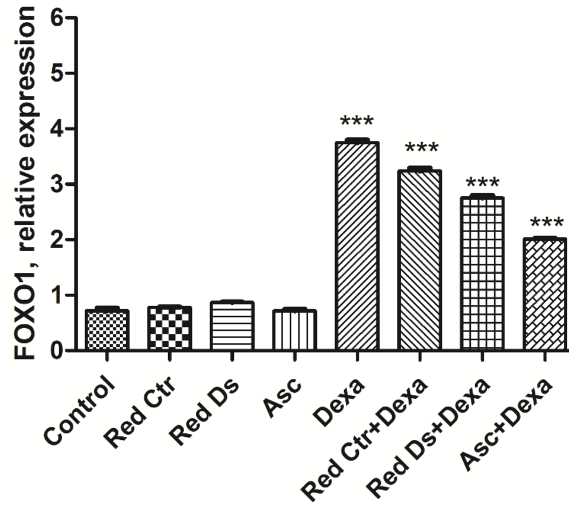


*Figure 17: Myosin heavy chain 2 (MYH2); Troponin T (TNNT1); Myogenin (MYOG) mRNA expression in samples (**a, b, c**). Untreated cells (Control); Myotubes (SkMy); ascorbic acid (Asc)*** p<0.0001 vs Control.*

To evaluate the intracellular pathway activated by dexamethasone, Protein kinase B (AKT1) and Forkhead Box O1 (FOXO1) mRNA expression were evaluated in control (HSMM differentiated in myotubes), myotubes pre-treated with Red Ctr, Red Ds, Dexa and ascorbic acid as shown in **Figure 18 a, b**. FOXO1 mRNA expression was significantly increased in Dexa and Red Ctr+Dexa vs control, but FOXO1 mRNA expression was significantly decreased in Red Ds+Dexa and Asc+Dexa (p <0.0001 vs control).Then dexamethasone lowers AKT1 mRNA expression and increases FOXO1 expression. AKT1 mRNA expression was increased in myotubes pre-treated with Red Ds and Dexa, Red Ds + Dexa, and Asc +Dexa (p <0.0001 vs control). Therefore, Red Ds protected myotubes by sarcopenia for the highly polyphenols content.

(a)



(b)

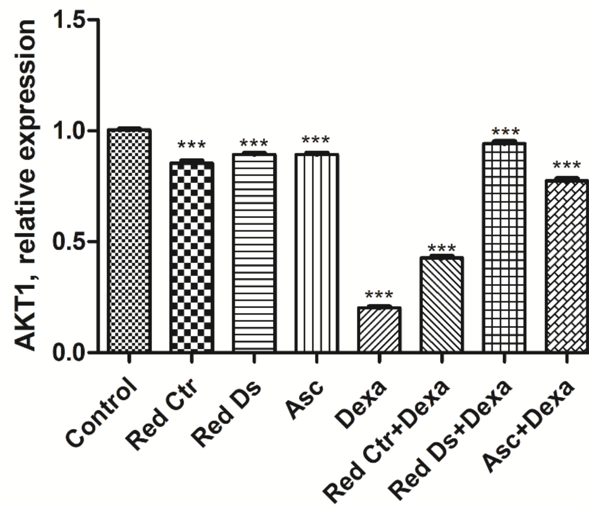


Figure 18: ***a,b****. FOXO1 and AKT1 mRNA expression in myotubes pre-treated with tomato peels extracts, ascorbic acid and Dexa (50µM). Pre-treatment with 5µg GAE/ml (TPC) of Rosso di Pitigliano (Red) extracts.*
*Protein kinase B (**AKT1**); Forkhead Box O1 (**FOXO1**); untreated myotubes (**Control**); ascorbic acid (**Asc**); dexamethasone (**Dexa**); plants grown in normal (**Red-Ctr**) or in drought stress condition (**Red-Ds**). p <0.0001 vs control.*

3.2.6 Antioxidant Enzymes Activity

The antioxidant activity was evaluated by measuring SOD, CAT and GPx enzymes activity in myotubes pre-treated with 5 µg GAE/mL of tomato peel extracts, ASC as

positive control and with DEXA (50 μM) for 48 h. In **Figure 19**, treatment with DEXA appears to significantly reduce the antioxidant enzyme activity of SOD compared with untreated cells ($p < 0.0001$). Dexamethasone triggers action like H_2O_2 [154]. Then pre-treatment of cells with RED-Ds prevented the negative effect of dexamethasone by acting SOD enzyme, which is known to be involved in the first line defense system against ROS. Furthermore, no substantial changes were shown for the activity of CAT and GPX enzymes. The defense system is different and should be investigated.

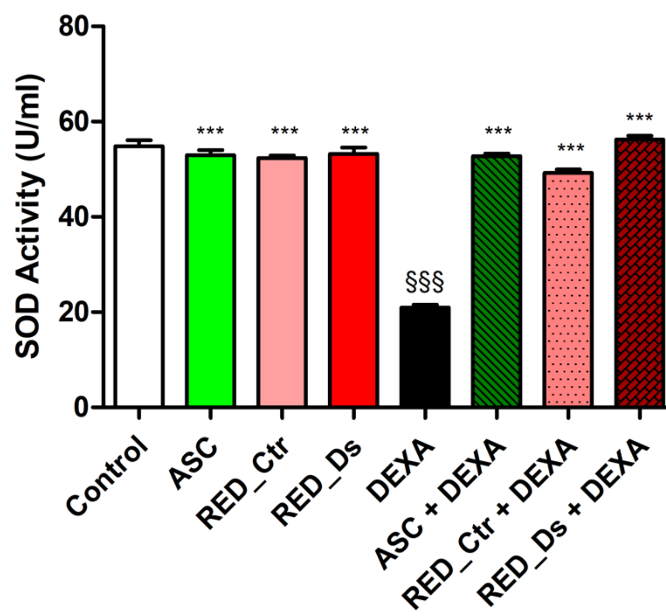


*Figure 19: Activity of SOD enzyme in myotubes pre-treated with 5μg GAE/mL of tomato peel extracts, or with 5 μg/mL ascorbic acid (ASC) as positive control, and with DEXA (50 μM) for 48 h. RED-Ctr: plants grown in normal condition; RED-Ds: plants grown in drought stress condition. ***p < 0.0001 vs DEXA; §§§ p < 0.0001 vs Control.*

3.3 Discussion

Tomato is one of the main sources of valuable nutrients in the Mediterranean diet. During tomato processing by-products obtained are unused, but these by-products contain a great variety of biologically active substances with beneficial properties [15]. The tomato variety, Rosso Di Pitigliano, was chosen by the Tuscan Regional Bank of the Germplasm to evaluate tomato peel extracts.

In tomato peel extracts the differences between the total content of phenols detected with LC-MS are 1201 µg / 100 g for the control vs 1073 µg / 100 g for the stressed sample. However, the variation within the classes of compounds is interesting. Phenolic acids increase in the stressed tomato peel extract, while flavonoids decrease. Stress affects plant metabolic pathways differently. When the plant is stressed, it activates the simpler metabolic pathways such as that of shikimic acid which leads to simple phenolic compounds rather than the one that leads to flavonoids which is a mixed pathway that includes the polyketide pathway [155]. This study was performed to evaluate the cytoprotective propensity of tomato peel polyphenols against sarcopenia. The human skeletal myoblasts were differentiated into myotubes, and they were responded appropriately to inducer of sarcopenia. Differentiated myotubes, pre-treated with tomato peel extracts obtained by plants grown in drought stress condition (Red Ds), were demonstrated a protective effect on the sarcopenia induced by DEXA treatment. This protective effect was determined by high content of polyphenols in tomato peels extract obtained in plants grown in drought stress condition. Chlorogenic acid, the main component of RED_DS extract, exhibited protective effects against sarcopenia suggesting its major contribution to the effects of the extracts. Chlorogenic acid, a type of polyphenolic compound formed by esterification of caffeic and quinic acids, is abundant in some fruits, dietary vegetables, and daily beverages like coffee, pineapple,

beans, strawberries, and apples [156]. Previous reports have demonstrated that chlorogenic acid exhibits a wide range of diverse pharmacological effects, including anti-inflammatory, anti-oxidative, and anti-carcinogenic activities [157-159]. Akila et al. [160] reported that chlorogenic acid is an effective protective agent to maintain the activities of enzymic antioxidants, including superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase. In this study, the differentiation of HSMM in myoblasts was evaluated. Then, sarcopenia was induced on myoblasts. Tomato peel extracts effect on sarcopenia was evaluated. Furthermore, after 6 days of differentiation was evaluated gene expression of later markers of muscle cell differentiation, Myosin heavy chain 2 (MYH2), troponin T (TNNT1), miogenin (MYOG). mRNA expression of these genes was higher in myotubes and thus the differentiation has taken place. Then intracellular pathway activated by dexamethasone was evaluated. Akt-mediated inhibition of the FoxO family of transcription factors is reduced by dexamethasone, which leads to the activation of FoxO transcription factors [63, 161]. Furthermore, dexamethasone increases FOXO1 mRNA expression, but Red Ds extract protects myotubes from sarcopenia by increasing AKT1 mRNA expression and reducing FOXO1 mRNA expression. The antioxidant activity was evaluated by measuring SOD, CAT and GPx enzymes activity in myotubes pre-treated with tomato peel extracts. Dexamethasone reduced the antioxidant enzyme activity of SOD, but in the cells pre-treated with tomato peel extract, RED_Ds was increased activity of SOD. No changes showed CAT and GPx activities.

CHAPTER 4 Future perspectives

Mediterranean vegetable local varieties are a valuable inheritance to be re-evaluated, since they constitute a source of more resilient cultivars under extreme climate conditions. In this study, tomato peel extracts of an ancient autochthonous Tuscan variety, obtained from plants grown in drought stress condition, showed enhanced antioxidant properties, which demonstrates new potential for biomedical applications.

Since the tomato peel extracts have shown an antioxidant action on endothelial cells even at low concentrations and that a good percentage was able to permeate intact through the isolated rat intestine, their supplementation in the diet may contribute to the prevention of cardiovascular diseases. For this reason, tomato peel extracts, even those grown in drought stress conditions, are a good source of bioactive molecules, with protective effect from oxidative stress even at low concentration and can be an added value in nutraceuticals. Moreover, the antioxidant action is present with free form of the plant extract also in the permeability studies.

The final results prove the protective effect of tomato peel extract in a cellular model of sarcopenia. With the diffusion of professional endurance sports activities, the emergence of sport sarcopenia is expected to increase in the future and since the role of oxidative stress in the pathogenesis of the disease further studies are needed to support the role of these compounds and to provide indications for their use as e a natural antioxidants supplementation.

For future clinical studies, tomato peels obtained in plants grown in drought stress conditions could be formulated in tablets, to be integrated into the diet of sarcopenic male and female athletes, for three months or six months. The data obtained will be useful to evaluate the protective effect of the tomato on the sarcopenia of athletes.

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REFERENCES

1. Kavitha, P.; Shivashankara, K.S.; Rao, V.K.; Sadashiva, A.T.; Ravishankar, K.V.; Sathish, G.J. Genotypic variability for antioxidant and quality parameters among tomato cultivars, hybrids, cherry tomatoes and wild species. *J. Sci. Food Agric.* **2014**, 94, 993–999. doi:10.1002/jsfa.6359.
2. Gomez-Romero, M.; Arraez-Roman, D.; Segura-Carretero, A.; Fernandez-Gutierrez, A. Analytical determination of antioxidants in tomato: Typical components of the Mediterranean diet. *J. Sep. Sci*. **2007**, 30, 452–461. doi:10.1002/jssc.200600400.
3. Ismea, **2017**. Report - I numeri della filiera del pomodoro da industria. Roma, June 2017
4. Cuna, D., Pallara, P., Miceli, V., **2018**. Sottoprodotti dell'industria di trasformazione del pomodoro e tecnologie per la loro valorizzazione. Available at: http:// openarchive.enea.it//handle/10840/9415. A
5. Kissoudis, C.; Sunarti, S.; Van deWiel, C.; Visser, R.G.F.; van der Linden, C.G.; Bai, Y. Responses to combined abiotic and biotic stress in tomato are governed by stress intensity and resistance mechanism. *J. Exp. Bot*. **2016**, 67, 5119–5132.
6. Mareri, L.; Faleri, C.; Romi, M.; Mariani, C.; Cresti, M.; Cai, G. Heat stress affects the distribution of JIM8-labelled arabinogalactan proteins in pistils of Solanum lycopersicum cv Micro-Tom. *Acta Physiol. Plant*. **2016**, 38, 184
7. Dai, A. Increasing drought under global warming in observations and models. *Nat. Clim. Chang*. **2013**, 3, 52–58
8. Morales, R.G.; Resende, L.V.; Maluf, W.R.; Peres, L.E.; Bordini, I.C. Selection of tomato plant families using characters related to water deficit resistance. *Hortic. Bras*. **2015**, 33, 27–33
9. Joshi, R.; Wani, S.H.; Singh, B.; Bohra, A.; Dar, Z.A.; Lone, A.A.; Pareek, A.; Singla-Pareek, S.L. Transcription Factors and Plants Response to Drought Stress: Current Understanding and Future Directions. *Front. Plant Sci.* **2016**, 7, 1029
10. Osakabe, Y.; Osakabe, K.; Shinozaki, K.; Tran, L.S. Response of plants to water stress. *Front. Plant Sci*. **2014**, 5, 86.
11. Skirycz, A.; Inze, D. More from less: Plant growth under limited water. *Curr. Opin. Biotechnol*. **2010**, 21, 197–203.
12. Wang, W.B.; Kim, Y.H.; Lee, H.S.; Kim, K.Y.; Deng, X.P.; Kwak, S.S. Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. *Plant Physiol. Biochem*. **2009**, 47, 570–577.
13. Yang, Y.; Liu, Q.; Wang, G.X.; Wang, X.D.; Guo, J.Y. Germination, osmotic adjustment, and antioxidant enzyme activities of gibberellin-pretreated Picea asperata seeds under water stress. *New For*. **2010**, 39, 231–243.
14. Sivakumar, R.; Srividhya, S. Impact of drought on flowering, yield and quality parameters in diverse genotypes of tomato (Solanum lycopersicum L.). Adv. *Hortic. Sci.* **2016**, 30, 3–11.
15. Viuda-Martos, M.; Sanchez-Zapata E.; Sayas-Barberá, E.; Sendra, E.; Pérez-Álvarez, JA.; Fernández-López, J. Tomato and Tomato Byproducts. Human Health Benefits of Lycopene and Its Application to Meat Products: A Review. *Critical Reviews in Food Science and Nutrition*, **2014** 54:1032–1049.
16. Szabo, K.; Catoi, A.F.; Vodnar, D.C. Bioactive Compounds Extracted from Tomato Processing by-Products as a Source of Valuable Nutrients. *Plant Foods Hum. Nutr*. **2018**, 73, 268–277.
17. Felice, F.; Fabiano, A.; De Leo, M.; Piras, A.M.; Beconcini, D.; Cesare, M.M.; Braca, A.; Zambito, Y.; Di Stefano, R. Antioxidant Effect of Cocoa By-Product

and Cherry Polyphenol Extracts: A Comparative Study. *Antioxidants* **2020**, 9, 132.

18. De la Ossa, J.G.; Felice, F.; Azimi, B.; Salsano, J.E.; Digiacomo, M.; Macchia, M.; Danti, S.; Di Stefano, R.Waste Autochthonous Tuscan Olive Leaves (Olea europaea var. Olivastra seggianese) as Antioxidant Source for Biomedicine. *Int. J. Mol. Sci.* **2019**, 20, 5918.
19. Sahin, S.; Bilgin, M. Olive tree (Olea europaea L.) leaf as a waste by-product of table olive and olive oil industry: A review. *J. Sci. Food Agric*. **2018**, 98, 1271–1279.
20. Pellicanò, T.M.; Sicari, V.; Loizzo, M.R.; Leporini, M.; Falco, T.; Poiana, M. Optimizing the supercritical fluid extraction process of bioactive compounds from processed tomato skin by-products. *J. Food Sci. Technol.* **2020**, 40, 692–697.
21. Cámara, M., Fernández-Ruiz, V., Cámara, R.M., Domínguez, L. and Sánchez-Mata, M.C. Tomato products and cardiovascular disease prevention. *Acta Hortic*. **2019**, 1233, 201-208.
22. Thies, F.; Masson, L.F.; Rudd, A.; Vaughan, N.; Tsang, C.; Brittenden, J.; Simpson,W.G.; Duthie, S.; Horgan, G.W.; Duthie, G. Effect of a tomato-rich diet on markers of cardiovascular disease risk in moderately overweight, disease-free, middle-aged adults: A randomized controlled trial. *Am. J. Clin. Nutr*. **2012**, 95, 1013–1022
23. Perveen, R.; Suleria, H.A.; Anjum, F.M.; Butt, M.S.; Pasha, I.; Ahmad, S. Tomato (Solanum lycopersicum) Carotenoids and Lycopenes Chemistry; Metabolism, Absorption, Nutrition, and Allied Health Claims—A Comprehensive Review. *Crit. Rev. Food Sci. Nutr*. **2015**, 55, 919–929.
24. Friedman, M. Anticarcinogenic, cardioprotective, and other health benefits of tomato compounds lycopene, alpha-tomatine, and tomatidine in pure form and in fresh and processed tomatoes. *J. Agric. Food Chem*. **2013**, 61, 9534–9550.
25. Pereira, B.L.B.; Arruda, F.C.O.; Reis, P.P.; Felix, T.F.; Santos, P.P.; Rafacho, B.P.; Gonçalves, A.F.; Claro, R.T.; Azevedo, P.S.; Polegato, B.F.; et al. Tomato (Lycopersicon esculentum) Supplementation Induces Changes in Cardiac miRNA Expression, Reduces Oxidative Stress and Left Ventricular Mass, and Improves Diastolic Function. *Nutrients* **2015**, 7, 9640–9649.
26. Steven, S.; Frenis, K.; Oelze, M.; Kalinovic, S.; Kuntic, M.; Bayo Jimenez, M.T.; Vujacic-Mirski, K.; Helmstädter, J.; Kröller-Schön, S.; Münzel, T.; et al. Vascular Inflammation and Oxidative Stress: Major Triggers for Cardiovascular Disease. *Oxid. Med. Cell. Longev*. **2019**, 7092151
27. Górecka, D.; Wawrzyniak,A.; Jędrusek-Golińska, A.; Dziedzic, K.; Hamułka, J.; Kowalczewski, P.L.; Walkowiak,J. Lycopene in tomatoes and tomato products. *Open Chemistry* **2020**, 18: 752–756.
28. Shen, Y.C.; Chen, S.L.;Wang, C.K. Contribution of tomato phenolics to antioxidation and down-regulation of blood lipids. *J. Agric. Food Chem.* **2007**, 55, 6475–6481.
29. Padalino, L.; Conte, A.; Lecce, L.; Likyova, D.; Sicari, V.; Pellicanò, T.M.; Poiana, M.; Del Nobile, M.A. Functional pasta with tomato by-product as a source of antioxidant compounds and dietary fibre. *Czech. J. Food Sci*. **2017**, 35, 48–56.
30. García-Valverde, V. Antioxidant Bioactive Compounds in Selected Industrial Processing and Fresh Consumption Tomato Cultivars. *Food Bioproc. Tech*. **2013**, 6, 391–402.

31. Frusciante, L.; Carli, P.; Ercolano, M.R.; Pernice, R.; Di Matteo, A.; Fogliano, V.; Pellegrini, N. Antioxidant nutritional quality of tomato. *Mol. Nutr. Food Res.* **2007**, 51, 609–617.
32. Kaliora, A. C., Dedoussis, G. V. Z., Schmidt, H. Dietary antioxidants in preventing atherogenesis. *Atherosclerosis* **2006**, 187 (1): 1 – 17.
33. Hernández, M.; Rodríguez, E.; Díaz, C. Free Hydroxycinnamic Acids, Lycopene, and Color Parameters in Tomato Cultivars. *J. Agric. Food Chem.* **2007**, 55, 8604–8615.
34. Slimestad, R.; Verheul, M. Review of flavonoids and other phenolics from fruits of different tomato (Lycopersicon esculentum Mill.) cultivars. *J. Sci. Food Agric.* **2009**, 89, 1255–1270.
35. Vallverdu-Queralt, A.; Jauregui, O.; Medina-Remon, A.; Andres-Lacueva, C.; Lamuela-Raventos, R.M. Improved characterization of tomato polyphenols using liquid chromatography/electrospray ionization linear ion trap quadrupole Orbitrap mass spectrometry and liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, 24, 2986–2992.
36. Jesus Periago, M.; Garcia-Alonso, J.; Jacob, K.; Belen Olivares, A.; Jose Bernal, M.; Dolores Iniesta, M.; Martinez, C.; Ros, G. Bioactive compounds, folates and antioxidant properties of tomatoes (Lycopersicum esculentum) during vine ripening. *Int. J. Food Sci. Nutr.* **2009**, 60, 694–708.
37. Martínez-Valverde, I.; Periago, M.J.; Provan, G.; Chesson, A. Phenolic compounds, lycopene and antioxidant activity in commercial varieties of tomato (Lycopersicum esculentum). *J. Sci. Food Agr.* **2002**, 82, 323–330.
38. Halliwell, B. Antioxidant activity and other biological effects of flavonoids. In International Congress and Symposium Series-Royal Society of Medicine, Proceedings of the Symposium Wake Up to Flavonoids, Location of London, UK, 8 July 1999; *Royal Society of Medicine* Press Ltd.: London, UK, **2000.**
39. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alex. J. Med.* **2018**, 54, 287–293.
40. Cao, Y.; Gong, Y.; Liu, L.; Zhou, Y.; Fang, X.; Zhang, C.; Li, Y.; Li, J. The use of human umbilical vein endothelial cells (HUVECs) as an in vitro model to assess the toxicity of nanoparticles to endothelium: A review. *J. Appl. Toxicol.* **2017**, 37, 1359–1369.
41. Felice, F.; Maragò, E.; Sebastiani, L.; Di Stefano, R. Apple juices from ancient Italian cultivars: A study on mature endothelial cells model. *Fruits* **2015**, 70, 361–369.
42. Beconcini, D.; Fabiano, A.; Zambito, Y.; Berni, R.; Santoni, T.; Piras, A.M.; Di Stefano, R. Chitosan-Based Nanoparticles Containing Cherry Extract from Prunus avium L. to Improve the Resistance of Endothelial Cells to Oxidative Stress. *Nutrients* **2018**, 10, 1598.
43. Hafizah, A.H.; Zaiton, Z.; Zulkhairi, A.; Mohd Ilham, A.; Nor Anita, M.M.; Zaleha, A.M. Piper sarmentosum as an antioxidant on oxidative stress in human umbilical vein endothelial cells induced by hydrogen peroxide. J. Zhejiang Univ. *Sci. B* **2010**, 11, 357–365.
44. Rosenberg, I.H. Sarcopenia: Origins and clinical relevance. *J. Nutr.* **1997**, 127, 990S–991S.

45. Sullivan-Gunn, M.; Lewandowski, P. Elevated hydrogen peroxide and decreased catalase and glutathione peroxidase protection are associated with aging sarcopenia. *BMC Geriatr*. **2013**, 13, 104.
46. Huang, J.; Zhu, X. The molecular mechanisms of calpains action on skeletal muscle atrophy. *Physiol Res* **2016**; 65(4):547-560.
47. Bodine, S.C.; Baehr, L.M. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. *Am J Physiol Endocrinol Metab* **2014**; 307 (6): E469-84.
48. Giordano, F.M.; Burattini, S.; Buontempo, F.; Canonico, B.; Martelli, A.M.; Papa, S.; Sampaolesi, M.; Falcieri, E.; Salucci, S. Diet Modulation Restores Autophagic Flux in Damaged Skeletal Muscle Cells. *J Nutr Health Aging* **2019**; 23(8):739-745.
49. Afroze, D.; Kumar, A. ER stress in skeletal muscle remodeling and myopathies. *FEBS* **2019**;286(2):379-398.
50. Anderson, L.J.; Liu, H.; Garcia, J.M. Sex Differences in Muscle Wasting. *Adv Exp Med Biol* **2017**; 1043:153-197.
51. Frati, A.; Landi, D.; Marinelli, C.; Gianni, G.; Fontana, L.; Migliorini, M.; Pierucci, F.; Garcia-Gil, M.; Meacci, E. Nutraceutical properties of chestnut flours: beneficial effects on skeletal muscle atrophy. *Food Funct* **2014**; 5(11): 2870-82.
52. Yoon M.S. mTOR as a Key Regulator in Maintaining Skeletal Muscle Mass. *Front Physiol* **2017**; 8: 788.
53. Laplante, M.; Sabatini, D.M. mTOR signaling in growth control and disease. *Cell* **2012**; 49(2): 274-93.
54. Mirzoev, T.; Tyganov, S.; Vilchinskaya, N.; Lomonosova, Y.; Shenkman, B. Key Markers of mTORC1-Dependent and mTORC1-Independent Signaling Pathways Regulating Protein Synthesis in Rat Soleus Muscle During Early Stages of Hindlimb Unloading. *Cell Physiol Biochem* **2016**; 39(3): 1011-20.
55. Bell, K.E.; Von Allmen, M.T.; Devries, M.C.; Phillips, S.M. Muscle Disuse as a Pivotal Problem in Sarcopenia-related Muscle Loss and Dysfunction. *J Frailty Aging* **2016**; 5(1): 33-41.
56. Auclair, D.; Garrel, D.R.; Chaouki Zerouala, A.; Ferland, L.H. Activation of the ubiquitin pathway in rat skeletal muscle by catabolic doses of glucocorticoids. *Am. J. Physiol*. **1997**, 272, C1007–C1016.
57. Jackman, R.W.; Kandarian, S.C. The molecular basis of skeletal muscle atrophy. *Am. J. Physiol. Cell Physiol*. **2004**, 287, C834–C843.
58. Klein, G.L. The Effect of Glucocorticoids On Bone And Muscle. *Osteoporos Sarcopenia* **2015**, 1, 39–45.
59. Bodine, S.C.; Latres, E.; Baumhueter, S.; Lai, V.K.; Nunez, L.; Clarke, B.A.; Poueymirou, W.T.; Panaro, F.J.; Na, E.; Dharmarajan, K.; et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **2001**, 294, 1704–1708.
60. Lecker, S.H.; Jagoe, R.T.; Gilbert, A.; Gomes, M.; Baracos, V.; Bailey, J.; Price, S.R.; Mitch, W.E.; Goldberg, A.L. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb. J*. **2004**, 18, 39–51.
61. Lagirand-Cantaloube, J.; Oner, N.; Csibi, A.; Leibovitch, M.P.; Batonnet-Pichon, S.; Tintignac, L.A.; Segura, C.T.; Leibovitch, S.A. The initiation factor eIF3-f is a major target for atrogin1/MAFbx function in skeletal muscle atrophy. *EMBO J*. **2008**, 27, 1266–1276.

62. Csibi, A.; Leibovitch, M.P.; Cornille, K.; Tintignac, L.A.; Leibovitch, S.A. MAFbx/Atrogin-1 controls the activity of the initiation factor eIF3-f in skeletal muscle atrophy by targeting multiple C-terminal lysines. *J. Biol. Chem.* **2009**, 284, 4413–4421.
63. Zhao, W.; Qin, W.; Pan, J.; Wu, Y.; Bauman, W.A.; Cardozo, C. Dependence of dexamethasone-induced Akt/FOXO1 signaling, upregulation of MAFbx, and protein catabolism upon the glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **2009**, 378, 668–672.
64. Stitt, T.N.; Drujan, D.; Clarke, B.A.; Panaro, F.; Timofeyva, Y.; Kline, W.O.; Gonzalez, M.; Yancopoulos, G.D.; Glass, D.J. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell* **2004**, 14, 395–403.
65. Sacheck, J.M.; Ohtsuka, A.; McLary, S.C.; Goldberg, A.L. IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1. *Am. J. Physiol. Endocrinol. Metab.* **2004**, 287, E591–E601.
66. Salucci, S.; Falcieri, E. Polyphenols and their potential role in preventing skeletal muscle atrophy. *Nutr Res*. **2020**; 74:10-22.
67. Davies, K.J.; Quintanilha, A.T.; Brooks, G.A.; Packer, L. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun*. **1982**, 107, 1198–1205.
68. Reid, M.B.; Li, Y.P. Cytokines and oxidative signalling in skeletal muscle. *Acta Physiol. Scand*. **2001**, 171, 225–232.
69. Steinbacher, P.; Eckl, P. Impact of oxidative stress on exercising skeletal muscle. *Biomolecules* **2015**, 5, 356–377.
70. He, F.; Li, J.; Liu, Z.; Chuang, C.-C.; Yang, W.; Zuo, L. Redox Mechanism of Reactive Oxygen Species in Exercise. *Front. Physiol*. **2016,** 7, 486.
71. Smith, M.A.; Reid, M.B. Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respir. Physiol. Neurobiol*. **2006**, 151, 229–241.
72. Kim, J.S.; Wilson, J.M.; Lee, S.R. Dietary implications on mechanisms of sarcopenia: Roles of protein, aminoacids and antioxidants. *J. Nutr. Biochem.* **2010**, 21, 1–13.
73. Scicchitano, B.M.; Pelosi, L.; Sica, G.; Musaro, A. The physiopathologic role of oxidative stress in skeletal muscle. *Mech. Ageing Dev*. **2018**, 170, 37–44.
74. Debold, E.P. Potential molecular mechanisms underlying muscle fatigue mediated by reactive oxygen and nitrogen species. *Front. Physiol*. **2015**, 6, 239.
75. Urso, M.L.; Clarkson, P.M. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* **2003**, 189, 41–54.
76. Ferreira, L.F.; Laitano, O. Regulation of NADPH oxidases in skeletal muscle. *Free Radic. Biol. Med*. **2016**, 98, 18–28.
77. Formanowicz, D.; Radom, M.; Rybarczyk, A.; Formanowicz, P. The role of Fenton reaction in ROS-induced toxicity underlying atherosclerosis - modeled and analyzed using a Petri net-based approach. *Biosystems* **2018**, 165, 71–87.
78. Brooks, S.V.; Vasilaki, A.; Larkin, L.M.; McArdle, A.; Jackson, M.J. Repeated bouts of aerobic exercise lead to reductions in skeletal muscle free radical generation and nuclear factor kappaB activation. *J. Physiol*. **2008**, 586, 3979–399.
79. Khassaf, M.; McArdle, A.; Esanu, C.; Vasilaki, A.; McArdle, F.; Griffiths, R.D.; Brodie, D.A.; Jackson, M.J. Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. *J. Physiol*. **2003**, 549, 645–652.

80. Damiano, S.; Muscariello, E.; La Rosa, G.; Di Maro, M.; Mondola, P.; Santillo, M. Dual Role of Reactive Oxygen Species in Muscle Function: Can Antioxidant Dietary Supplements Counteract Age-Related Sarcopenia? *Int. J. Mol. Sci*. **2019**, 20, 3815.
81. Oldford, C.; Kuksal, N.; Gill, R.; Young, A.; Mailloux, R.J. Estimation of the hydrogen peroxide producing capacities of liver and cardiac mitochondria isolated from C57BL/6N and C57BL/6J mice. *Free Radic. Biol. Med*. **2019**, 135, 15–27.
82. Del Rio, L.A.; Lopez-Huertas, E. ROS Generation in Peroxisomes and its Role in *Cell Signaling. Plant. Cell Physiol*. **2016**, 57, 1364–1376.
83. Ji, L.L. Antioxidants and oxidative stress in exercise. *Proc. Soc. Exp. Biol. Med*. **1999**, 222, 283–292.
84. Gonchar, O. Muscle fiber specific antioxidative system adaptation to swim training in rats: Influence of intermittent hypoxia. *J. Sports Sci. Med*. **2005**, 4, 160–169.
85. Berger, M.M. Can oxidative damage be treated nutritionally? *Clin. Nutr*. **2005**, 24, 172–183.
86. Gomez-Cabrera, M.C.; Salvador-Pascual, A.; Cabo, H.; Ferrando, B.; Vina, J. Redox modulation of mitochondriogenesis in exercise. Does antioxidant supplementation blunt the benefits of exercise training? *Free Radic. Biol. Med*. **2015**, 86, 37–46.
87. Pingitore, A.; Lima, G.P.; Mastorci, F.; Quinones, A.; Iervasi, G.; Vassalle, C. Exercise and oxidative stress: Potential effects of antioxidant dietary strategies in sports. *Nutrition* **2015**, 31, 916–922.
88. Boots, A.W.; Haenen, G.R.; Bast, A. Health effects of quercetin: From antioxidant to nutraceutical. *Eur. J. Pharmacol*. **2008**, 585, 325–337.
89. Pellavio, G.; Rui, M.; Caliogna, L.; Martino, E.; Gastaldi, G.; Collina, S.; Laforenza, U. Regulation of Aquaporin Functional Properties Mediated by the Antioxidant E effects of Natural Compounds. *Int. J. Mol. Sci.* **2017**, 18, 2665.
90. Tresserra-Rimbau, A.; Arranz, S.; Vallverdu-Queralt, A. New Insights into the Benefits of Polyphenols in Chronic Diseases. *Oxid. Med. Cell Longev.* **2017**, 1432071.
91. Damiano, S.; Sasso, A.; Accetta, R.; Monda, M.; De Luca, B.; Pavone, L.M.; Belfiore, A.; Santillo, M.; Mondola, P. Effect of Mutated Cu, Zn Superoxide Dismutase (SOD1(G93A)) on Modulation of Transductional Pathway Mediated by M1 Muscarinic Receptor in SK-N-BE and NSC-34 Cells. *Front. Physiol*. **2018**, 9, 611.
92. Padayatty, S.J.; Katz, A.;Wang, Y.; Eck, P.; Kwon, O.; Lee, J.H.; Chen, S.; Corpe, C.; Dutta, A.; Dutta, S.K.; Levine, M. Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *J. Am. Coll. Nutr*. **2003**, 22, 18–35.
93. Mandl, J.; Szarka, A.; Banhegyi, G. Vitamin C: Update on physiology and pharmacology. *Br. J. Pharmacol*. **2009**, 157, 1097–1110.
94. Welch, A.A.; Jennings, A.; Kelaiditi, E.; Skinner, J.; Steves, C.J. Cross-Sectional Associations Between Dietary Antioxidant Vitamins C, E and Carotenoid Intakes and Sarcopenic Indices in Women Aged 18-79 Years. *Calcif. Tissue Int*. **2020**, 106, 331–342.
95. Fenech, M.; Amaya, I.; Valpuesta, V.; Botella, M.A. Vitamin C Content in Fruits: Biosynthesis and Regulation. *Front. Plant. Sci*. **2018**, 9, 2006.
96. Neubauer, O.; Yfanti, C. Antioxidants in Athlete's Basic Nutrition: Considerations towards a Guideline for the Intake of Vitamin C and Vitamin E; CRC Press/Taylor & Francis: Boca Raton, FL, USA, **2015.**

97. Aggarwal, B.B.; Sundaram, C.; Prasad, S.; Kannappan, R. Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. *Biochem. Pharmacol*. **2010**, 80, 1613–1631.
98. Szymanska, R.; Nowicka, B.; Kruk, J. Vitamin E—Occurrence, Biosynthesis by Plants and Functions in Human Nutrition. *Mini Rev. Med. Chem*. **2017**, 17, 1039–1052.
99. Miyazawa, T.; Burdeos, G.C.; Itaya, M.; Nakagawa, K. Vitamin E: Regulatory Redox Interactions. *IUBMB Life* **2019**, 71, 430–441.
100. Rokitzki, L.; Logemann, E.; Sagredos, A.N.; Murphy, M.; Wetzel-Roth,W.; Keul, J. Lipid peroxidation and antioxidative vitamins under extreme endurance stress. *Acta Physiol. Scand*. **1994**, 151, 149–158.
101. Vitale, K.; Getzin, A. Nutrition and Supplement Update for the Endurance Athlete: Review and Recommendations. *Nutrients* **2019**, 11, 1289.
102. Bohn, T. Carotenoids and Markers of Oxidative Stress in Human Observational Studies and Intervention Trials: Implications for Chronic Diseases. *Antioxidants (Basel)* **2019**, 8, 179.
103. Sandmann, G. Antioxidant Protection from UV- and Light-Stress Related to Carotenoid Structures. *Antioxidants (Basel)* **2019**, 8, 219.
104. De Vries, J.H.; Hollman, P.C.; Meyboom, S.; Buysman, M.N.; Zock, P.L.; van Staveren, W.A.; Katan, M.B. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. *Am. J. Clin. Nutr*. **1998**, 68, 60–65.
105. Wenzel, E.; Somoza, V. Metabolism and bioavailability of trans-resveratrol. *Mol. Nutr. Food Res*. **2005**, 49, 472–481.
106. Andres-Lacueva, C.; Macarulla, M.T.; Rotches-Ribalta, M.; Boto-Ordonez, M.; Urpi-Sarda, M.; Rodriguez,V.M.; Portillo, M.P. Distribution of resveratrol metabolites in liver, adipose tissue, and skeletal muscle in rats fed different doses of this polyphenol. *J. Agric. Food Chem*. **2012**, 60, 4833–4840.
107. Jackson, J.R.; Ryan, M.J.; Hao, Y.; Alway, S.E. Mediation of endogenous antioxidant enzymes and apoptotic signaling by resveratrol following muscle disuse in the gastrocnemius muscles of young and old rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2010**, 299, R1572–R1581.
108. Jackson, J.R.; Ryan, M.J.; Alway, S.E. Long-term supplementation with resveratrol alleviates oxidative stress but does not attenuate sarcopenia in aged mice. *J. Gerontol. Series A Biol. Sci. Med Sci*. **2011**, 66, 751–764.
109. Tung, B.T.; Rodriguez-Bies, E.; Thanh, H.N.; Le-Thi-Thu, H.; Navas, P.; Sanchez, V.M.; Lopez-Lluch, G. Organ and tissue-dependent effect of resveratrol and exercise on antioxidant defenses of old mice. *Aging Clin. Exp. Res.* **2015**, 27, 775–783.
110. Muhammad, M.H.; Allam, M.M. Resveratrol and/or exercise training counteract aging-associated decline of physical endurance in aged mice; targeting mitochondrial biogenesis and function. *J. Physiol. Sci*. **2018**, 68, 681–688.
111. Myburgh, K.H. Polyphenol supplementation: Benefits for exercise performance or oxidative stress? *Sports Med*. **2014**, 44 (Suppl. 1), S57–S70.
112. Landi, S.; Nurcato, R.; De Lillo, A.; Lentini, M.; Grillo, S.; Esposito, S. Glucose-6-phosphate dehydrogenase plays a central role in the response of tomato (Solanum lycopersicum) plants to short and long-term drought. *Plant Physiol. Biochem.* **2016**, 105, 79–89.

113. Nuruddin, M.; Madramootoo, C.; Dodds, G. Effects of Water Stress at Different Growth Stages on Greenhouse Tomato Yield and Quality. *HortScience* **2003**, 38, 1389–1393.
114. Tokusoˇglu, Ö.; Ünal, M.; Yildirim, Z. HPLC-UV and GC-MS characterization of the flavonol aglycons quercetin, kaempferol, and myricetin in tomato pastes and other tomato-based products. *Acta Chromatogr*. **2003**, 196–207.
115. Kumar, N.; Bhandari, P.; Singh, B.; Gupta, A.P.; Kaul, V.K. Reversed phase-HPLC for rapid determination of polyphenols in flowers of rose species. *J. Sep. Sci.* **2008**, 31, 262–267.
116. Olives Barba, A.I.; Camara Hurtado, M.; Sanchez Mata, M.C.; Fernandez Ruiz, V.; Lopez Saenz de Tejada, M. Application of a UV-vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chem*. **2006**, 95, 328–336.
117. Scherer, R.; Rybka, A.C.P.; Ballus, C.A.; Meinhart, A.D.; Filho, J.T.; Godoy, H.T. Validation of a HPLC method for simultaneous determination of main organic acids in fruits and juices. *Food Chem*. **2012**, 135, 150–154.
118. Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem*. **1996**, 239, 70–76.
119. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* **1999**, 299, 152–178.
120. Jaffe, E.A.; Nachman, R.L.; Becker, C.G.; Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Investig*. **1973**, 52, 2745–2756.
121. Zambito, Y.; Di Colo, G. Thiolated quaternary ammonium-chitosan conjugates for enhanced precorneal retention, transcorneal permeation and intraocular absorption of dexamethasone. *Eur. J. Pharm. Biopharm*. **2010**, 75, 194–199.
122. Fabiano, A.; Brilli, E.; Fogli, S.; Beconcini, D.; Carpi, S.; Tarantino, G.; Zambito, Y. Sucrosomial® iron absorption studied by in vitro and ex-vivo models. *Eur. J. Pharm. Sci*. **2018**, 111, 425–431.
123. Felice, F.; Zambito, Y.; Di Colo, G.; D'Onofrio, C.; Fausto, C.; Balbarini, A.; Di Stefano, R. Red grape skin and seeds polyphenols: Evidence of their protective effects on endothelial progenitor cells and improvement of their intestinal absorption. *Eur. J. Pharm. Biopharm*. **2012**, 80, 176–184.
124. Legen, I.; Salobir, M.; Kerc, J. Comparison of different intestinal epithelia as models for absorption enhancement studies. *Int. J. Pharm*. **2005**, 291, 183–188.
125. Harel, D.; Sofer, M.; Bruner, M.; Gantz, D. Growth-Stage-Specific Kc of Greenhouse Tomato Plants Grown in Semi-Arid Mediterranean Region. *J. Agric. Sci.* **2014**, 6, 132–142.
126. Kapoor, D.; Bhardwaj, S.; Landi, M.; Sharma, A.; Ramakrishnan, M.; Sharma, A. The Impact of Drought in Plant Metabolism: How to Exploit Tolerance Mechanisms to Increase Crop Production. *Appl. Sci*. **2020**, 10, 5692.
127. Hussain, S.; Rao, M.J.; Anjum, M.A.; Ejaz, S.; Zakir, I.; Ali, M.A.; Ahmad, N.; Ahmad, S. Oxidative Stress and Antioxidant Defense in Plants Under Drought Conditions. *Plant Abiotic Stress Tolerance: Agronomic, Molecular and Biotechnological Approaches*; Hasanuzzaman, M., Hakeem, K., Nahar, K.,

Alharby, H., Eds.; Springer International Publishing: Cham, Switzerland, **2019**; 207–219.
128. Klunklin,W.; Savage, G. Effect on Quality Characteristics of Tomatoes Grown UnderWell-Watered and Drought Stress Conditions. *Foods* **2017**, 6, 56.
129. Theobald, J.; Bacon, M.; Davies, W. Delivering enhanced fruit quality to the UK tomato industry through implementation of partial root-zone drying. *Comp. Biochem. Physiol. Mol. Integr. Physiol*. **2007**, 146, S241.
130. Conti, V.; Mareri, L.; Faleri, C.; Nepi, M.; Romi, M.; Cai, G.; Cantini, C. Drought Stress Affects the Response of Italian Local Tomato (Solanum lycopersicum L.) Varieties in a Genotype-Dependent Manner. *Plants* **2019**, 8, 336.
131. Barbagallo, R.N.; Di Silvestro, I.; Patanè, C. Yield, physicochemical traits, antioxidant pattern, polyphenol oxidase activity and total visual quality of field-grown processing tomato cv. Brigade as affected by water stress in Mediterranean climate. *J. Sci. Food Agric*. **2013**, 93, 1449–1457.
132. Chandra, H.M.; Ramalingam, S. Antioxidant potentials of skin, pulp, and seed fractions of commercially important tomato cultivars. *Food Sci. Biotechnol*. **2011**, 20, 15–21.
133. Tommonaro, G.; De Prisco, R.; Abbamondi, G.R.; Nicolaus, B. Bioactivity of tomato hybrid powder: Antioxidant compounds and their biological activities. *J. Med. Food* **2013**, 16, 351–356.
134. Minoggio, M.; Bramati, L.; Simonetti, P.; Gardana, C.; Iemoli, L.; Santangelo, E.; Mauri, P.L.; Spigno, P.; Soressi, G.P.; Pietta, P.G. Polyphenol pattern and antioxidant activity of different tomato lines and cultivars. *Ann. Nutr. Metab.* **2003**, 47, 64–69.
135. Riggi, E.; Patanè, C.; Ruberto, G. Content of carotenoids at different ripening stages in processing tomato in relation to soil water availability. *Aust. J. Agric. Res*. **2008**, 59, 348–353.
136. Atkinson, N.J.; Dew, T.P.; Orfila, C.; Urwin, P.E. Influence of combined biotic and abiotic stress on nutritional quality parameters in tomato (Solanum lycopersicum). *J. Agric. Food Chem.* **2011**, 59, 9673–9682.
137. Delazari, F.T.; Gomes, R.S.; Laurindo, B.S.; Laurindo, R.D.F.; Giovanelli, L.B.; de Freitas, D.S.; Mantovani, E.C.; da Silva, D.J.H. Establishing irrigation levels targeting higher content of lycopene and water use efficiency in tomato. *J. Agric. Sci.* **2019**, 11, 1.
138. Nasri, H.; Baradaran, A.; Shirzad, H.; Rafieian-Kopaei, M. New concepts in nutraceuticals as alternative for pharmaceuticals. *Int. J. Prev. Med*. **2014**, 5, 1487–1499.
139. Yiran, Z.; Chenyang, J.; Jiajing, W.; Yan, Y.; Jianhong, G.; Jianchun, B.; Xuezhong, L.; Zongping, L. Oxidative stress and mitogen-activated protein kinase pathways involved in cadmium-induced BRL 3A cell apoptosis. *Oxid. Med. Cell. Longev. 2013*,**2013**, 516051.
140. Milani, A.; Basirnejad, M.; Shahbazi, S.; Bolhassani, A. Carotenoids: Biochemistry, pharmacology and treatment. *Br. J. Pharmacol*. **2017**, 174, 1290–1324.
141. Balestrieri, M.L.; De Prisco, R.; Nicolaus, B.; Pari, P.; Moriello, V.S.; Strazzullo, G.; Iorio, E.L.; Servillo, L.; Balestrieri, C. Lycopene in association with alpha-tocopherol or tomato lipophilic extracts enhances acyl-platelet-activating factor biosynthesis in endothelial cells during oxidative stress. *Free Radic. Biol. Med*. **2004**, 36, 1058–1067.

142. Mozos, I.; Stoian, D.; Caraba, A.; Malainer, C.; Horbanczuk, J.O.; Atanasov, A.G. Lycopene and Vascular Health. *Front. Pharmacol*. **2018**, 9, 521.
143. Liu, D.; Shi, J.; Colina Ibarra, A.; Kakuda, Y.; Jun Xue, S. The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and β-carotene mixtures on the DPPH free radical. LWT–*Food Sci. Technol*. **2008**, 41, 1344–1349.
144. Kong, K.W.; Khoo, H.E.; Prasad, K.N.; Ismail, A.; Tan, C.P.; Rajab, N.F. Revealing the power of the natural red pigment lycopene. *Molecules* **2010**, 15, 959–987.
145. Hajimehdipoor, H.; Shahrestani, R.; Shekarchi, M. Investigating the synergistic antioxidant effects of some flavonoid and phenolic compounds. *Res. J. Pharmaco*. **2014**, 1, 35–40.
146. Alkhatib, A. Antiviral Functional Foods and Exercise Lifestyle Prevention of Coronavirus. *Nutrients* **2020**, 12, 2633.
147. Macaluso, M.; Bianchi, A.; Sanmartin, C.; Taglieri, I.; Venturi, F.; Testai, L.; Flori, L.; Calderone, V.; De Leo, M.; Braca, A.; Ciccone, V.; Donnini, S.; Guidi, L.; Zinnai, A. By-products from winemaking and olive mill value chains for the enrichment of refined olive oil: Technological challenges and nutraceutical features. *Foods* **2020**, 9, 1390.
148. Barros, L.; Dueñas, M.; Pinela, J.; Carvalho, A.M.; Buelga, C.S.; Ferreira, I.C. Characterization and quantification of phenolic compounds in four tomato (Lycopersicon esculentum L.) farmers' varieties in northeastern Portugal homegardens. *Plant Foods Hum. Nutr*. **2012**, 67, 229-234.
149. Moco, S.; Bino, R.J.; Vorst, O.; Verhoeven, H.A.; De Groot, J.; Van Beek, T.A.; Vervoort, J.; De Vos, C.H. A liquid chromatography-mass spectrometry-based metabolome database for tomato. *Plant Physiol.* **2006**, 141, 1205-1218.
150. Owens, J.; Moreira, K.; Bain, G. Characterization of primary human skeletal muscle cells from multiple commercial sources. *In vitro cell. Dev Biol-Animal* **2003**, 49, 695-705.
151. Lecomte, V.; Meugnier, E.; Euthine, V.; Durand, C.; Freyssenet, D.; Nemoz, G.; Rome, S.; Vidal, H.; Lefai, E. A new role for sterol regulation of muscle mass and muscle cell differentiation. *Mol Cell Biol* **2010**, 30, 1182-1198.
152. Ozernyuk, N.; Balan, O. Muscle satellite cells and regulation of recovery potential of muscle. *Biol. Bull*. **2007**, 34, 542-550.
153. Burattini, S.; Ferri, P.; Battistelli, M.; Curci, P.; Luchetti, F.; Falcieri, E. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur. J. Histochem* **2004**, 392, 110-116.
154. Orzechowski, A.; Grizard, J.; Jank, M.; Gajkowska, B.; Lokociejewska, M.; Zaron-Teperek, M.; Godlewski, M. Dexamethasone-mediated regulation of death and differentiation of muscle cells. Is hydrogen peroxide involved in the process? *Reprod Nutr Dev*, **2002**, 42, 3,197-216.
155. Santos-Sánchez, N.F.; Salas-Coronado, R.; Hernández-Carlos, B.; Villanueva-Cañongo, C. Shikimic Acid Pathway in Biosynthesis of Phenolic Compounds. *Plant Physiological Aspects of Phenolic Compounds*, **2019**. DOI: http://dx.doi.org/10.5772/intechopen.83815
156. Upadhyay, R.; Mohan Rao, L.J. An outlook on chlorogenic acids-occurrence, chemistry, technology, and biological activities. *Crit Rev Food Sci Nutr,***2013**, 53, 968–984.

157. Liu, Y.J.; Zhou, C.Y.; Qiu, C.H.; Lu, X.M.; Wang, Y.T. Chlorogenic acid induced apoptosis and inhibition of proliferation in human acute promyelocytic leukemia HL60 cells. *Mol Med Rep*. **2013**, 8,1106–1110.
158. Tsang, M.S.; Jiao, D.; Chan, B.C.; Hon, K.L.; Leung, P.C.; Lau, C.B.; Wong, E.C.; Cheng, L.; Chan, C.K.; Lam, C.W.; Wong, C.K. Anti-inflammatory activities of pentaherbs formula, berberine, gallic acid and chlorogenic acid in atopic dermatitis-like skin inflammation. *Molecules*. **2016**, 21,519.
159. Ye, H.Y.; Li, Z.Y.; Zheng, Y.; Chen, Y.; Zhou, Z.H.; Jin, J. The attenuation of chlorogenic acid on oxidative stress for renal injury in streptozotocin-induced diabetic nephropathy rats. *Arch Pharm Res*. **2016**, 39, 989–997.
160. Akila, P.; Vennila, L. Chlorogenic acid a dietary polyphenol attenuates isoproterenol induced myocardial oxidative stress in rat myocardium: An in vivo study. *Biomed Pharmacother* **2016**, 84, 208-214.
161. Xu, M.; Chen, X.; Chen, D.;Yu, B.; Huang, Z. FoxO1: a novel insight into its molecular mechanisms in the regulation of skeletal muscle differentiation and fiber type specification. *Oncotarget*, **2017**, 8, 10662-74.